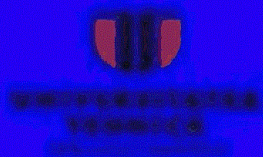




CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

Special Issue

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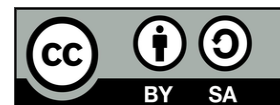
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MESSAGE OF CONFERENCE CO-CHAIRS

It is our great pleasure, as the Conference Co-Chairs of NUTRICON 2025, to reflect on the valuable academic discussions, collaborations, and knowledge exchange fostered through this global platform. The conference brought together researchers, academics, practitioners, industry experts, and policymakers to explore innovative solutions across food science, nutrition, health, lifestyle, and sustainability. Through inspiring presentations and meaningful discussions, NUTRICON 2025 strengthened global research connections and highlighted the role of scientific knowledge in advancing sustainable practices. This Special Issue of the *Carpathian Journal of Food Science and Technology* extends these contributions, supporting continued innovation and collaboration toward a sustainable future.

Prof. Dr. Nurpudji A Taslim, MD, MPH, SPGK (K), FRSPH

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MESSAGE OF KEYNOTE SPEAKER

It is the first time for me to join NUTRICON 2025 on November 17-18, 2025, in Bangkok, Thailand. I am very impressive on the program arrangement and also interesting sessions from different visions. The theme of the conference is future-focused nutrition, which is very attractive and innovative. All the invited speakers give very nice presentations. All the participants also get involved and pay attention very much. There are so many people from academy, industry and government of so many countries to join together. I ever attend so many conferences around the world. This conference is one of the best. I believe this is the best platform to exchange and cooperate for the experts around the world. By the way, I also know some new friends and have some advanced cooperation now. I will attend NUTRICON 2027 in Bali. I am sure it is a very good chance again for me. I greatly recommend NUTRICON to all the related experts around the world. And also wish it can extend the influence the nutrition issue in the future.

Distinguish Prof. Chin-Kun Wang

Chung Shan Medical University (CSMU)
Taiwan

MESSAGE OF GUEST EDITORS

Food science and nutrition research increasingly demands an integrated approach that considers nutritional quality, safety, consumer acceptance, processing efficiency, and environmental sustainability. Successful food development requires balancing these interconnected factors to ensure products are nutritious, safe, appealing, feasible, and sustainable. The sixteen articles in this Special Issue collectively address these priorities and are grouped into four key themes: food processing and preservation, extraction and characterization of bioactive compounds, development of functional and enhanced foods, and sustainable materials and food safety technologies.

Contemporary food science and nutrition research is increasingly interdisciplinary, integrating food processing, nutrition, chemistry, biotechnology, microbiology, and sensory science to develop safer, healthier, and sustainable food systems. Future research should emphasize validation of findings, storage stability, biological relevance, consumer acceptance, safety evaluation, process optimization, scalability, and environmental impact assessment. Integrating technological, nutritional, safety, consumer, and sustainability perspectives will be essential to transform innovative research into practical solutions for industry, public health, and sustainable food development.

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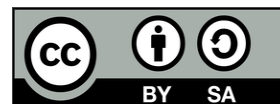
MESSAGE OF CONFERENCE CONVENER

It is a pleasure to introduce this Special Issue of the *Carpathian Journal of Food Science and Technology*, developed in connection with the International Conference on Food, Nutrition, Health and Lifestyle (NUTRICON).

Food, nutrition, health, and sustainability are interconnected global priorities requiring interdisciplinary research, collaboration, and knowledge translation. NUTRICON provides a platform for researchers, practitioners, policymakers, and industry professionals to exchange ideas and strengthen partnerships. Through its Global Knowledge Ecosystem Platform, TIIKM promotes research, innovation, and global collaboration. This Special Issue reflects our commitment to advancing knowledge, sustainable solutions, and meaningful societal impact. I sincerely thank all editors, reviewers, authors, contributors, and partners for their valuable support.

Isanka P. Gamage

Co-Founder & Managing Director
The International Institute of Knowledge Management (TIIKM)
Sri Lanka



Research Article

EFFECT OF pH AND PRECURSOR TYPE ON GREEN-SYNTHESISED ZINC OXIDE PARTICLES USING MANGO SEED EXTRACT

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Abstract

Zinc oxide (ZnO) possesses optical and antimicrobial characteristics relevant to functional material applications. Plant-assisted synthesis provides an alternative to conventional ZnO synthesis routes that may involve chemical-intensive and energy-demanding processing. In this study, mango (*Mangifera indica*) seed extract (MSE) was used as a plant-derived synthesis medium to investigate the effects of pH and precursor type on ZnO characteristics. Zinc acetate dihydrate (ZA) and zinc nitrate hexahydrate (ZN) were separately mixed with MSE, adjusted to pH 5, 7, 8, 10, 12 or 14, subjected to controlled thermal and agitation treatments and subsequently recovered as dried ZnO products. The synthesised products were characterised using dynamic light scattering (DLS), zeta-potential analysis, scanning electron microscopy–energy-dispersive X-ray spectroscopy (SEM–EDX), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and ultraviolet–visible (UV–Vis) spectroscopy. ZA at pH 12 exhibited the most negative measured zeta potential (–25 mV) and the smallest DLS-derived hydrodynamic size of approximately 649 nm. SEM–EDX showed that all samples contain Zn and O elements and revealed predominantly spherical-to-irregular particle features for ZA, whereas ZN at pH 12 produced distinctive flower-like bundles. XRD confirmed the characteristic hexagonal wurtzite ZnO phase, with Scherrer-derived apparent crystallite sizes of approximately 17–39 nm. The larger DLS-derived hydrodynamic sizes relative to the XRD-derived apparent crystallite sizes reflected the different physical characteristics measured by the two techniques. FTIR showed Zn–O-associated absorption within approximately 400–600 cm^{–1}, while UV–Vis analysis showed pronounced absorption within approximately 250–300 nm, with the highest measured absorbance within the ZA series observed at pH 8. Overall, pH and precursor chemistry produced property-dependent responses, and no single synthesis condition provided the most favourable response across all evaluated characteristics.

1. Introduction

Zinc oxide (ZnO) is a multifunctional inorganic material with optical and antimicrobial characteristics that support its use in a range of functional material systems. Its optical behaviour enables effective interaction with ultraviolet radiation, while ZnO nanomaterials have also been widely investigated for antimicrobial functions (Irede et al., 2024). Conventional physical and chemical ZnO synthesis routes may involve hazardous reagents and substantial energy consumption, contributing to increasing interest in plant-assisted approaches that use naturally derived constituents during particle formation (Mutukwa et al., 2022).

Plant extracts contain phytochemical constituents that can participate in nanoparticle formation and act as capping or stabilising agents during green synthesis (Villagrán et al., 2024). Mango (*Mangifera indica*) seed is an agro-industrial by-product rich in bioactive compounds, including polyphenols, flavonoids and tannins (Cárdenas-Hernández et al., 2024). Aqueous mango seed extract has also been previously used as a plant-derived medium for ZnO synthesis (Rajeshkumar et al., 2023). These findings support the use of mango seed extract (MSE) as a plant-derived synthesis medium.

Reaction pH is an important synthesis variable because changes in alkalinity alter hydroxide availability and the chemical environment governing ZnO growth (Abdulrahman et al., 2021). Recent ZnO studies have demonstrated pH-dependent differences in particle morphology, crystal characteristics and optical behaviour, although the magnitude and direction of these responses vary among synthesis systems (Mechai et al., 2024). The common finding across these studies is therefore the sensitivity of ZnO formation to reaction pH rather than a universal response to increasing alkalinity. This variability suggests that pH should be interpreted together with the surrounding chemical environment rather than as an isolated determinant of the resulting ZnO characteristics.

Precursor chemistry represents another component of the synthesis environment

because different zinc salts have been shown to produce differences in ZnO morphology, crystallite size and particle-size distribution under comparable preparation conditions (Gatou et al., 2022). Plant-assisted systems introduce an additional chemical environment because phytochemicals derived from plant extracts can participate in nanoparticle formation and stabilisation (Jeevanandam et al., 2022). This is particularly relevant to MSE-assisted synthesis, in which interactions between Zn-containing species and plant-derived constituents may contribute to the resulting particle characteristics. Systematic comparison of zinc acetate and zinc nitrate across a broad pH range within the same MSE-assisted framework remains limited. The novelty of the present study therefore lies in evaluating both variables under a common synthesis environment and determining whether their effects remain consistent across colloidal, morphological, structural and optical characteristics.

Accordingly, this study investigated the effects of pH 5–14 and precursor type, namely zinc acetate dihydrate (ZA) and zinc nitrate hexahydrate (ZN), on MSE-assisted ZnO synthesis. The resulting products were evaluated in terms of DLS-derived hydrodynamic size, zeta potential, particle morphology and elemental composition, functional groups, XRD-derived apparent crystallite size and optical absorption behaviour. The objective was to determine how pH and precursor type influenced these physicochemical characteristics and whether a common synthesis condition produced favourable responses across the measured properties or whether the preferred condition was property dependent.

2. Materials and Methods

2.1. Materials

Mango (*Mangifera indica*) seeds were obtained from a local fruit-processing facility in Shah Alam, Selangor, Malaysia. Zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) and zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) from Sigma-Aldrich® were used as Zn^{2+} precursor salts. Sodium hydroxide (NaOH), distilled water and ethanol were used for pH adjustment,

solution preparation, washing and sample preparation.

2.2. Preparation of Mango Seed Extract (MSE)

The mango seeds were cleaned, washed with tap and distilled water and finally rinsed with diluted ethanol with dilution ratio of 1:4. The seeds were oven-dried at 60 °C until constant mass, ground and sieved before storage at 4 °C.

For extraction, 1 g of mango seed powder was dispersed in 100 mL of distilled water (1:100, w/v) and stirred at 500 rpm at 70 °C for 1 h. These extraction conditions were adapted from a previously reported aqueous mango-seed-extraction approach used for plant-assisted ZnO synthesis (Rajeshkumar et al., 2023). The mixture was subsequently cooled to room temperature and filtered through Whatman No. 1 filter paper. The resulting MSE was collected and stored at 4 °C in amber glass bottles until use.

2.3. Green Synthesis of ZnO

ZA and ZN solutions were separately prepared at 0.05 M. For each synthesis condition, 10 mL of MSE was mixed with 75 mL of zinc precursor solution under magnetic stirring at 500 rpm. The pH of each mixture was adjusted to 5, 7, 8, 10, 12 or 14 by the dropwise addition of 0.1 M NaOH under continuous stirring. The mixtures were maintained at 70 °C for 1 h, followed by orbital shaking at 40 °C and 200 rpm for 1 h. These thermal and agitation conditions were adapted from a previously reported MSE-assisted ZnO synthesis procedure and were maintained consistently across all pH and precursor treatments to permit direct comparison of the two investigated synthesis variables (Kamalia et al., 2025). After standing undisturbed at room temperature for 1 h, the precipitates were recovered by filtration, washed three times with distilled water, rinsed with diluted ethanol and dried at 60 °C for 24 h. The dried products were gently ground and stored in a desiccator before characterisation.

2.4. Characterisation of Synthesised ZnO

2.4.1. Hydrodynamic Size and Zeta Potential

Hydrodynamic size and zeta potential were measured using a ZN3600 Zetasizer (Malvern Instruments Ltd., UK). Prior to measurement, 0.025 g of dried ZnO powder was dispersed in 10 mL of distilled water and ultrasonicated for 30 min. Measurements were conducted at 25 °C. The refractive index of ZnO was set to 2.0, while that of distilled water was approximately 1.333. DLS was used to determine the hydrodynamic size of redispersed particle entities, whereas folded capillary cells were used for zeta-potential measurements. Accordingly, the DLS-derived hydrodynamic size was not interpreted as the physical diameter of individual ZnO crystallites, and the measured zeta potential was not treated as a direct measurement of colloidal stability during the original synthesis reaction.

2.4.2. SEM–EDX Analysis

Surface morphology and elemental composition were examined using SEM–EDX (SU3500, Hitachi High-Technologies Corporation, Tokyo, Japan). Samples were mounted on carbon stubs, gold-coated and examined at 15–20 kV. SEM was used for qualitative comparison of external particle morphology and visible particle association. Quantitative SEM-derived particle-size distributions were not generated because image-based particle segmentation was not performed. EDX provides local elemental information from the analysed region and, particularly when standardless quantification is used, should be interpreted cautiously as qualitative or semi-quantitative compositional information rather than as definitive bulk composition (Hodoroaba, 2020).

2.4.3. FTIR Analysis

FTIR spectra were obtained using a PerkinElmer spectrometer over 4000–400 cm⁻¹ at a resolution of 4 cm⁻¹ and evaluated for Zn–O-associated absorption and residual surface-associated functional groups. FTIR is commonly used in plant-mediated ZnO characterisation to identify surface-associated functional groups, with Zn–O-related vibrations typically occurring in the lower-wavenumber region of approximately 400–600 cm⁻¹ (Fagier, 2021).

2.4.4. XRD Analysis

XRD analysis was performed using a D/MAX-2000/PC diffractometer (Rigaku Corporation, Tokyo, Japan) equipped with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) over $20\text{--}80^\circ 2\theta$. Diffraction patterns were compared with JCPDS No. 36-1451 for hexagonal wurtzite ZnO.

Apparent crystallite size (D) was estimated from the (101) reflection using the Scherrer equation (Hassanzadeh-Tabrizi, 2023):

$$D = \frac{k\lambda}{\beta \cos\theta} \quad (1)$$

where K is the shape factor (0.9), λ is the X-ray wavelength ($1.5406 \text{ \AA}$), β is the full width at half maximum (FWHM) of the selected diffraction reflection expressed in radians, and θ is the corresponding Bragg angle. The Scherrer-derived values represent the size of coherently diffracting crystalline domains and were therefore reported as apparent crystallite sizes rather than particle sizes.

2.4.5. UV-Vis Analysis

Optical absorption was evaluated using a UV-1900i UV-visible spectrophotometer (Shimadzu) over $200\text{--}800 \text{ nm}$, and the spectra were compared among pH and precursor conditions. UV-Vis spectroscopy is commonly used to evaluate the optical response of biosynthesised ZnO, including changes in ultraviolet absorption associated with different synthesis conditions (Suciyati et al., 2024). The resulting spectra were compared among pH and precursor conditions.

2.5. Data Analysis

All synthesis conditions were prepared using three independent synthesis replicates, and

instrumental analyses were conducted using three technical replicates for each independent synthesis replicate. Quantitative data for which replicate measurements were available were summarised as mean $\pm$ standard deviation. Comparisons among pH conditions and between the ZA and ZN precursor systems were performed descriptively. No inferential statistical tests were conducted; therefore, differences among synthesis conditions were interpreted as observed trends and were not described as statistically significant. Technical replicates were used to assess measurement repeatability and were not treated as independent experimental units.

3. Results and Discussion

3.1. Physical Observation

Visible precipitation varied with synthesis pH and precursor system (Fig. 1). Only a small amount of precipitate was observed at pH 5, whereas greater white precipitate formation occurred under more alkaline conditions, particularly between pH 8 and 12. Increasing alkalinity (OH^-) modifies the $\text{Zn}^{2+}/\text{OH}^-$ chemical environment and can promote zinc hydrolysis, supersaturation and subsequent nucleation and crystal development (Mechai et al., 2024). However, intermediate zinc species, supersaturation and nucleation rates were not measured directly in the present study. The visual observations therefore demonstrate pH-dependent precipitation behaviour but do not independently establish the reaction pathway responsible for ZnO formation. The composition and crystalline characteristics of the recovered products were subsequently evaluated using EDX, FTIR and XRD.

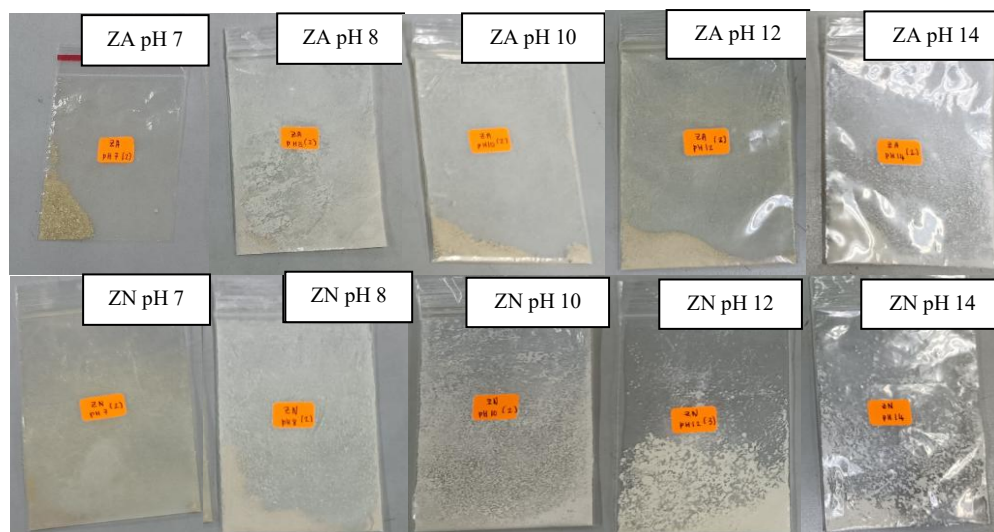


Figure 1. ZnO products synthesised using MSE at different pH conditions and precursor types.

3.2. Zeta Potential and Hydrodynamic Size

Zeta potential and DLS revealed descriptive differences between precursor and pH conditions (Table 1). ZA at pH 12 exhibited the most negative measured zeta potential (-25 mV) and the smallest hydrodynamic size of

approximately 649 nm. Zeta-potential magnitudes within approximately the ± 20 to ± 30 mV range are commonly associated with appreciable electrostatic stabilisation, although they do not necessarily indicate complete colloidal stability (Sultan et al., 2024).

Table 1. Zeta potential and DLS-derived hydrodynamic size of ZnO synthesised using zinc acetate dihydrate (ZA) and zinc nitrate hexahydrate (ZN) at different pH values.

ZA					ZN				
pH	Zeta potential (mV)	SD	Hydrodynamic size (nm)	SD	pH	Zeta potential (mV)	SD	Hydrodynamic size (nm)	SD
7	-4.99	0.71	4218.00	845.99	7	-4.61	0.22	1923.33	264.23
8	-21.07	0.38	1143.33	87.80	8	-15.40	2.63	1618.33	189.47
10	-8.17	0.46	3440.00	972.12	10	-13.50	0.10	1610.33	237.39
12	-25.00	0.10	649.20	40.63	12	-6.39	1.14	2156.67	408.16
14	4.01	0.24	2426.33	485.15	14	-18.00	0.62	2252.33	200.24

Note: Values are presented as mean $\pm$ standard deviation from three independent synthesis replicates, with instrumental measurements conducted in triplicate. No inferential statistical comparisons were performed; therefore, differences among conditions are interpreted descriptively.

The most negative measured zeta potential and smallest hydrodynamic size within the ZA series were both observed at pH 12. This coincidence is consistent with greater electrostatic repulsion and reduced association of the redispersed particle entities under the measurement conditions. However, the present descriptive dataset does not establish a statistically significant relationship or a direct causal relationship between zeta potential and hydrodynamic size. Particle morphology, surface chemistry and dispersion history may

also contribute to the measured hydrodynamic response. The response also differed between precursor systems. ZN-derived products exhibited zeta potentials of approximately -4 to -18 mV and predominantly micrometre-scale hydrodynamic dimensions, whereas ZA showed a broader response and reached approximately 649 nm at pH 12. Precursor-dependent differences in DLS-derived size, morphology and other physicochemical characteristics have similarly been reported for ZnO synthesised from different zinc salts (Gatou et al., 2022).

Therefore agreement lies in the sensitivity of ZnO particle behaviour to precursor chemistry rather than in reproduction of an identical precursor-specific size response. Since the present measurements were performed after dried ZnO had been redispersed in water, the results describe the hydrodynamic behaviour of the redispersed products rather than particle dimensions or colloidal stability during the original synthesis reaction.

3.3. Surface Morphology and Elemental Composition

SEM showed predominantly spherical-to-irregular associated particle structures, with visible clustering under several synthesis conditions (Fig. 2). The most evident precursor-dependent difference occurred at pH 12, at

which ZA exhibited predominantly spherical-to-irregular features whereas ZN formed distinctive flower-like bundles despite both being processed at the same nominal pH and Zn^{2+} concentration. Previous work comparing zinc acetate- and zinc nitrate-containing ZnO synthesis systems has likewise demonstrated that precursor composition can modify ZnO microstructure, aggregation and morphology (Kaenphakdee et al., 2022). However, the specific morphologies reported in that study were not identical to those observed in the present MSE-assisted system. This comparison indicates that precursor chemistry can influence ZnO morphology, but the resulting morphology also depends on the surrounding synthesis environment rather than being determined solely by the identity of the zinc salt.

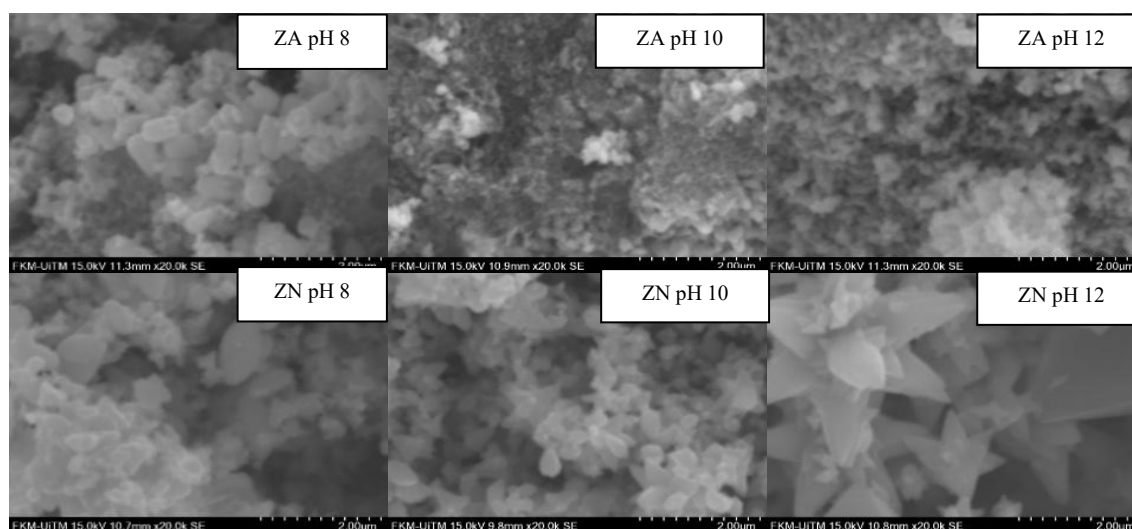


Figure 2. SEM micrographs of ZnO synthesised at different pH conditions using ZA and ZN at 20,000 $\times$ magnification.

A plausible interpretation is that precursor chemistry modified the ionic and coordination environment surrounding Zn-containing species during nucleation and subsequent particle development. However, precursor-specific Zn^{2+} complexation, intermediate hydroxo species and crystallographic growth rates were not measured directly. The SEM evidence therefore demonstrates a precursor-dependent morphological response but does not establish the molecular mechanism responsible for that response. Quantitative image segmentation was

not performed; therefore, numerical SEM-derived particle-size distributions were not generated. SEM was consequently used to compare external particle morphology and visible particle association rather than to quantify particle diameter. These observations were interpreted together with the DLS-derived hydrodynamic sizes and XRD-derived apparent crystallite sizes because the three techniques describe different levels of particle organisation.

EDX spectra were dominated by Zn and O, with local semi-quantitative compositions of

approximately 86–89 wt% Zn and 11–13 wt% O. These elemental signals complement the Zn–O-associated FTIR absorption and wurtzite reflections observed by XRD. EDX provides spatially localised elemental information and its quantitative interpretation for nanoparticles is subject to analytical and sampling limitations. Therefore, EDX commonly used as qualitative

or semi-quantitative compositional evidence rather than as an automatic measure of bulk composition (Hodoroaba, 2020). Accordingly, the measured compositions were interpreted as evidence for the local presence of Zn and O rather than as quantitative confirmation of bulk chemical purity.

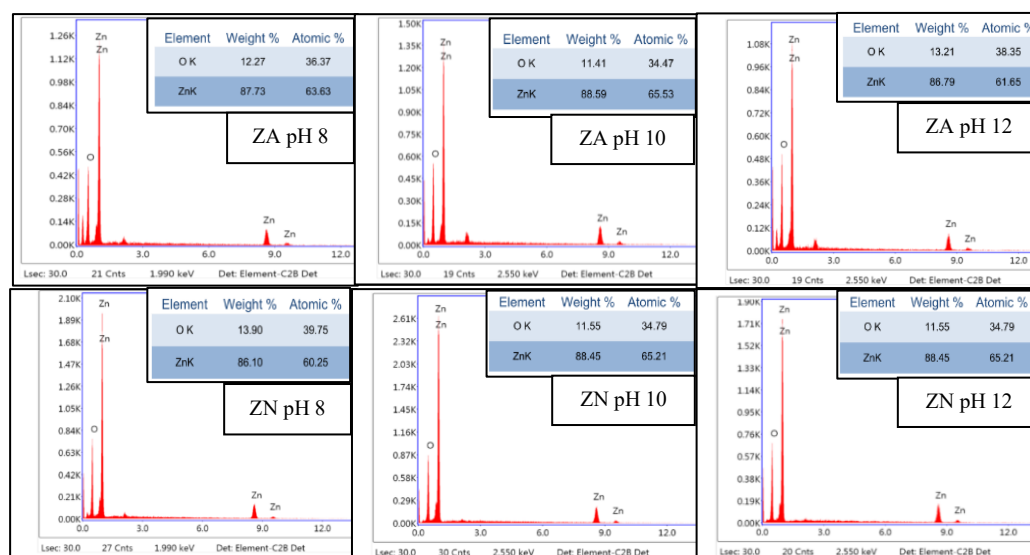


Figure 3. EDX spectra of ZnO synthesised at different pH conditions using ZA and ZN.

3.4. Functional Group Analysis

As shown in Fig. 4, FTIR spectra showed broad O–H-associated absorption within approximately 3200–3600 cm^{-1} and organic-associated bands within approximately 2800–3000 and 1400–1650 cm^{-1} . Their persistence after particle recovery and washing is consistent with residual MSE-derived constituents remaining associated with the recovered material. Plant-mediated ZnO systems commonly exhibit FTIR bands associated with hydroxyl- and other organic functional groups originating from plant-derived constituents, which may participate in interactions with Zn-containing species during synthesis (Fagier, 2021).

Hydroxyl- and carbonyl-containing groups have been proposed to participate in Zn^{2+} coordination and surface stabilisation during plant-assisted ZnO synthesis. In the present study, however, FTIR did not identify the

individual phytochemical compounds responsible for these bands or directly demonstrate their role during nucleation. Their involvement should therefore be regarded as a mechanistically plausible interpretation rather than evidence of a specific synthesis pathway.

Zn–O-associated absorption was observed within approximately 400–600 cm^{-1} . Zn–O stretching vibrations in plant-mediated ZnO are commonly reported within this lower-wavenumber region (Fagier, 2021). FTIR alone does not demonstrate crystalline ZnO formation; consequently, these bands were interpreted together with the local Zn and O signals detected by EDX and the characteristic wurtzite reflections identified by XRD. Convergence of the three independent characterisation techniques provides stronger evidence for ZnO formation than interpretation based on any single measurement.

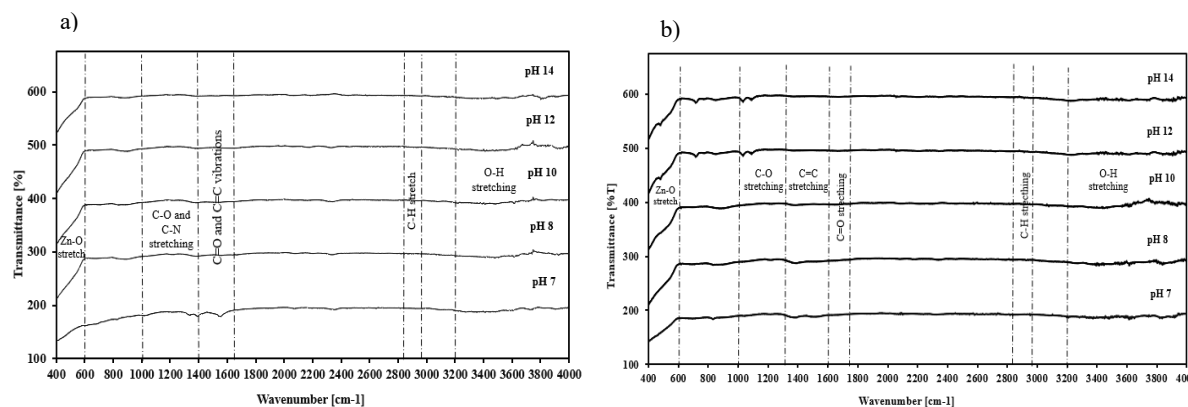


Figure 4. FTIR spectra of ZnO synthesised using MSE at different pH conditions with (a) zinc acetate dihydrate and (b) zinc nitrate hexahydrate.

3.5. Structural Characterisation

XRD patterns exhibited characteristic reflections at approximately 31.5° , 34.1° , 36.0° , 47.2° , 56.2° , 62.5° , 66.0° , 67.6° and 68.8° 2θ , corresponding to the principal crystallographic planes of hexagonal wurtzite ZnO (Fig. 5). Comparable ZnO reflections at approximately 31.7° , 34.3° , 36.2° , 47.5° , 56.5° , 62.8° and 67.9° 2θ have been reported for mango-assisted ZnO and assigned to the characteristic hexagonal

wurtzite structure (Endah et al., 2023). Importantly, both ZA- and ZN-derived products retained the same principal wurtzite phase despite the precursor-dependent morphological differences observed by SEM. Thus, precursor chemistry affected particle development without producing an observable change in the principal ZnO crystalline phase under the investigated conditions.

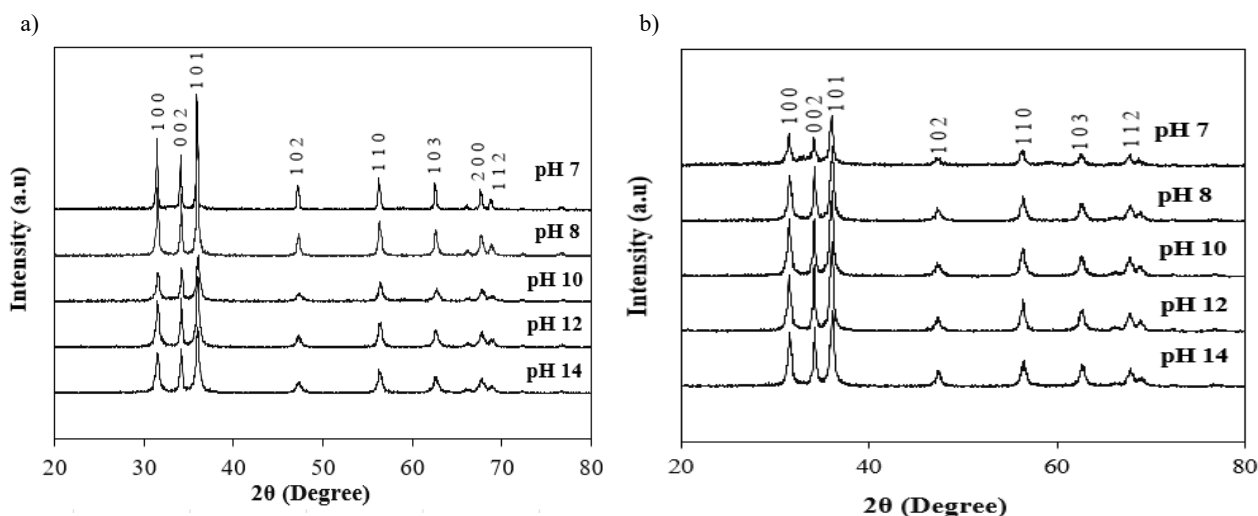


Figure 5. XRD patterns of ZnO synthesised using MSE at pH 7–14 with (a) zinc acetate dihydrate and (b) zinc nitrate hexahydrate.

Apparent crystallite sizes varied descriptively with both pH and precursor type (Table 2). For ZA, the apparent crystallite size decreased from 38.517 nm at pH 7 to 18.488 nm at pH 14, although the response was non-monotonic, with an increase from 18.995 nm at

pH 10 to 21.333 nm at pH 12. The ZN series showed a narrower range from 20.676 nm at pH 7 to 16.706 nm at pH 14, also with intermediate fluctuations. Changes in alkaline synthesis conditions and NaOH concentration have previously been shown to modify ZnO crystal

development and apparent crystallite dimensions in biosynthesis systems (Suciyati et al., 2024). Increased OH^- availability may therefore contribute to altered supersaturation and nucleation behaviour, providing a plausible explanation for the generally smaller coherent crystalline domains observed at higher pH.

However, because neither nucleation density nor crystal-growth kinetics was directly measured, the present XRD data demonstrate variation in apparent crystallite sizes but do not establish the kinetic mechanism responsible for that variation.

Table 2. Apparent crystallite sizes of ZnO synthesised under different pH conditions using zinc acetate dihydrate and zinc nitrate hexahydrate, estimated from the (101) reflection using the Scherrer equation

Precursor	pH	Apparent crystallite size (nm)	Precursor	pH	Apparent crystallite size (nm)
Zinc acetate dihydrate	7	38.517	Zinc nitrate hexahydrate	7	20.676
	8	30.814		8	17.552
	10	18.995		10	18.008
	12	21.333		12	19.809
	14	18.488		14	16.706

The comparison between ZA and ZN further demonstrates that precursor effects were not constant across the pH range. At pH 7, the apparent crystallite sizes were 38.517 nm for ZA and 20.676 nm for ZN, whereas at pH 14 the corresponding values were 18.488 and 16.706 nm. The difference between the two precursor systems therefore narrowed under the more alkaline condition. Independent comparison of zinc acetate and zinc nitrate has also demonstrated precursor-dependent differences in ZnO morphology and crystallite size (Limón-rocha et al., 2022). Taken together, the studies agree that precursor chemistry influences crystal development, but the specific magnitude and direction of the response depend on the surrounding synthesis environment. The present results therefore do not support the assumption that either ZA or ZN inherently produces smaller ZnO crystallites independent of pH.

The XRD-derived apparent crystallite sizes of approximately 17–39 nm were substantially smaller than the DLS-derived hydrodynamic sizes of approximately 649–4218 nm. This difference does not represent disagreement between the techniques because they quantify different physical characteristics. The Scherrer equation estimates the size of a coherently diffracting crystalline domain, whereas DLS

determines the hydrodynamic behaviour of dispersed entities that may contain multiple associated crystallites, surface-associated species and the surrounding solvation layer. SEM independently showed visible particle association under several synthesis conditions. Taken together, the evidence supports the interpretation that nanoscale ZnO crystalline domains occurred within larger associated particulate structures following redispersion. XRD, DLS and SEM were therefore interpreted as complementary assessments of apparent crystallite size, hydrodynamic association and external morphology, respectively, rather than as interchangeable particle-size measurements.

3.6. Optical Properties

All investigated ZnO products exhibited pronounced UV absorption within approximately 250–300 nm (Fig. 6). Strong ultraviolet absorption is characteristic of ZnO materials, while the exact absorption position and intensity can vary with synthesis route and resulting physicochemical characteristics; green-synthesised ZnO systems have likewise shown synthesis-dependent UV–Vis absorption responses (Aalami et al., 2024). The agreement therefore lies in the presence of a pronounced

UV response rather than in an identical absorbance trend across synthesis systems.

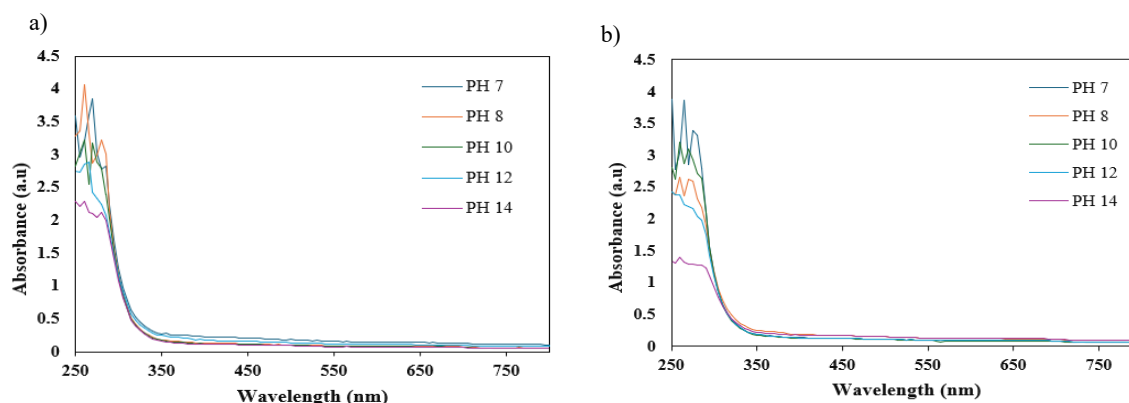


Figure 6. UV–Vis absorption spectra of ZnO synthesised using MSE at pH 7–14 with (a) zinc acetate dihydrate and (b) zinc nitrate hexahydrate.

Within the ZA series, the highest measured absorbance occurred at pH 8, whereas pH 14 produced the lowest response. In contrast, the ZN series showed its highest absorbance at pH 7 and generally lower responses at increasing pH. The differing trends between ZA and ZN under the same investigated pH range indicate that increasing alkalinity did not produce a common optical response for both precursor systems. Particle morphology, degree of association and crystalline characteristics can influence ZnO optical behaviour, although their individual contributions cannot be separated from absorbance measurements alone. Accordingly, their individual contributions could not be resolved quantitatively in the present study.

Importantly, the condition associated with the most favourable measured colloidal response was not the same condition associated with the highest measured optical response. ZA at pH 12 exhibited the most negative zeta potential and smallest hydrodynamic size, whereas ZA at pH 8 produced the highest measured absorbance within the ZA series. ZN at pH 12 additionally exhibited a distinctive flower-like morphology while retaining the principal wurtzite phase. These cross-characterisation differences provide direct evidence that the effects of pH and precursor chemistry were property dependent. Consequently, no single pH–precursor condition

can be designated as universally optimal from the present dataset.

4. Conclusion

MSE-assisted ZnO synthesis produced pH- and precursor-dependent differences across the measured physicochemical characteristics. ZA at pH 12 exhibited the most negative measured zeta potential of -25 mV and the smallest DLS-derived hydrodynamic size of approximately 649 nm, whereas ZN at pH 12 exhibited distinctive flower-like particle structures. XRD confirmed the characteristic hexagonal wurtzite ZnO phase with apparent crystallite sizes of approximately 17–39 nm. The substantially larger DLS-derived hydrodynamic sizes relative to the XRD-derived apparent crystallite sizes, together with visible particle association observed by SEM, support the interpretation that nanoscale crystalline domains occurred within larger associated particulate structures following redispersion. UV–Vis analysis showed pronounced absorption within approximately 250–300 nm, with the highest measured absorbance within the ZA series observed at pH 8.

The principal finding was that the favourable synthesis condition depended on the physicochemical characteristic being considered. ZA at pH 12 provided the most favourable measured colloidal response, ZA at

pH 8 produced the highest measured absorbance within the ZA series, and ZN at pH 12 exhibited a distinctive precursor-dependent morphology. Because the comparisons were descriptive and no inferential statistical tests were conducted, these differences should be interpreted as observed trends rather than statistically demonstrated treatment effects. Nevertheless, the combined DLS, SEM–EDX, FTIR, XRD and UV–Vis evidence indicates that pH and precursor chemistry were associated with non-identical responses across the evaluated characteristics.

The present study did not determine intermediate zinc species, quantitative surface chemistry, antimicrobial activity, safety or functional behaviour following incorporation into a polymer matrix. The powder-level findings therefore provide a basis for selecting ZnO conditions for subsequent property-specific validation rather than evidence of direct food-packaging or biomedical performance. Future research should evaluate antimicrobial activity, safety, polymer-matrix compatibility and whether the physicochemical differences observed among the ZnO products are retained following incorporation into biodegradable films.

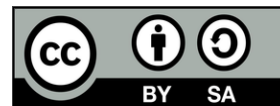
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Research article

EFFECT OF DEHYDRATION PROCESS OPTIMIZATION ON PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF GUYABANO (*ANNONA MURICATA* L.) LEAF TEA

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Abstract

Guyabano (*Annona muricata* L.) leaves are used in herbal infusions, but dehydration conditions can alter phenolic retention, radical-scavenging activity, and sensory quality. This study evaluated the effects of forced-air dehydration temperature and time on guyabano leaf tea using an I-optimal response surface design over 55-75 degrees C and 2-6 h. Independent processing batches were analyzed for proximate composition, total phenolic content (TPC), and DPPH radical-scavenging activity, while selected high-temperature formulations were evaluated for sensory acceptability and microbiological quality. Formulation significantly affected all testable proximate and bioactive responses ($p < 0.05$). GT12 showed the highest observed TPC (119.39 ± 2.52 mg GAE/100 g dried sample), whereas GT04 and GT11 showed the highest DPPH inhibition group, with GT12 showing moderate DPPH activity ($56.61 \pm 1.21\%$). The TPC RSM model showed strong fit and predictive performance ($R^2 = 0.977$; predicted $R^2 = 0.856$), while the DPPH model was statistically significant but less predictive ($R^2 = 0.788$; predicted $R^2 = 0.325$). Multi-response desirability identified 75 degrees C for 6 h as the model-predicted optimum, with predicted TPC of 125.99 mg GAE/100 g dried sample and DPPH inhibition of 61.39%. Sensory testing of GT10-GT12 showed acceptable hedonic scores, with GT12 numerically highest in overall acceptability. The selected formulations tested for microbiological quality met the referenced criteria. The optimum represents a model-predicted condition requiring independent confirmation.

1. Introduction

Guyabano (*Annona muricata* L.) leaves are used locally as a herbal infusion and are valued

because soursop leaves contain phenolic compounds and other phytochemical constituents associated with antioxidant

potential (Hasmila et al., 2019; Santos et al., 2023). For a dried-leaf tea product, however, infusion quality depends not only on the plant material but also on the processing conditions used before storage and brewing (Nhi et al., 2020).

Forced-air dehydration is necessary to lower product moisture and support handling stability; in this study, ASEAN tea moisture guidance was treated only as an analogous benchmark for the dried herbal-infusion product (ASEAN, 2014; Liu et al., 2022). Thermal exposure can alter bioactive quality. Moderate heating may improve phenolic extractability through tissue disruption and enzyme inactivation, whereas excessive temperature or prolonged drying can promote oxidation, polymerization, or degradation of heat-sensitive compounds (Antony & Farid, 2022; Duan et al., 2021; ElGamal et al., 2023). Similar drying-parameter effects have been reported for herbs and tea leaves (Roslan et al., 2020; Thamkaew et al., 2021).

Although guyabano leaf tea and related soursop-leaf products have been studied as herbal infusions or processed extracts, limited evidence is available on how forced-air dehydration temperature and time jointly affect phenolic retention, DPPH radical-scavenging activity, sensory acceptability, and microbial quality under controlled processing conditions (De Castro et al., 2018; Nhi et al., 2020). This distinction is important because total phenolic content and DPPH inhibition are related but not interchangeable measures of antioxidant behavior (Dobrinás et al., 2021; Platzer et al., 2022).

The present study tested the hypothesis that dehydration temperature and time exert significant linear, interaction, and nonlinear effects on the bioactive quality of guyabano leaf tea. The study aimed to identify a process condition that balances phenolic retention, DPPH activity, product moisture, sensory acceptability, and microbiological quality within the tested dehydration region.

Specifically, the study sought to: (1) quantify proximate composition, TPC, and DPPH radical-scavenging activity across the I-

optimal design runs; (2) model the effects of dehydration temperature and time using response surface methodology; (3) evaluate sensory acceptability and microbiological quality for selected high-temperature formulations; and (4) identify a model-predicted optimum for future independent validation.

2. Materials and Methods

2.1. Gathering of raw materials

Guyabano leaves were collected during rainy season from approximately five source tree(s) within the vicinity of Calamba, Laguna. Leaves used for the experiment were obtained from multiple trees and pooled before allocation to the experimental batches. Visually immature or overly mature leaves, as well as leaves showing yellowing, browning, physical damage, or apparent disease, were excluded. The selected leaves were transported to the processing area under clean handling conditions before preparation.

2.2. Preparation of Guyabano Leaves

The leaves were washed repeatedly with running water to remove dust, soluble impurities, and adhering dirt. The petiole and midrib were removed using clean kitchen scissors, and each leaf was cut into smaller pieces to improve drying uniformity. The prepared leaves were patted dry with clean absorbent material before loading into the dehydrator.

2.3. Preparation of Dehydrated Guyabano Leaves

The trimmed guyabano leaves were dried using a forced-air cabinet dehydrator (LJ Industrial Fabrication, Model CFD-12T, Philippines) at the temperature-time combinations specified in the I-optimal response surface design. Before each run, the dehydrator was preheated for 30 min, and chamber temperature was verified using an independently calibrated digital thermometer placed at the center tray. Fresh leaves were loaded at 250 g per tray, equivalent to 0.69 kg/m², with two trays used per experimental batch for a total loading of 500 g. The leaves were arranged in a single

layer with minimal overlap; leaf-layer thickness was not independently quantified. The internal fan operated continuously at a fixed setting, with an average air velocity of 1.2 ± 0.1 m/s. Relative humidity within the drying chamber was not independently controlled or recorded. Each run was terminated after the dehydration time specified by the experimental design rather than at a predetermined final-moisture endpoint. Initial moisture content was $68.4 \pm 1.9\%$ wet basis, determined from triplicate 5 g subsamples oven-dried at 105°C to constant weight, while final moisture content was subsequently determined for each dried formulation. After

dehydration, samples were cooled in a desiccator for 30 min, ground using an Osterizer 10-speed blender (Model BLSTMG-W00, 450 W), passed through a 20-mesh stainless-steel sieve, packed in food-grade laminated aluminum pouches, protected from light, and stored at $25 \pm 2^\circ\text{C}$ until analysis within 7 days.

The I-optimal response surface design used for the dehydration runs is summarized in Table 1. The duplicated low-temperature point and triplicated mid-region point were retained to support model estimation, pure-error assessment, and lack-of-fit evaluation.

Table 1. I-optimal response surface design for guyabano leaf dehydration

Run	Formulation	Temperature ($^\circ\text{C}$)	Time (h)	Design Note
1	GT01	55	4.54	Replicated low-temperature point
2	GT02	55	4.54	Replicated low-temperature point
3	GT03	55	5.98	High-time boundary
4	GT04	58	2.00	Low-time interior point
5	GT05	62	6.00	High-time interior point
6	GT06	66	2.00	Low-time interior point
7	GT07	67	3.72	Replicated mid-region point
8	GT08	67	3.72	Replicated mid-region point
9	GT09	67	3.72	Replicated mid-region point
10	GT10	75	2.00	High-temperature boundary
11	GT11	75	3.68	High-temperature interior point
12	GT12	75	5.42	High-temperature, near-optimum point

Note. The design comprised 12 design runs representing 9 unique temperature-time points. Runs at 55 degrees C for 4.54 h and 67 degrees C for 3.72 h were duplicated or triplicated within the design to support model estimation.

I-optimality was selected because the study emphasized prediction across the specified temperature-time region while maintaining an efficient number of experimental runs and replicated design points for estimation of pure error. Unlike fixed geometric response surface designs such as central composite and Box-Behnken designs, the I-optimal approach allowed the experimental points to be allocated within the defined factor region to improve prediction precision. The experimental design and response surface analyses were performed using Design-Expert software version 23.1, and the statistical outputs and model diagnostics were independently cross-checked using Python.

2.4. Evaluation of Dehydrated Guyabano Leaves

The proximate composition of the dehydrated guyabano leaf tea samples was determined following AOAC methods (AOAC, 2016). Moisture, ash, crude fat, protein, and fiber were measured for each formulation, and nitrogen-free extract (NFE) was treated as a calculated descriptive value rather than as an independently replicated analytical response.

$$\text{Moisture (\%)} = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

Where:

W₁: weight (g) of Guyabano tea leaves sample before drying

W₂: weight (g) of Guyabano tea leaves sample after drying

$$\text{Total ash (\%)} = \frac{W_1}{W} \times 100 \quad (2)$$

Where:

W: weight (g) of the dried Guyabano tea leaves sample

W₁: weight (g) of the ash Guyabano tea leaves sample after drying

$$\text{protein (\%)} = \frac{(A-B) \times N \times 1.4007 \times F}{W} \quad (3)$$

Where:

A = volume (ml) of 0.2 N HCl used in the sample
B = volume (ml) of 0.2 N HCl used in the blank titration

N = Nitrogen of HCl

W = weight (g) of sample

14.007 = atomic weight of nitrogen

F = the protein-nitrogen conversion factor

$$\text{Fat (\%)} = \frac{W_{fat}}{W_{GT}} \times 100 \quad (4)$$

Where:

W_{fat}: weight (g) of fat present in the Guyabano tea leaves

W_{GT}: weight (g) of Guyabano tea leaves sample

Nitrogen-free extract was calculated by difference from the measured proximate components following the food-composition approach described by FAO (2003). Because NFE was calculated by difference and showed no within-formulation variation, it was reported descriptively and excluded from inferential comparison.

$$\text{Total Carbohydrates} = 100 - (\text{protein} + \text{fat} + \text{water} + \text{ash} + \text{alcohol}) \quad (5)$$

2.4.5. Sample preparation for determination of total phenolic content and antioxidant activity

For TPC and DPPH analysis, 3 g of dried guyabano leaf tea sample was infused in 100 mL of boiling deionized water and held for 20 min following the adapted extraction procedure used for tea antioxidant assessment (Tong et al., 2019). The extract was cooled, filtered, and analyzed under the same extraction conditions for all formulations.

2.4.6. Determination of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method. The Folin-Ciocalteu approach remains widely used for estimating total phenolics in tea matrices, although it measures Folin-reactive reducing capacity rather than individual phenolic compounds (Sun et al., 2022). Absorbance was measured at 760 nm using a Shimadzu UV-1900i UV-Vis spectrophotometer with 1 cm quartz cuvettes. Gallic acid working standards of 0, 20, 40, 60, 80, and 100 mg/L were used to generate the calibration curve (absorbance = 0.0087 concentration + 0.021, R² = 0.997). Results were expressed as mg gallic acid equivalent per 100 g dried sample.

2.4.7. Determination of antioxidant activity

DPPH radical-scavenging activity was measured using a procedure adapted from antioxidant-assay protocols for food extracts (Tong et al., 2014). Freshly prepared 0.1 mM DPPH in methanol was protected from light. The DPPH control absorbance at 517 nm was accepted when between 0.700 and 0.900. Absorbance was measured after 30 min of dark incubation, and results were expressed as percent inhibition. Each formulation was produced in three independent processing batches; each batch was extracted once and read in triplicate, and triplicate absorbance readings were averaged to produce one batch-level value for statistical analysis.

$$\% \text{ inhibition} = \left[\frac{(Abs_0 - Abs_1)}{(Abs_0)} \right] \times 100 \quad (6)$$

Where:

Abs₀: absorbance without samples

Abs₁: absorbance in the presence of the sample

2.4.8. Sensory and microbiological evaluation of selected formulations

GT10, GT11, and GT12 were selected for sensory acceptability and microbiological quality assessment because they represented high-temperature formulations near the response surface optimum region. The 75°C for 6 h condition was treated as a model-predicted

numerical solution rather than an independently confirmed treatment condition.

The selected high-temperature formulations evaluated for sensory acceptability and microbiological quality are listed in Table 2. These formulations represented the observed high-temperature treatments nearest to the predicted optimum region.

Table 2. Selected high-temperature formulations evaluated for sensory and microbiological quality

Formulation	Temperature (°C)	Time (h)
GT10	75	2.00
GT11	75	3.68
GT12	75	5.42

Sensory acceptability was evaluated using complete responses from 35 analyzable raters on a 9-point hedonic scale (Heymann & Ebeler, 2016; Yang & Lee, 2019). Microbiological assessment for the selected formulations included aerobic plate count, coliform, *E. coli*, *Salmonella*, and yeast and mold count.

2.5. Statistical Treatment of Data

Data were summarized as mean $\pm$ standard deviation. Formulation effects on proximate composition, TPC, and DPPH radical-scavenging activity were evaluated using one-way analysis of variance followed by Tukey HSD post-hoc comparison at $\alpha = 0.05$. ANOVA assumptions were assessed using residual diagnostics, the Shapiro-Wilk test for residual normality, and the Brown-Forsythe test for homogeneity of variance. Variance homogeneity was supported for all analyzed responses, while a modest departure from residual normality was observed for TPC. NFE was treated as a derived descriptive variable because it was calculated by difference rather than measured as an independent analytical response.

Response surface models were fitted using coded factors for temperature (A) and time (B), where $A = (\text{Temperature} - 65)/10$ and $B = (\text{Time} - 4)/2$. A quadratic model was fitted for TPC,

while a two-factor interaction model was fitted for DPPH inhibition. Model adequacy was evaluated using R^2 , adjusted R^2 , predicted R^2 , RMSE, lack-of-fit testing, observed-versus-predicted plots, and residual diagnostics. Numerical optimization was performed by simultaneously maximizing predicted TPC and DPPH inhibition within the experimental ranges of 55-75°C and 2-6 h. Individual response desirabilities were combined into an overall desirability score ranging from 0 to 1, with higher values indicating better simultaneous attainment of the two response goals. Candidate conditions were ranked according to overall desirability.

3. Results and Discussion

Results are presented by proximate composition, bioactive response, response surface modeling, numerical optimization, sensory acceptability, and microbiological quality.

Proximate Composition and Bioactive Responses

The proximate composition of the dehydrated guyabano leaf tea formulations is summarized in Table 3. This table reports the measured composition by formulation and separates NFE as a calculated descriptive value.

Table 3. Proximate composition of guyabano leaf tea formulations

Formulation	Moisture (%)	Ash (%)	Crude Fat (%)	Protein (%)	Fiber (%)	NFE (%)
GT01	1.10 ± 0.08	8.39 ± 0.17	8.53 ± 0.72	32.96 ± 0.73	21.65 ± 2.11	28.47 ± 0.00
GT02	3.02 ± 0.01	7.86 ± 0.24	11.05 ± 0.02	37.05 ± 0.33	37.36 ± 0.89	6.68 ± 0.00
GT03	1.09 ± 0.05	7.92 ± 0.28	7.70 ± 0.13	37.17 ± 3.32	25.53 ± 2.33	21.67 ± 0.00
GT04	1.26 ± 0.09	8.16 ± 0.16	9.33 ± 0.31	34.56 ± 2.14	19.94 ± 1.57	28.00 ± 0.00
GT05	0.76 ± 0.08	7.06 ± 0.11	7.63 ± 0.21	39.55 ± 0.25	23.09 ± 3.20	22.66 ± 0.00
GT06	0.68 ± 0.06	7.10 ± 0.20	7.82 ± 0.20	36.14 ± 1.37	21.51 ± 1.41	27.43 ± 0.00
GT07	1.04 ± 0.07	8.24 ± 0.08	8.88 ± 0.46	34.87 ± 2.38	18.22 ± 1.83	29.79 ± 0.00
GT08	1.84 ± 0.05	7.89 ± 0.51	8.83 ± 0.47	37.15 ± 1.76	20.36 ± 1.05	25.77 ± 0.00
GT09	2.15 ± 0.13	8.19 ± 0.28	10.01 ± 0.23	30.91 ± 1.16	17.70 ± 0.52	33.18 ± 0.00
GT10	1.20 ± 0.02	7.05 ± 0.34	8.87 ± 0.53	36.47 ± 1.57	30.38 ± 2.93	17.23 ± 0.00
GT11	1.78 ± 0.06	7.44 ± 0.74	8.92 ± 0.37	37.72 ± 0.77	19.74 ± 1.05	26.19 ± 0.00
GT12	1.40 ± 0.06	7.83 ± 0.10	8.98 ± 0.10	38.48 ± 1.65	25.79 ± 2.10	18.92 ± 0.00

Note. Values are mean ± SD from three independent processing batches (n = 3). NFE was calculated by difference and was analyzed descriptively.

The bioactive responses for TPC and DPPH inhibition are presented in Table 4. The table also shows Tukey groupings so that numerical

differences can be distinguished from statistically supported differences.

Table 4. Bioactive-response values with Tukey groupings

Formulation	TPC (mg GAE/100 g dried sample)	TPC Tukey Group	DPPH (% Inhibition)	DPPH Tukey Group
GT01	81.69 ± 2.13	c	41.43 ± 1.32	e
GT02	78.16 ± 1.29	c	37.07 ± 1.35	f
GT03	79.46 ± 2.17	c	35.50 ± 0.09	f
GT04	98.89 ± 4.08	b	69.51 ± 1.10	a
GT05	106.26 ± 6.64	b	45.49 ± 0.58	d
GT06	108.19 ± 6.59	b	59.43 ± 0.45	b
GT07	107.12 ± 1.63	b	55.75 ± 1.12	c
GT08	102.39 ± 0.70	b	47.69 ± 2.32	d
GT09	105.06 ± 5.16	b	56.29 ± 1.21	bc
GT10	116.69 ± 1.45	a	53.97 ± 0.63	c
GT11	114.62 ± 1.19	a	68.12 ± 0.49	a
GT12	119.39 ± 2.52	a	56.61 ± 1.21	bc

Note. Values are mean ± SD from three independent processing batches (n = 3). Within each response, formulations sharing the same Tukey letter are not significantly different at $\alpha = 0.05$.

The inferential summary for formulation effects is provided in Table 5. Table 5 summarizes formulation effects and indicates

which responses were eligible for inferential comparison.

Table 5. ANOVA and post-hoc summary

Response	df	F-value	p-value	Interpretation Boundary
Moisture	11, 24	274.69	< 0.0001	Formulations differed significantly; report results as mean $\pm$ SD.
Ash	11, 24	6.66	0.0001	Formulations differed significantly; interpret as a quality and mineral-content indicator.
Crude Fat	11, 24	21.10	< 0.0001	Formulations differed significantly; avoid unsupported claims regarding storage stability.
Protein	11, 24	6.16	0.0001	Formulations differed significantly; protein functionality was not directly measured.
Fiber	11, 24	26.26	< 0.0001	Formulations differed significantly; discussion should be limited to compositional differences.
NFE	Not valid	Not valid	Not valid	Derived value with zero within-formulation standard deviation; ANOVA could not be validly performed.
TPC	11, 24	47.93	< 0.0001	Significant differences were observed; GT12, GT10, and GT11 belonged to the highest Tukey group.
DPPH	11, 24	284.08	< 0.0001	Significant differences were observed; GT04 and GT11 belonged to the highest Tukey group, whereas GT12 was not among the strongest DPPH-performing formulations.

Note. Statistical significance was assessed at $\alpha = 0.05$. NFE was excluded from inferential analysis because it was calculated by difference.

The final moisture content of the dried samples ranged from $0.68 \pm 0.06\%$ in GT06 to $3.02 \pm 0.01\%$ in GT02, indicating low residual moisture across all formulations (ASEAN, 2014; Liu et al., 2022). Ash, crude fat, protein, and fiber also differed among formulations, but these compositional differences should be interpreted descriptively because the study did not directly measure mineral speciation, protein functionality, or shelf-life stability.

The bioactive-response table corrects the previous omission of GT08 TPC and DPPH values and applies consistent units. GT12 had the highest numerical TPC (119.39 ± 2.52 mg GAE/100 g dried sample) and shared the top Tukey group with GT10 and GT11. DPPH inhibition showed a different pattern: GT04 and GT11 shared the highest Tukey group, whereas GT12 showed moderate DPPH activity ($56.61 \pm 1.21\%$). The divergence between TPC and DPPH responses indicates that antioxidant behavior cannot be inferred from total phenolic

concentration alone. DPPH inhibition depends on the chemical structure of reducing compounds, phenolic transformation during radical neutralization, extractability, and possible interactions with non-phenolic antioxidants (Olszowy-Tomeczyk & Typek, 2023; Platzer et al., 2022; Zeb, 2020). Therefore, GT12 is best described as a high-TPC and multi-response desirability candidate, not as the strongest DPPH formulation.

NFE was treated as a derived descriptive variable because it was calculated by difference and showed no within-formulation variation. Consequently, NFE was excluded from inferential comparison.

Response Surface Modeling and Model-Predicted Optimization

The ANOVA for the fitted quadratic TPC response surface model is shown in Table 6. The model terms provide the statistical basis for interpreting the effects of temperature, time, interaction, and curvature on phenolic retention.

Table 6. ANOVA for the quadratic TPC response surface model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	2213.79	5	442.76	51.35	< 0.0001
A – Temperature	1721.83	1	1721.83	199.70	< 0.0001
B – Time	0.4846	1	0.4846	0.0562	0.8205
AB	61.70	1	61.70	7.16	0.0368
A ²	67.14	1	67.14	7.79	0.0316
B ²	114.77	1	114.77	13.31	0.0107
Residual	51.73	6	8.62	—	—
Lack of Fit	34.21	3	11.40	1.95	0.2983
Pure Error	17.52	3	5.84	—	—
Cor Total	2265.53	11	—	—	—

Note. Model terms with $p < 0.05$ were considered statistically significant.

For TPC, the quadratic model was statistically significant ($F = 51.35$, $p < 0.0001$), with significant temperature, temperature-time interaction, and curvature terms. The non-significant lack-of-fit result ($p = 0.2983$) supports the model's adequacy within the tested design region.

The DPPH response was fitted using a two-factor interaction model, and the corresponding ANOVA is shown in Table 7. This table should be read together with the weaker predicted R^2 reported in Table 8.

Table 7. ANOVA for the two-factor interaction DPPH response surface model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1061.02	3	353.67	9.91	0.0045
A – Temperature	188.26	1	188.26	5.28	0.0507
B – Time	273.78	1	273.78	7.67	0.0243
AB	263.52	1	263.52	7.39	0.0263
Residual	285.39	8	35.67	—	—
Lack of Fit	229.48	5	45.90	2.46	0.2444
Pure Error	55.91	3	18.64	—	—
Cor Total	1346.41	11	—	—	—

Note. Model terms with $p < 0.05$ were considered statistically significant.

Table 8. Coded response surface equations and model diagnostics

Model	Coded Equation	R ²	Adjusted R ²	Predicted R ²	RMSE	Interpretation
TPC Quadratic	TPC = 102.347 + 16.939A + 0.316B + 4.827AB – 5.465A ² + 7.021B ²	0.977	0.958	0.856	2.94 mg GAE/100 g	Strong model fit with good predictive performance within the experimental design region. Statistically significant model, but limited predictive capability; results should be interpreted with caution.
DPPH 2FI	DPPH = 53.572 + 5.563A – 7.265B + 9.522AB	0.788	0.709	0.325	5.97% inhibition	

The DPPH model was statistically significant ($F = 9.91$, $p = 0.0045$), with significant time and temperature-time interaction effects. However, the model's predicted R^2 was weak, so DPPH predictions should be interpreted more cautiously than TPC predictions.

The coded model equations and diagnostic indices for both response surface models are consolidated in Table 8. This table provides the main basis for comparing the stronger TPC model with the less predictive DPPH model.

The TPC response was well described by a quadratic model, with $R^2 = 0.977$, adjusted $R^2 = 0.958$, predicted $R^2 = 0.856$, and $RMSE = 2.94$ mg GAE/100 g dried sample. In contrast, the DPPH model was statistically significant but less reliable for prediction, with $R^2 = 0.788$, adjusted $R^2 = 0.709$, predicted $R^2 = 0.325$, and $RMSE = 5.97\%$ inhibition. These results indicate that the DPPH model is useful for describing observed trends within the design region but should be used cautiously for

prediction outside the observed experimental support.

Figure 1 shows the response-surface contour plots for TPC, DPPH inhibition, and overall desirability. The TPC surface increases toward the high-temperature, longer-time region of the tested design space, whereas the DPPH surface is less uniform because DPPH inhibition was affected by the temperature-time interaction. The desirability surface therefore identifies 75 degrees C for 6 h as the best model-predicted compromise within the studied range. The response-surface pattern is consistent with the dual role of heating in plant matrices. Thermal treatment can improve release of bound or compartmentalized phenolics, but excessive exposure may accelerate oxidation and degradation of thermolabile compounds, causing nonlinear or interaction effects between temperature and time (Antony & Farid, 2022; Duan et al., 2021; ElGamal et al., 2023; Taokaenchan et al., 2021).

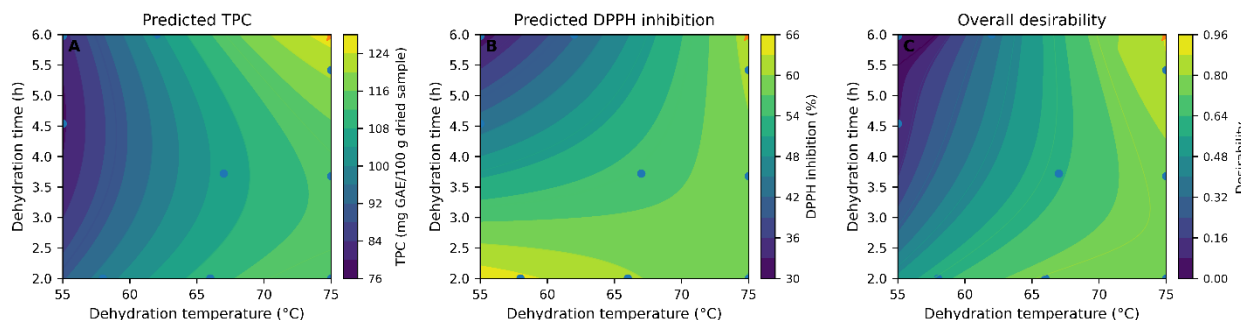


Figure 1. Response-surface contour plots for (A) total phenolic content, (B) DPPH inhibition, and (C) overall desirability. Points represent the I-optimal design runs; the marked boundary point indicates the model-predicted optimum at 75°C for 6 h.

Model agreement between observed and predicted responses is shown in Figure 2. Figure

2 compares observed and predicted values for the TPC and DPPH response mode

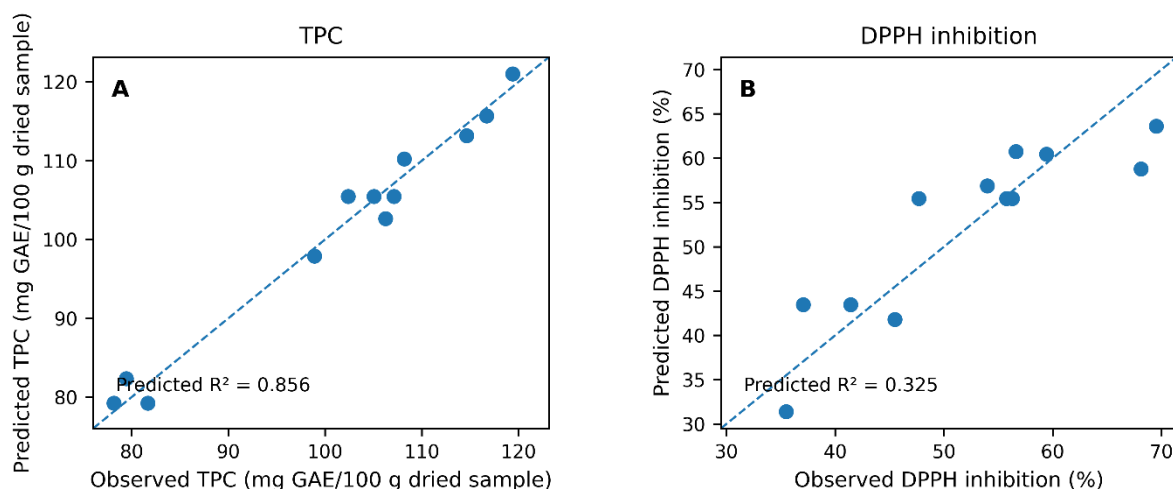


Figure 2. Observed-versus-predicted plots for (A) total phenolic content and (B) DPPH inhibition. The diagonal line represents perfect agreement between observed and predicted values.

The observed-versus-predicted plots support the different levels of confidence assigned to the two response models. TPC observations clustered more closely around the diagonal reference line, consistent with the strong predicted R^2 . DPPH observations showed greater dispersion, which supports the cautious interpretation of DPPH predictions and

reinforces the need for independent confirmation of the predicted optimum.

The numerical desirability solutions generated from the fitted models are listed in Table 9. These solutions rank candidate temperature-time combinations by combined desirability for TPC and DPPH responses.

Table 9. Model-predicted numerical solutions from multi-response desirability optimization

Solution	Temperature (°C)	Time (h)	Predicted TPC (mg GAE/100 g dried sample)	Predicted DPPH (% Inhibition)	Desirability
1	75.0	6.0	125.99	61.39	0.886
2	75.0	5.8	124.54	61.22	0.883
3	75.0	5.7	123.44	61.08	0.881
4	74.7	6.0	125.70	60.99	0.880
5	75.0	5.2	119.73	60.54	0.873
6	71.4	2.0	114.59	58.28	0.781
7	71.4	2.0	114.55	58.31	0.781
8	71.6	2.0	114.65	58.23	0.781
9	71.3	2.0	114.50	58.36	0.781
10	72.0	2.0	114.85	58.07	0.781
11	74.9	2.0	115.69	56.92	0.773

The numerical optimization identified 75 degrees C for 6 h as the highest-desirability condition within the tested design region, with predicted TPC of 125.99 mg GAE/100 g dried sample and predicted DPPH inhibition of 61.39%. This condition is interpreted as a

model-predicted optimum requiring experimental confirmation. The weaker predictive performance of the DPPH model further supports cautious interpretation of the numerical optimum.

The difference between TPC and DPPH responses indicates that total phenolic concentration alone did not determine radical-scavenging performance (Dobrinás et al., 2021; Olszowy-Tomczyk & Typek, 2023; Platzer et al., 2022). Figure 3 shows the formulation-level relationship between mean TPC and mean DPPH inhibition. The moderate positive correlation (Pearson $r = 0.716$, $p = 0.0088$) supports the interpretation that phenolic content contributes to radical-scavenging activity but

does not fully determine it, explaining about 51% of the formulation-level variation in DPPH. This pattern suggests that dehydration affected not only the amount of Folin-reactive phenolics but also phenolic composition, extractability, oxidation state, and possible interactions with non-phenolic antioxidants. The result also explains why GT12 had the highest observed TPC but did not show the highest observed DPPH inhibition.

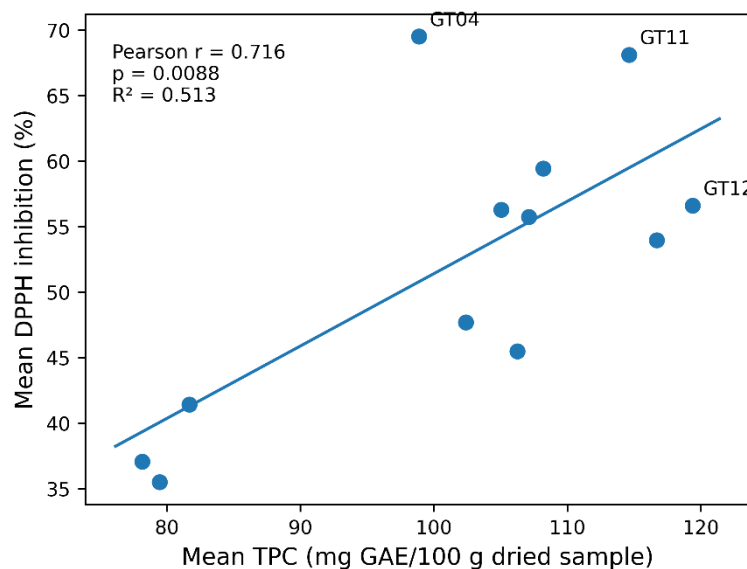


Figure 3. Relationship between formulation-level mean total phenolic content and DPPH inhibition (Pearson $r = 0.716$, $p = 0.0088$; $R^2 = 0.513$).

Sensory Acceptability and Microbiological Quality

The sensory mixed-model results for the selected high-temperature formulations are summarized in Table 10. This table reports consumer acceptability scores and the omnibus formulation effect for each sensory attribute. GT12 had the highest numerical flavor and overall acceptability scores, but no Holm-adjusted pairwise contrast remained significant. The sensory results therefore support comparable acceptability among GT10-GT12, with GT12 numerically favorable for overall acceptability rather than statistically preferred.

The sensory findings should be interpreted as consumer acceptability data rather than trained descriptive profiling. The absence of significant adjusted pairwise contrasts indicates that GT10-GT12 were broadly comparable in liking, while individual preference variation may explain why numerical differences did not translate into robust pairwise significance (Feeney et al., 2024; Heymann & Ebeler, 2016; Yang & Lee, 2019).

The microbiological quality results for the selected tested formulations are shown in Table 11. It reports the microbiological status of GT10-GT12 under the assays performed.

Table 10. Sensory mixed-model summary for selected high-temperature formulations (n = 35 analyzable raters)

Attribute	GT10 (Mean ± SD)	GT11 (Mean ± SD)	GT12 (Mean ± SD)	Wald χ^2	p-value	Interpretation
Color	6.34 ± 1.63	7.03 ± 1.12	6.86 ± 1.14	8.002	0.0183	Significant omnibus formulation effect; however, no Holm-adjusted pairwise comparison was significant.
Aroma	6.31 ± 1.32	6.74 ± 1.22	6.66 ± 1.31	4.732	0.0939	No significant differences among formulations.
Taste	5.74 ± 1.72	6.06 ± 1.41	6.03 ± 1.01	1.911	0.3845	No significant differences among formulations.
Flavor	5.77 ± 1.73	6.00 ± 1.39	6.06 ± 1.28	1.546	0.4618	No significant differences among formulations.
Overall Acceptability	6.00 ± 1.55	6.40 ± 1.38	6.43 ± 1.04	6.031	0.0490	Borderline significant omnibus effect; however, no Holm-adjusted pairwise comparison was significant.

Table 11. Microbiological quality results for selected tested formulations

Formulation	Aerobic Plate Count (CFU/g)	Coliform (MPN/g)	<i>E. coli</i> (MPN/g)	<i>Salmonella</i>	Yeast and Mold Count (CFU/g)
GT10	30,000	79	< 1.8	Absent	< 10
GT11	96,000	240	< 1.8	Absent	10
GT12	62,000	240	< 1.8	Absent	20

The selected formulations tested for microbiological quality (GT10-GT12) met the referenced microbiological criteria under the assays performed (Tea & Herbal Infusions Europe [THIE], 2021). This interpretation applies only to GT10-GT12. The low final moisture values are also consistent with the general principle that moisture reduction supports microbial-risk control in low-moisture foods, although shelf-life testing remains necessary for storage claims (Liu et al., 2022).

4. Conclusion

Within the tested forced-air dehydration range of 55-75 degrees C and 2-6 h, temperature and time significantly influenced the physicochemical and bioactive properties of guyabano leaf tea. GT12 showed the highest observed TPC and acceptable DPPH radical-scavenging activity, while GT04 and GT11 showed stronger observed DPPH inhibition. The multi-response desirability analysis identified 75 degrees C for 6 h as the best model-predicted condition within the design space, but this optimum should not be treated as experimentally

validated until independent confirmation runs are completed.

Sensory evaluation showed that GT10-GT12 were generally acceptable, with GT12 numerically highest in overall acceptability, although adjusted pairwise differences were not significant. Microbiological findings apply only to the selected tested formulations. Future work should validate the predicted optimum using independent processing batches and assess storage stability and phenolic-profile changes during shelf life.

5. Study Limitations and Recommendations

The model-predicted optimum of 75°C for 6 h was not independently validated, and the DPPH model showed lower predictive performance than the TPC model. Sensory and microbiological assessments were limited to GT10-GT12, and sensory evaluation focused on hedonic acceptability rather than descriptive profiling. Individual phenolic compounds and changes during storage were also not characterized.

Future studies should confirm the predicted optimum using independent processing batches, evaluate shelf-life stability, and characterize phenolic-profile changes associated with dehydration and storage. Quantitative descriptive analysis may also be used to extend the sensory characterization of guyabano leaf tea.

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Conflict of interest:

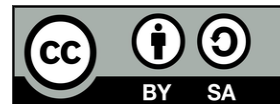
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The study received ethics review clearance from the PUP Research Ethics Committee through the College/Campus Research Ethics Committee under PHREB requirements, with UREC Code CL-F-2026-033. Sensory evaluation was conducted with informed consent from all participating panelists.



Research article

DEVELOPMENT OF SYNBIOTIC MARSHMALLOW USING INDIGENOUS PROBIOTIC *LACTOBACILLUS PLANTARUM* DAD-13 AND KONJAC GLUCOMANNAN

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Abstract

A synbiotic food is a dietary product that combines probiotic and prebiotic properties. People prefer healthy food and snacks that offer health benefits. A synbiotic marshmallow incorporating *L. plantarum* Dad-13 and konjac glucomannan has been developed, yet sensory acceptance and characterization have not been conducted. This study aimed to examine consumer acceptance and the effect of konjac glucomannan substitution on the characteristics and sensory acceptance of synbiotic marshmallows. A consumer survey was conducted to evaluate consumers' knowledge of probiotics, prebiotics, and synbiotics and their intention to buy the product. Synbiotic marshmallows were produced by combining a sugar solution (sucrose and glucose) with gelatin and konjac glucomannan in certain concentration ratios of P1 (9%:0%), P2 (8.5%:0.5%), P3 (8%:1%), P4 (7.5%:1.5%), and P5 (7%:2%) (w/v), followed by the addition of probiotic powder *L. plantarum* Dad-13. The experiment was designed using a Completely Randomized Design, and the non-parametric data were analyzed using Kruskal-Wallis followed by the Mann-Whitney U Test. Sensory evaluation, including Just-About-Right (JAR) and penalty analyses, and the viability of *L. plantarum* Dad-13, were also evaluated. Survey results indicated that consumers had knowledge of the health benefits of probiotics, prebiotics, and synbiotics. The substitution of gelatin and konjac glucomannan at 8.5%:0.5% was the most preferred formula for all sensory attributes (color, aroma, texture, and taste). The addition of konjac glucomannan did not affect the viability, which remained at 6.33 ± 0.07 log CFU/g. However, JAR and penalty analyses indicated that the sweetness intensity of P2 required adjustment to improve consumer acceptance.

1. Introduction

People who follow a modern diet prefer foods high in fat and low in fiber. This condition can alter the average balance of the microflora in the large intestine, leading to various

disorders. Consuming probiotics is one strategy to combat the disruption of the natural microbiota of the large intestine (Zhang et al., 2019). Probiotics have physiological effects, including antihypertensive, anticholesterol,

anticarcinogenic, anti-lactose intolerance, protection against digestive tract diseases, and allergy prevention (Kechagia et al., 2013). Most probiotic strains are lactic acid bacteria from *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Streptococcus* (Ringø et al., 2020). Probiotics reduce harmful bacteria by producing antimicrobial components and vying for resources and colonization sites (Chen et al., 2020a). Fermented foods, such as yogurt, kefir, and tape (fermented cassava), are well-known probiotic foods, particularly in Indonesia. The demand for fermented products has increased because consumers believe that only fermented foods can offer health benefits. To enhance diversity and creativity in probiotic foods, products that contain probiotics without requiring fermentation must be developed; however, this remains challenging.

Variations of popular products are common in nibbles, which are often consumed as snacks. Gummy probiotic *L. plantarum* Dad-13 has been developed and can be used as a carrier for undernourished children's treatment (Kamil et al., 2021, 2022). Further studies have investigated how confectionery items, such as chocolate (Rahayu et al., 2024) and jelly candy (Kahraman et al., 2023), can be used to deliver probiotics. In addition, marshmallow is a popular snack among the general population and are soft confections shaped like a light, soft, and chewy foam and come in various flavors, smells, and colors. Previous research has demonstrated the potential of marshmallows as a carrier matrix for probiotic bacteria, including *Lactobacillus rhamnosus* (Colmenares-Cuevas et al., 2024). Marshmallows are manufactured with gelatin, which can form gels and foams. Gelatin is a derivative protein from bone and skin collagen hydrolysis with gelling, stabilizing, emulsifying, thickening, and foaming properties (Mardani et al., 2019). To improve the texture of marshmallows, gelling agents can be added, including konjac glucomannan. Konjac glucomannan is a polysaccharide consisting of D-mannose and D-glucose at a ratio of 1.6:1 and β (1-4) linkages and can be used as a gelling agent and dietary fiber with reported prebiotic potential (Al-Ghazzewi & Tester, 2012).

Therefore, the concentrations of gelatin and konjac glucomannan influence marshmallow quality.

Probiotic products must have a desirable and viable probiotic cell. The efficacy dose of probiotic food is in the range of 6 to 9 log CFU/g or /mL (Barbosa et al., 2015). In addition, processing can affect bacterial cell viability (Tripathi & Giri, 2014). The combination of probiotics and prebiotics is defined as synbiotics, and in this research, *L. plantarum* Dad-13 and konjac glucomannan are used to develop synbiotic marshmallows. According to Indriyanti et al. (2015), a synbiotic product contains probiotics as living microorganisms and their substrates, namely, prebiotics, to promote the growth of probiotic bacteria. *L. plantarum* Dad-13 was utilized in this research because it is a local probiotic bacterium isolated from spontaneously fermented buffalo milk.

A marshmallow formulation incorporating konjac glucomannan and the probiotic *L. plantarum* Dad-13 has been successfully developed (Bintoro et al., 2024). The optimal composition consists of 8.5% gelatin and 0.5% konjac glucomannan. However, an evaluation of consumer acceptance for this product has yet to be conducted. Although konjac glucomannan is recognized for its potential prebiotic properties and its ability to act as a synbiotic when combined with probiotics, this evidence does not universally confirm its prebiotic functionality in all food formulations. Therefore, this research focuses on sensory evaluation relative to commercial marshmallows and the characterization of the optimal formula. Consumer acceptance surveys are necessary to determine whether the community can accept food products. This is because consumers are the focal point of the marketing process, and understanding their needs leads to appropriate and efficient product development (Montossi et al., 2013). Consumer expectations are believed to substantially influence product quality and satisfaction (Chen et al., 2020b). Therefore, this study aimed to evaluate the effect of two gelling agent combinations, gelatin and konjac glucomannan, on the characteristics of synbiotic marshmallows and their sensory properties.

2. Material and Methods

2.1. Material

The bacterial strain used in this study was a skim milk powder containing the probiotic *L. plantarum* Dad-13, which was supplied by the Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia, and was produced following the method of Kamil et al. (2020). The production of skim milk powder containing *L. plantarum* Dad-13 involved mixing the *L. plantarum* Dad-13 pellet with 10% (w/v) skim milk and 1% (w/v) sucrose. The resulting suspension was frozen at -40°C for 24 hours, then freeze-dried for 72 hours. The initial viable cell count of the probiotic powder was 3×10^9 CFU/g, and the skim milk powder was stored at refrigerated temperature (4°C) before use. The ingredients for marshmallow production were as follows: bovine gelatin (Hakiki, Indonesia), konjac glucomannan (El Masta, Indonesia), sucrose (Gulaku, Lampung, Indonesia), glucose (Fortune, Indonesia). In addition, de Man, Rogosa, and Sharpe (MRS) agar (Merck, Germany) was used for lactic acid bacteria enumeration.

2.2. Methods

2.2.1. Synbiotic marshmallow production

Synbiotic marshmallows were prepared following the method of Bintoro et al. (2024). Bovine gelatin (Hakiki) and konjac glucomannan (El Masta) were soaked in 20 mL of warm water (35°C) for 15 minutes for each treatment: P1: 9:0 % w/v, P2: 8.5:0.5 % w/v, P3: 8:1 % w/v, P4: 7.5:1.5 % w/v, and P5: 7:2 % w/v. A sugar solution was prepared by boiling (105°C–115°C) 150 g sucrose (Gulaku), 45 g glucose (Fortune), and 45 mL water until they completely dissolved. The soaked gelatin and konjac glucomannan were added to the sugar solution and whipped at high speed with a mixer (Philips, China) until foaming was observed. Fifteen-gram skim milk powder containing *L. plantarum* Dad-13 was added to the mixture at 40°C, then slowly whipped until evenly incorporated. The mixture was then molded, left to cool at room temperature ($\pm 25^\circ\text{C}$) for 1 hour, and stored in the chiller (4°C) for 24 hours. The marshmallow was then cut and packed in an

aluminum-sealed pouch and stored until further use for analysis. The production was conducted 3 batches for each formula.

2.2.2. Consumer survey of synbiotic knowledge

A consumer survey was conducted following the method of Kamil et al. (2021). This survey aimed to determine consumers' knowledge regarding the health benefits of synbiotic products and consumers' intention to purchase synbiotic marshmallows. The survey was conducted using an online form targeting teenage to adult consumers (19–60 years old), as they represent the target consumer group. Before participating in the survey, respondents were informed that their personal data would be collected solely for research purposes, treated confidentially, and not disclosed to third parties. Participation was voluntary, and respondents provided their consent before completing the questionnaire. Each correct answer was scored 1, and each wrong answer was scored 0. The obtained score was then classified as good (>75% correct answers), fair (50%–75% correct answers), and low (<50% correct answers). Consumers' intention to buy the product was assessed using yes/no questions.

2.2.3. Viability of *L. plantarum* Dad-13

The viability of *L. plantarum* Dad-13 was quantified by the pour plate method. In brief, 1 g of the sample was homogenized with 9 mL of 0.85% NaCl. At the appropriate dilution series, 1 mL of bacterial suspension was placed in a Petri dish and covered with de Man, Rogosa, and Sharpe (MRS) agar medium (Merck). NaCl solution and MRS medium were sterilized using an autoclave (Hiramaya, Japan) before use. Once the medium solidified, the inoculated Petri dish was incubated at 37°C for 48 hours using an incubator (Mermmert, Germany).

2.2.4. Sensory evaluation of synbiotic marshmallows

Sensory evaluation of synbiotic marshmallows was conducted at the sensory laboratories of the food technology department, Universitas Diponegoro, in accordance with ISO 8586:2012, which provides guidelines for the selection, training, and monitoring of assessors and expert sensory assessors. Participation in the sensory evaluation was voluntary. Prior to the

test, participants were informed about the study objectives and provided their consent. The product tasting was not conducted for medical intervention purposes, but solely for sensory evaluation to obtain product descriptions and assess consumer acceptance by the panelists. All collected data were treated confidentially and used only for research purposes. All the panelists involved in this research had no allergies to the synbiotic marshmallows ingredients, i.e., gelatin and skim milk. They also had no history of lactose intolerance.

2.2.5. Hedonic and preference tests of synbiotic marshmallows

Hedonic and preference tests were conducted with 50 panelists aged 20 and older to evaluate the sensory attributes of synbiotic marshmallows. A preference test was conducted to determine the best formula. In both tests, no control sample (no addition of konjac glucomannan) was used to evaluate the effect of konjac glucomannan addition on the panelists' preferences. This was because the study was primarily designed to focus on the sensory acceptance and perception of marshmallows formulated with konjac glucomannan as the gelling agent, rather than to compare them with a conventional formulation. In the hedonic test, the panelists were served freshly made synbiotic marshmallows, each with three random codes. The panelists were asked to evaluate their overall liking regarding the specific sensory attributes of synbiotic marshmallows, namely, color, taste, texture, and flavor. The hedonic scale consists of seven levels: dislike very much (1), dislike (2), slightly dislike (3), neither like nor dislike (4), slightly like (5), like (6), and like very much (7). The hedonic data were represented as mean $\pm$ SD and categorized into three different groups following the study of Lim et al. (2022) to determine the panelists' liking: T3B liking (Top 3 Box Rating), B3B liking (Bottom 3 Box Rating), and neutral. The preference was determined by calculating the percentage of panelists who selected the formula.

2.2.6. Descriptive and penalty analysis of synbiotic marshmallows

A descriptive test was performed to evaluate the sensory profile of the selected synbiotic marshmallow formula. Penalty analysis was also conducted to determine specific sensory attributes that must be improved and to verify which sensory attributes are essential for product acceptability. In both tests, control (P1) and commercial marshmallow (Corniche) were used for comparison. The observed sensory profile reflected the sensory attributes the panelists expected from the products, based on the discussion. Twenty panelists were briefed before the actual evaluation and informed of the definition of evaluated sensory attributes for each sample, namely, appearance (balanced proportions, softness, glossiness, and brightness), texture (hardness, chewiness, gumminess, porosity, and sandy), flavor (sweet, vanilla, and foreign), and taste (sweet, milky, sour, fermented, and foreign). All the panelists had experience and were familiar with sensory evaluation. A five-point just-about-right (JAR) scale was used, following Lim et al. (2022), with 3 representing just right, 1 the lowest intensity, and 5 the highest. For example, for foreign taste, the JAR scale was as follows: 1, too weak; 2, slightly weak; 3, just right; 4, slightly strong; and 5, too strong. The JAR data were further analyzed using penalty analysis.

2.2.7. Proximate analysis of synbiotic marshmallows

Proximate analysis was performed on the most preferred formula and followed the method specified in the Indonesian Standard for Chewy Candy (SNI 3547.2-2008). The Gross energy of synbiotic marshmallows was estimated using Atwater's conversion factors.

2.2.8. Texture analysis of synbiotic marshmallows

Texture analysis was performed for the most preferred formula using texture profile analysis (Micro-Vickers Hardness Testing Machines), with the conditions as follows: trigger (2.0g), deformation (10 mm), and speed (1mm/s). The used probe was a cylindrical probe with dimensions (L: 20 mm, D: 38.1 mm).

2.2.9. Data analysis

Statistical analysis was conducted using IBM Statistics SPSS 26.0 for Windows. Analysis of non-parametric data was performed using Kruskal-Wallis followed by the Mann-Whitney U Test. Penalty analysis was performed using XLSTAT programs (version 2021.4; Addinsoft, Paris, France). Data were considered statistically different when the p-value <0.05.

3. Results and Discussion

3.1. Consumer behavior and synbiotic products knowledge

A total of 198 individuals (58.5% female and 41.5% male) with an average age of 21 years participated in this survey. The cumulative data from the survey indicated that most of the respondents (90.50%) had good knowledge regarding the health benefits of prebiotics, probiotics, and synbiotic, who received information from the Internet (56%), followed by television advertisements or social media (16.5%), product packaging (11%), books (7%), printed media (7%), and other resources (8.5%). According to Kamil et al. (2021), aggressive media information of the health benefits of probiotic consumption is affecting consumers' knowledge and awareness of a healthy lifestyle. Consequently, the trend in consuming probiotics, prebiotics, and synbiotics has expanded significantly. Lerner et al. (2019) stated that the probiotic market is still rising and will exceed 3 billion US dollars by 2024.

The questionnaire also asked respondents why they consume probiotics, prebiotics, and synbiotics. The results showed that 68.5% of respondents cited health benefits, and 22.5% cited good taste. Probiotics, prebiotics, and synbiotics are usually fermented products in the form of desserts and snacks (i.e., yogurt, kefir, and fermented cassava). The survey on snack consumption frequency indicated that 39.5% of respondents answered "frequently" (more than once a week), 27.5% answered "once a week," and 24% answered "rarely." By a margin of 33.5%, people preferred chips over other types of snacks. Confectionary products came in second (27%), followed by cake and bread (17.5%), biscuits (12.5%), and other types of

snacks (5%). This result indicated a promising future for the development of confectionery goods.

Furthermore, the intention to buy symbiotic marshmallows was high, with 86% of the 200 respondents indicating they would. The high degree of knowledge and customer intention to buy drives the development and introduction of novel synbiotic products to the market in the form of marshmallows. Therefore, innovations of marshmallow synbiotics are challenging yet have high potential.

3.2. Hedonic and preference tests of synbiotic marshmallows

Table 1 lists the results of the hedonic and preference tests for synbiotic marshmallows. No significant differences in any of the evaluated sensory attributes were observed among the treatments. The combination of gelatin and konjac glucomannan at an 8.5%:0.5% (P2) ratio scored higher on T3B liking for color, texture, and taste attributes than all other treatments. However, the highest T3B score for flavor characteristics was achieved by the treatment with P5, a combination of gelatin and konjac glucomannan at a 7%:2% ratio. Despite the lack of significant differences among gelling agent ratios, P2 was the most-liked synbiotic marshmallow among the panelists, as indicated by the T3B results. This finding also aligned with P2 receiving the highest percentage of preference. According to Samant et al., (2017), consumer preference could be used to characterize product acceptance. Therefore, P2 treatment was selected for further sensory profiling.

Color is one of the factors that determine product acceptance. Overall, the synbiotic marshmallows were white, indicating that advanced caramelization did not occur during production. According to Kurt et al. (2022), the brownish color of the confectionery product is a result of carbohydrate degradation, particularly sugar, at high temperatures. Whipped gelatin gave the synbiotic marshmallow a white color, which indicates its excellent quality (Koli et al., 2012). The absence of differences in color attributes suggested that the distinguishing

gelling-agent ratio has minimal effect on color and therefore does not influence the panelists' preference scores. The aroma of marshmallows comes from boiling sugar, which creates a

distinct flavor and aroma. In this research, the same sugar and vanilla flavor were used for all treatments. Therefore, no significant difference in flavor and taste was observed.

Table 1. Results of hedonic and preference tests of synbiotic marshmallows

		P2	P3	P4	P5
Color	B3B (%)	2	2	2	4
	Neutral (%)	22	32	28	28
	T3B (%)	76	66	70	68
	Mean $\pm$ SD ^{ns}	5.40 $\pm$ 1.03	5.32 $\pm$ 1.08	5.34 $\pm$ 1.12	5.22 $\pm$ 1.04
Flavor	B3B (%)	16	16	14	16
	Neutral (%)	26	20	38	10
	T3B (%)	58	64	48	74
	Mean $\pm$ SD ^{ns}	4.84 $\pm$ 1.35	5.12 $\pm$ 1.45	4.62 $\pm$ 1.29	5.12 $\pm$ 1.22
Texture	B3B (%)	20	32	28	26
	Neutral (%)	22	14	24	30
	T3B (%)	58	54	48	44
	Mean $\pm$ SD ^{ns}	4.76 $\pm$ 1.38	4.52 $\pm$ 1.42	4.38 $\pm$ 1.58	4.30 $\pm$ 1.30
Taste	B3B (%)	8	16	18	12
	Neutral (%)	10	12	18	16
	T3B (%)	82	72	64	72
	Mean $\pm$ SD ^{ns}	5.42 $\pm$ 1.18	5.08 $\pm$ 1.34	4.80 $\pm$ 1.43	4.94 $\pm$ 1.30
Preference		36%	24%	24%	16%

Data presented as mean $\pm$ standard deviation.

^{ns} mean no significant difference, analyzed by Kruskal-Wallis

P2 (8.5% gelatin:0.5% konjac glucomannan). P3 (8% gelatine:1% konjac glucomannan). P4 (7.5% gelatine:1.5% konjac glucomannan). P5 (7% gelatine:2% konjac glucomannan).

Gelatin, a common ingredient in marshmallows, acts as a gelling and foaming agent and provides a chewy and non-sticky texture (Lin et al., 2017). Despite its gelling ability, konjac glucomannan can stabilize the gel, thereby affecting texture properties (Jian et al., 2016). This polysaccharide also has a high water-holding capacity. The combination of two gelling agents can modify their rheological properties. Research by Tomczyńska-Mleko et al. (2014) indicates that when using the combination of konjac and gelatin gel, increasing the konjac glucomannan concentration elevated the elastic moduli but

slightly reduced the fracture stress. In addition, the increment in konjac glucomannan does not change the mixture gel's thermal properties. Therefore, the melt-in-mouth texture of the mixture gel can be retained. This phenomenon may explain the lack of a significant difference in the texture of synbiotic marshmallows across all treatments. The other result revealed that the obtained gelatin and konjac glucomannan gels were separated. Gelatin appeared as the continuous phase, and konjac glucomannan acted as large filler domains (Tomczyńska-Mleko et al., 2014).

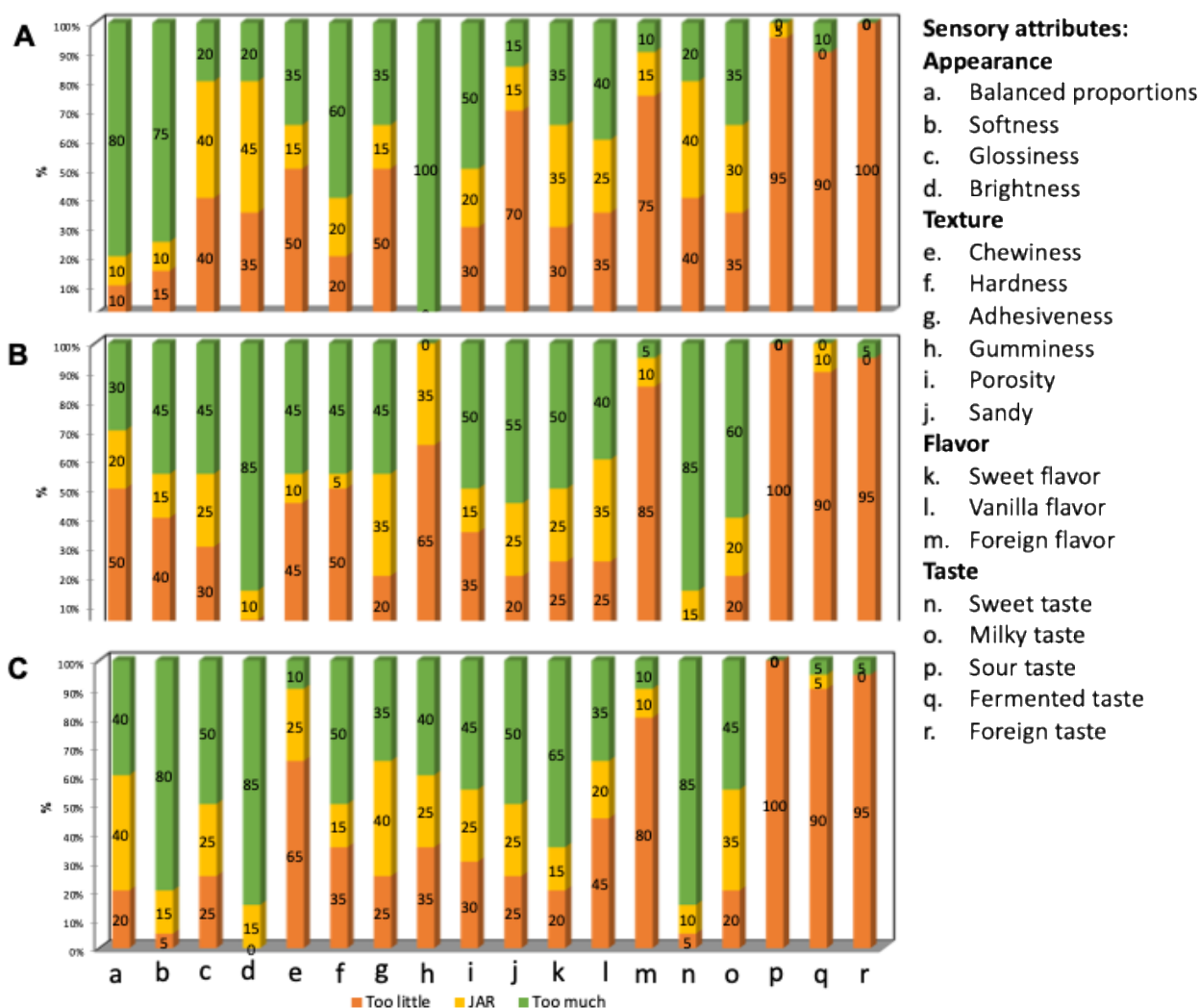


Figure 1. JAR score percentage.

A (Commercial marshmallows). B (P1: 9% gelatin:0% konjac glucomannan). C (P2: 8.5% gelatin:0.5% konjac glucomannan).

3.3. Sensory profile and penalty analysis of synbiotic marshmallows

The panelists' favorable sensory attributes cannot be determined from the hedonic test results; therefore, a descriptive test is required for precise elaboration. According to Narayanan et al. (2014), an analysis using two types of data, namely, consumer preference data and descriptive data, is required to understand consumers' acceptance. The JAR scores for sensory attributes are shown in Figure 1. The high JAR scores for commercial marshmallows were obtained for the attributes of brightness, hardness, porosity, sweet flavor, and sweet taste. Meanwhile, most panelists rated the control marshmallows (P1) at the JAR level for

glossiness, adhesiveness, gumminess, vanilla flavor, and milky taste. In addition, balanced proportions, adhesiveness, vanilla flavor, and milky taste received the highest JAR score for marshmallows using an 8.5%:0.5% gelatin and konjac glucomannan combination (P2). However, the sensory attributes of sour, fermented, and foreign tastes received low scores across all samples. This finding indicated that most panelists could not recognize these sensory attributes across all samples. No fermentation was performed in this study, and only microencapsulated probiotics were used. The sensory acceptance of fermented foods may decline due to the formation of organic acids, volatile sulfur compounds, biogenic amines,

aldehydes, ketones, alcohols, and ammonia. At elevated concentrations, these compounds can impart excessively sour, bitter, rancid, pungent, or otherwise unpleasant sensory characteristics

(Hu et al., 2025; McFeeters, 2004). Therefore, the specific sensory attributes of the fermented-like products were not recognized.

Table 2. Penalty analysis results of commercial marshmallows, control, and synbiotic marshmallows

Samples	Variable	Level	Selection%	Mean (Liking scores)	Mean drops	Weighted penalty
Commercial marshmallows	Sandy	Too little	70.00%	5.000	1.000	1.118
		JAR	15.00%	6.000		
		Too much	<i>15.00%</i>	4.333	1.667	
	Sweet flavor	Too little	30.00%	4.667	1.048	1.022
		JAR	35.00%	5.714		
		Too much	35.00%	4.714	1.000	
P1	Brightness	Too little	<i>5.00%</i>	3.000	3.000	2.444
		JAR	10.00%	6.000		
		Too much	85.00%	3.588	2.412	
	Chewiness	Too little	45.00%	3.778	1.222	1.333
		JAR	10.00%	5.000		
		Too much	45.00%	3.556	1.444	
	Sandy	Too little	<i>20.00%</i>	3.500	1.900	2.133
		JAR	25.00%	5.400		
		Too much	55.00%	3.182	2.218	
	Milky taste	Too little	<i>20.00%</i>	4.500	0.250	1.188
		JAR	20.00%	4.750		
		Too much	60.00%	3.250	1.500	
P2	Sweet taste	Too little	<i>5.00%</i>	3.000	2.500	1.167
		JAR	10.00%	5.500		
		Too much	85.00%	4.412	1.088	

The weighted penalty and mean drop data below 1 are not shown. The complete results of the penalty analysis are available in the supplementary data. Weighted penalty indicates the rank of the most impactful sensory attributes for improvement. Data in italics indicate the percentage selection below or equal to the threshold (20%). P1 (9% gelatin:0% konjac glucomannan), P2 (8.5% gelatin:0.5% konjac glucomannan).

The sensory attributes that significantly affect the synbiotic marshmallows' acceptability were determined by penalty analysis and calculated as a mean drop (Figure 2 and Table 2). The dashed line is a 20% threshold, commonly used in the industry (Lim et al., 2022). Therefore, the penalty analysis did not consider the sensory attributes below the threshold. A high x value indicates that most panelists assumed that these sensory attributes

were too high or too low. Sensory attributes that have either high y or x mapping (critical corner) must be considered. According to Ortega-Heras et al. (2019), attributes with a mean drop higher than 1 can affect a product's acceptability. The upper-right-hand corner mapping (mean drop higher than 1) indicated low acceptability of sensory attributes and therefore the need for improvement.

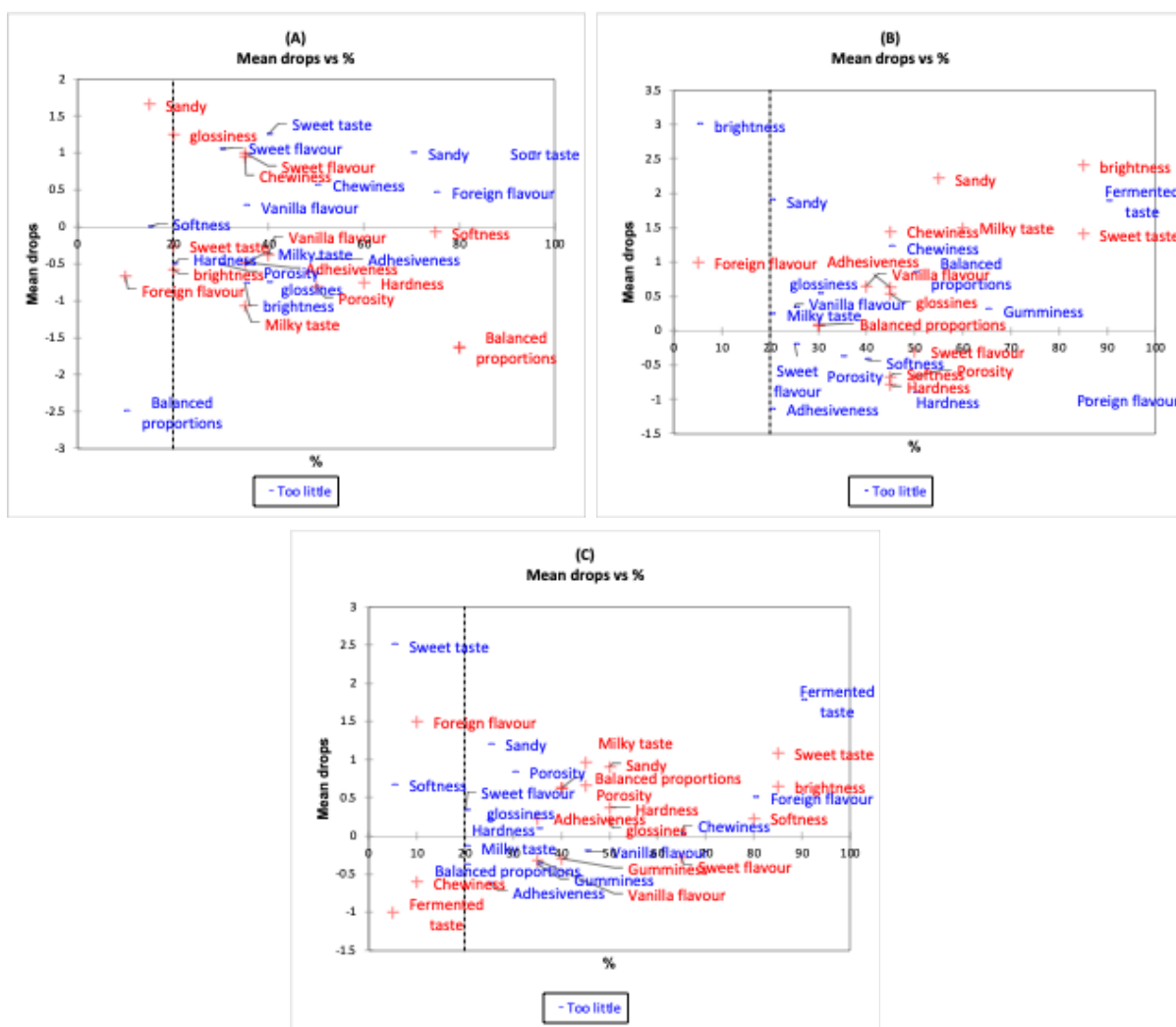


Figure 2. Mean drop plot.

A (Commercial marshmallows), B (P1: 9% gelatin:0% konjac glucomannan), and C (P2: 8.5% gelatin:0.5% konjac glucomannan). The dashed line represents 20% of the threshold.

According to the mean drop plot and penalty analysis, commercial marshmallows had two sensory attributes with penalty scores higher than 1: sandy and sweet flavor. However, only the mean drop value of “too little” for sweet flavor passed the threshold. Therefore, the most troublesome sensory attribute of commercial marshmallows was their sweet flavor. This attribute was rated “too little” by 30% of the panelists, with a mean drop value of 1.048 in the overall liking score. By contrast, for the control (P1), the following four sensory attributes required improvement: brightness, chewiness, sandy, and milky taste. Among these, brightness and sandy required the most improvement. Brightness was found to be “too much” with a mean drop value of 2.412. The sandy texture

was strongly penalized by the panelists as “too much” with a mean drop of 2.218. For chewiness, the panelists penalized it as “too much” with a mean drop of 1.444. Meanwhile, the milky taste was also penalized as “too much” with a mean drop of 1.500. The reformulation of the control by substituting 0.5% konjac glucomannan for gelatin still required some adjustments, primarily to the product's sweetness. Approximately 85% of the panelists considered the product (P2) as “too much” for sweet taste, with a mean drop value of 1.088.

Based on the mean drop and penalty analysis, the reformulation of marshmallows by substituting 0.5% konjac glucomannan improved the acceptance of “too much” brightness, chewiness, sandy, and milky taste.

Konjac glucomannan has low brightness on its own, and its addition may affect the product's brightness (Akesowan, 2016). In addition, using gelatin alone as a gelling agent produces a chewy texture, which can be modified by adding other gelling agents, such as carrageenan and konjac glucomannan, to improve texture, flexibility, and gel strength. Combining gelatin with konjac glucomannan reduces the product's hardness (Bintoro et al., 2024; Tomczyńska-Mleko et al., 2014). Chewiness is defined as the energy required to chew solid food until it is ready to be swallowed; this feature is calculated as a product of hardness, cohesiveness, and elasticity, as described by texture profile analysis. Therefore, a linear correlation exists between chewiness and hardness, such that a decrease in hardness is associated with a decrease in product chewiness (Smirnov et al., 2024; Vickers, 1982).

The sandy texture of the control sample results from sugar crystallization. Hence, high viscosity can delay crystallization by slowing the rate of mass transfer (Richard & Speck, 2015). Konjac glucomannan can retain water, thereby increasing the gel's viscosity (Tomczyńska-Mleko et al., 2014). Even though the reformulation of the control improved the acceptance of certain sensory attributes, the substitution of 0.5% konjac glucomannan still needs improvement regarding its "too much" sweet taste. This finding indicated that the use of sugar (sucrose and glucose) in the formulation must be further adjusted.

3.4. Synbiotic marshmallow characteristics

Table 3 shows the characteristics of the selected synbiotic marshmallow in terms of proximate composition, texture characteristics, and viable cells of *L. plantarum* Dad-13. In addition, the calorie intake from confectionery products should not be over 100 kcal/g.

Therefore, it can be assumed that the serving size of synbiotic marshmallows is 30 g/day (99.6 kcal/30g). The moisture and ash content of synbiotic marshmallows comply with the Indonesian Standard for chewy candy (SNI 3547.2-2008), with maximum limits of 20% and 3%, respectively. Gelatin, a main ingredient in marshmallows, can hold water. High moisture content affects the product's characteristics and shelf life. Since probiotics were incorporated into the marshmallows, the low moisture content is preferred to maintain their viability during storage.

Furthermore, the viability of *L. plantarum* Dad-13 is important to its efficacy, and marshmallow synbiotics meet the minimum viability required for probiotic products. However, there is a 3-log-cycle reduction in the number of viable cells relative to the initial level. The loss of probiotics' viability can occur during processing mainly due to high temperatures (Asaithambi et al., 2021). This finding is two log cycles lower than that reported for the viability of *L. plantarum* incorporated in gummy candy (Kamil et al., 2021). This is because during marshmallow production, high-speed mixing was used, which may increase the number of injured or dead cells. In this study, the microencapsulated probiotic was added to the mixture at 40°C to maintain bacterial viability. In addition, microencapsulation prevents the loss of probiotics' viability in harsh conditions. This technique extends the shelf-life of probiotics by protecting them with polysaccharides, proteins, and lipids (Kamil et al., 2020). Hence, further research is needed to assess the stability of *L. plantarum* Dad-13 viable cells during storage. Although it has no effect on probiotic viability, konjac glucomannan can still affect gut microbiota diversity and the probiotics inhabiting the colon.

Table 3. Characteristics of synbiotic marshmallows (P2: 8.5% gelatin:0.5% konjac glucomannan)

Proximate content	
Moisture (%)	16.69 ± 0.14
Ash (%)	0.50 ± 0.01
Crude fat (%)	0.06 ± 0.02
Crude protein (%) [#]	3.97 ± 0.01

Available carbohydrate (%)	78.78 ± 0.15
Energy (Kcal/g)	3.32 ± 0.01
Texture characteristics	
Hardness (g)	1155.00 ± 230.11
Gumminess	337.34 ± 45.48
Chewiness	1150.07 ± 155.63
Viability	
Log CFU/g	6.33 ± 0.07

Data presented as mean ± standard deviation and are obtained from 3 batches and replications.

#Conversion factor: 6.25

According to Zeng et al. (2018), konjac glucomannan has prebiotic properties in vitro and in vivo. Prebiotics can stimulate the growth of probiotic bacteria, and supplementing a product with prebiotics can boost their survival in the digestive tract. According to Peters et al. (2019), prebiotics are effective as substrates for probiotic bacteria and inhibit the growth of harmful bacteria in the intestine. When a high concentration of konjac glucomannan was added, the prebiotic activity in the digestive tract increased. According to Carlson et al. (2018), prebiotics can increase the number of *Bifidobacteria* and *Lactobacilli*, produce beneficial metabolites, help calcium absorption, and decrease the number of pathogenic bacteria. However, the investigation into the role of konjac glucomannan as a prebiotic and synbiotic in this product remains incomplete, which represents a limitation of this study. This research primarily concentrates on the sensory evaluation of the product.

4. Conclusion

Based on the results of this research, consumers demonstrated good knowledge of the health benefits of probiotics, prebiotics, and synbiotics, indicating an opportunity for the development of health-oriented food products. In this work, synbiotic marshmallows were developed by incorporating probiotic *L. plantarum* Dad-13 and konjac glucomannan. The developed product showed good consumer acceptance based on the consumer survey. The substitution of gelatin with konjac glucomannan did not compromise the viability of *L. plantarum* Dad-13, which remained within the minimum viable cell level required for probiotic products.

The formulation containing 8.5% gelatin and 0.5% konjac glucomannan was the most preferred among the evaluated formulations. However, JAR and penalty analyses indicated that the sweetness of the preferred formulation requires further adjustment. These findings demonstrate the potential of synbiotic marshmallows as a functional confectionery product that combines consumer acceptability with probiotic functionality. Further research is warranted to evaluate probiotic stability during storage, verify the prebiotic and synbiotic effects of konjac glucomannan, and investigate the use of low-calorie sweeteners as alternatives to sucrose and glucose. In vivo studies are also needed to confirm the functional effects of the product before its potential application for commercial development.

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Research article

EFFECT OF DRYING TECHNIQUES ON THE PRESERVATION OF ANTIOXIDANT PROPERTIES IN *ANANAS COMOSUS*

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Abstract

Preservation of antioxidant properties in pineapple (*Ananas comosus*) is crucial to maintain its nutritional value and health benefits. Pineapple is rich in polyphenols, flavonoids and other bioactive compounds that combat oxidative stress and reduce the risk of chronic diseases. However, the drying process which is essential in preserving the fruit's shelf life has the potential to reduce antioxidants contents due to the sensitivity of these compounds to heat and oxidative stress. This study analysed the impact of three drying techniques namely microwave, oven, and infrared drying on the retention of antioxidant properties in pineapple. The antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and Total Phenolic Content (TPC). To ensure consistency and to facilitate an accurate comparison across the techniques, all drying experiments were conducted in triplicate (n=3) and standardized at 80 °C with microwave drying operated at 300 W. Statistical analysis revealed significant differences ($p < 0.05$) among all three drying methods. Microwave drying was the most effective in preserving antioxidant properties, resulting in the fastest moisture removal and significantly higher antioxidant retention with a TPC 729.41 ± 12.35 mg GAE/100g and 56.03 ± 1.12 %DPPH inhibition. By comparison, infrared drying demonstrated moderate antioxidant retention (516.28 ± 9.84 mg GAE/100g TPC ; 44.03 ± 0.95 %DPPH), while oven drying was the least effective, resulting in the lowest retention (331.37 ± 8.15 mg GAE/100g TPC; 40.20 ± 0.88 %DPPH). The relatively poor antioxidant retention observed in oven dried samples is attributed to slower drying kinetics and extended heat exposure, which accelerates the thermal degradation of phenolic compounds.

1.Introduction

Pineapple (*Ananas comosus*), a tropical fruit which belongs to the *Bromeliaceae* family, is widely recognized for its rich nutritional and bioactive compounds. It is a

significant source of vitamins, particularly vitamin C and essential minerals such as manganese and potassium. These essential minerals contribute to immune function, bone health and metabolic processes (Mohd Ali et

al., 2020). Pineapple also contains antioxidants, including polyphenols and flavonoids which plays a crucial role in combating oxidative stress and lowering the risk of chronic diseases such as cardiovascular disorders and cancerous diseases (Sun et al., 2015). Additionally, pineapple is a source of bromelain, an enzyme known for its anti-inflammatory properties and digestive health benefits (Mohd Ali et al., 2020).

Drying is a common preservation method that reduces the moisture content of the food in which inhibits the microbial growth and enzymatic activity. By converting fresh pineapple into a more stable form through drying, it enhances the durability and usability of the pineapple in various food applications. However, the drying process can significantly impact the nutritional and antioxidant properties of pineapple. The thermal and oxidative stresses involved in drying can degrade heat-sensitive bioactive compounds where it can reduce the fruit's nutritional value and health benefits (Izli et al., 2018). For example, microwave drying has been shown to preserve antioxidants effectively due to its shorter drying time and it reduced thermal exposure (Chauhan et al., 2021). On the other hand, despite the simplicity of oven drying and its widespread application, it often leads to higher nutrient loss when operated at elevated temperature (Vidinamo et al., 2022). Meanwhile, infrared drying, which provides direct and efficient heating, is gaining attention for its potential to retain nutritional quality while improving drying rates (Adak et al., 2017).

This study focuses on three drying methods: microwave drying, oven drying, and infrared drying. Each method utilizes distinct mechanism and operating conditions, which influence the drying efficiency and the extent of the nutrient preservation. Microwave drying utilizes electromagnetic waves to heat and dry pineapple slices internally which offers faster

drying times but requires careful control to prevent thermal degradation (Zaki et al., 2017). Oven drying, a conventional method, relies on hot air circulation but often results in slower drying rates and potential nutrient loss (Vidinamo et al., 2022). Infrared drying, which uses infrared radiation to heat the fruit directly promises an efficient moisture removal but risks to an uneven heating and degradation of sensitive compounds (Adak et al., 2017).

Although microwave, infrared, and oven drying are widely used individually, comparative studies evaluating their performance under standardized thermal conditions while simultaneously assessing both drying kinetics and antioxidant retention in tropical fruits remain limited. Therefore, this study aimed to evaluate and compare the effects of microwave, infrared, and hot-air oven drying on the moisture removal kinetics and bioactive compound preservation (TPC and DPPH radical scavenging activity) of pineapple slices under identical processing parameters. Antioxidant activity was assessed using DPPH radical scavenging activity and Total Phenolic Content (TPC), while drying performance was evaluated based on moisture content, moisture ratio, and drying rate. The findings provide a comprehensive comparison of the drying techniques in terms of antioxidant preservation and drying efficiency, thereby contributing to the development of high-quality dried pineapple products with improved nutritional value.

2. Methodology

2.1. Material selection & preparation

Fresh and naturally ripened pineapples were sourced from a supermarket in Shah Alam, ensuring consistency in color and variety as Figure 1(a). Prior to the drying process, the pineapples were thoroughly cleaned, peeled, and manually sliced to uniform dimensions of 1 cm in thickness and 3 cm × 3 cm as Figure 1(b).

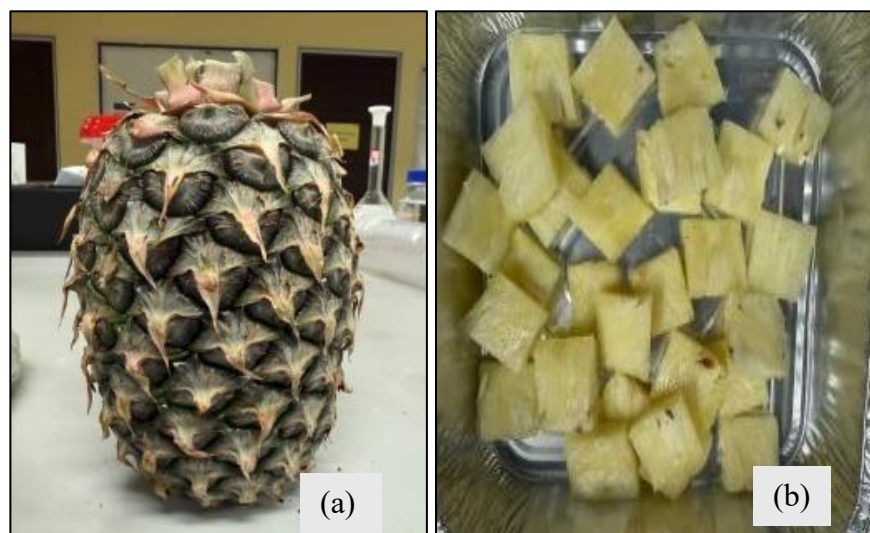


Figure 1. (a) Fresh pineapple fruit, (b) Diced pineapple flesh

2.2. Sample Extraction

The extraction method from Abd Hashib et al., (2019) was applied with modification. Dried pineapple samples were ground into a paste like consistency. Each sample was weighed at 5 grams and extracted using 80% ethanol in a 1:10 (w/v) ratio as illustrated in Figure 2. The mixtures were placed in a shaker incubator (Infors AG, Ritttergasse 27, 4103 Bottmingen Switzerland) and was left overnight at a room temperature and 150 rpm

to promote the extraction of antioxidants compounds. Following incubation, the extracts were filtered using a syringe-mounted membrane filter (0.45 μm pore size) to remove solids and obtained a clear solution. The filtered extracts were kept at room temperature prior to immediate use for determination of antioxidant activity using 2,2 diphenyl 1 picrylhydrazyl (DPPH) radical scavenging assay and Total Phenolic Content (TPC) analyses.

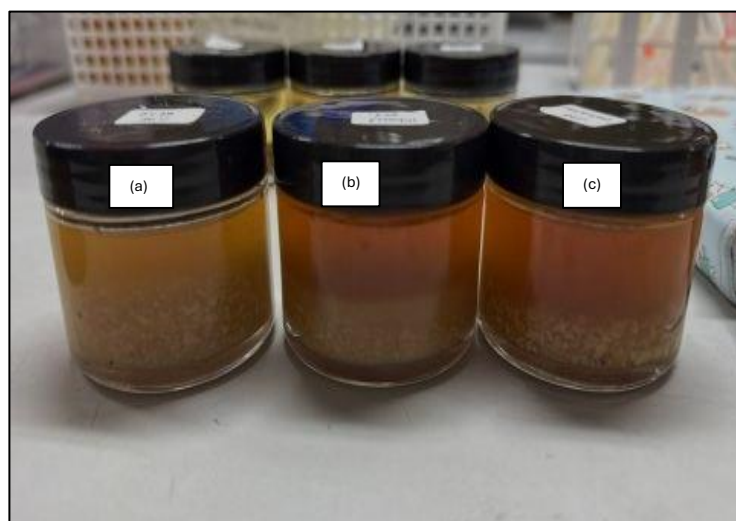


Figure 2. Sample extraction from oven drying (a), infrared (b) and microwave drying(c)

2.3. Microwave Drying

For the microwave drying process, the prepared pineapple slices were subjected to microwave drying at a power level of 300 W. The drying process was monitored by weighing the samples at 5 minutes intervals

until equilibrium moisture content was achieved.

A microwave power level of 300 W was selected to achieve efficient moisture removal while minimizing thermal degradation of heat-sensitive antioxidant compounds.

2.4.Oven Drying

For the oven drying process, the prepared sliced were arranged in a single layer on stainless steel trays and placed in a preheated hot-air oven at 80°C for the drying process. Samples were weighed at 30-minutes intervals to monitor moisture loss, and the drying process continued until three consecutive weights of reading remained constant, indicating the completion of drying. Following the drying, the weight data were utilised to compute the drying rates and moisture ratios.

2.5.Infrared Drying

For the infrared drying process, the pineapple slices were evenly arranged on trays within the infrared dryer without overlapping to ensure uniform drying. The dryer was operated at 80°C, a temperature selected to promote efficient moisture removal while minimizing thermal degradation of heat-sensitive antioxidant compounds. The samples were weighed at 15-minute intervals throughout the drying process to monitor moisture loss and evaluate the drying behaviour of the pineapple slices.

2.6.Antioxidant Activity Analysis

The antioxidant activity of the samples was determined using a modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay adapted from Harith et al. (2023). A DPPH solution was prepared by dissolving 10 mg of DPPH powder in 100 mL of 80% methanol and storing the solution in the dark at room temperature. For the analysis, 1.0 mL of fruit extract was mixed with 2.0 mL of DPPH solution. The reaction mixture was incubated in the dark for 60 min, after which the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. An 80% methanol solution was used as the blank, while the control solution consisted of 4.0 mL of 80% methanol and 1.0 mL of DPPH solution. According to Šturm et al. (2019), the spectrophotometer has an accuracy of ± 0.01 AU. The DPPH radical scavenging activity was expressed as percentage inhibition (%) and calculated using Equation (1) (Chaudhuri et al., 2012):

$$\text{Inhibition (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (1)$$

Where:

A_0 = the absorbance control DPPH

A_1 = the absorbance of the sample

2.7.Total Phenolic Content (TPC) Analysis

The Total Phenolic Content (TPC) of the samples was determined using the *Folin-Ciocalteu* method as described by Harith et al. (2023) with slight modifications. A gallic acid stock solution (1000 mg/L) was prepared by dissolving 30 mg of gallic acid in 10 mL of 80% ethanol, and the volume was subsequently made up to 100 mL using the same solvent. A series of gallic acid standard solutions of different concentrations were then prepared by appropriate dilution of the stock solution using 80% ethanol.

For both standard and sample analyses, 0.3 mL of the solution was transferred into a test tube, followed by the addition of 1.5 mL of diluted Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 10 min before adding 1.2 mL of 7.5% (w/v) sodium carbonate solution. The reaction mixture was then incubated in the dark for 30 min. Absorbance was measured at 765 nm using a UV-Vis spectrophotometer, with 80% ethanol serving as the blank.

A calibration curve was established using the gallic acid standard solutions. The absorbance values obtained from the standards were plotted against their corresponding concentrations to generate the calibration equation, as shown in Equation (2).

$$y = 0.1027 x + 0.0595, R^2 = 0.9812 \quad (2)$$

Where:

y represents the absorbance and x represents the concentration of gallic acid standard solution.

The total phenolic content of the sample extracts was determined by interpolating their absorbance values into the calibration equation. The TPC was calculated using Equation (3) and expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dried pineapple

$$\text{TPC} = C \times V/M \quad (3)$$

Where:

TPC = total phenolic content (mg GAE/g)

C = concentration of gallic acid established from the calibration curve (mg/mL)

V = volume of extract solution (mL)

M = weight of extract (g)

2.8. Moisture Content Analysis

The moisture content (MC) was recorded at fixed intervals times following respective drying methods. The moisture content is calculated by using Equation (4):

$$\text{Moisture Content (\%)} = \frac{(\text{Weight}_1 \text{ (g)} - \text{Weight}_2 \text{ (g)})}{(\text{Weight}_2 \text{ (g)})} \times 100 \quad (4)$$

Where:

Weight₁ = Initial weight of powder (g)

Weight₂ = Final weight of powder (g)

2.9. Drying Kinetics

The drying kinetics can be obtained based on method described by Izli et al., (2018) with some modifications. The collected data for moisture content is used to calculate the drying rate and drying time for each technique. The drying rate will be calculated using equation (5):

$$\text{Drying Rate (gram H}_2\text{O/gram dry matter.time)} = \frac{(X_1 - X_2)}{(t_1 - t_2)} \quad (5)$$

Where :

X₁ = gram dry matter moisture content of sample at time t₁ ,

X₂ = moisture content of sample at time , t₂

t₁ , t₂ = time in minute

Moisture ratio (MR), defined as the ratio of current moisture content to the initial moisture content, represented moisture removal over time and essentially important for analysing drying kinetics. The moisture ratio data were calculated using Equation (6):

$$\text{MR} = \frac{(M_t - M_e)}{(M_o - M_e)} \quad (6)$$

Where:

MR = Moisture Ratio

M_t = moisture content at a given time

M_o = initial moisture content

M_e = equilibrium moisture content

In this study, the equilibrium moisture content (M_e) was not directly used in the calculation of moisture ratio (MR). This approach is consistent with common practice in many drying studies, especially under high temperatures conditions where the equilibrium moisture content is very low and the drying curve did not reach a stable plateau. Therefore, the drying process is stopped before reaching equilibrium to avoid thermal degradation. Following (Samad et al., 2024) , M_e was assumed negligible (M_e ≈ 0) and MR equation (6) was simplified into equation (7) followed with drying curves plot (for drying rate and moisture ratio against time).

$$\text{MR} = M_t/M_o \quad (7)$$

Where :

MR = Moisture Ratio

M_t = moisture content at a given time

M_o = initial moisture content

2.10. Replication and Data Analysis

All experimental procedures and analyses were performed in triplicate (n = 3) unless otherwise stated, and the results were expressed as mean ± standard deviation (SD). Analytical instruments, including the analytical balance, spectrophotometer, and temperature sensors, were calibrated prior to measurement according to standard procedures. One-way Analysis of Variance (ANOVA) was performed to determine whether significant differences existed among the treatment groups followed with t-tests to identify specific differences. Statistical significance was evaluated at p < 0.05 using Microsoft Excel.

3. Results and Discussion

3.1. Drying Kinetics of Pineapple Slices

The drying behavior of the pineapple slices exposed to microwave, infrared, and oven drying was analyzed based on changes in moisture ratio and drying rate over time. The moisture ratio is a dimensionless quantity that are being used to represent the relative amount of moisture remained in a material compared to their initial and equilibrium moisture contents (Zeng et al., 2024). As presented in

Figure 3, the moisture ratio for all drying techniques consistently decreased over time. This indicates that a progressive moisture removal. Among all drying techniques, microwave drying demonstrated the most rapid reduction in moisture ratio where it reaches a near-equilibrium within approximately 85 minutes. This can be explained by microwaves' volumetric heating effect in which microwaves allow for internal

moisture movement. It also allows the heat to penetrate the material uniformly which delivers energy directly into the entire volume rather than relying on the surface-based conduction and convection (Parit et al., 2018). As for infrared drying, it shows a moderately fast decline in moisture ratio as it took around 130-150 minutes to reach the final moisture level.

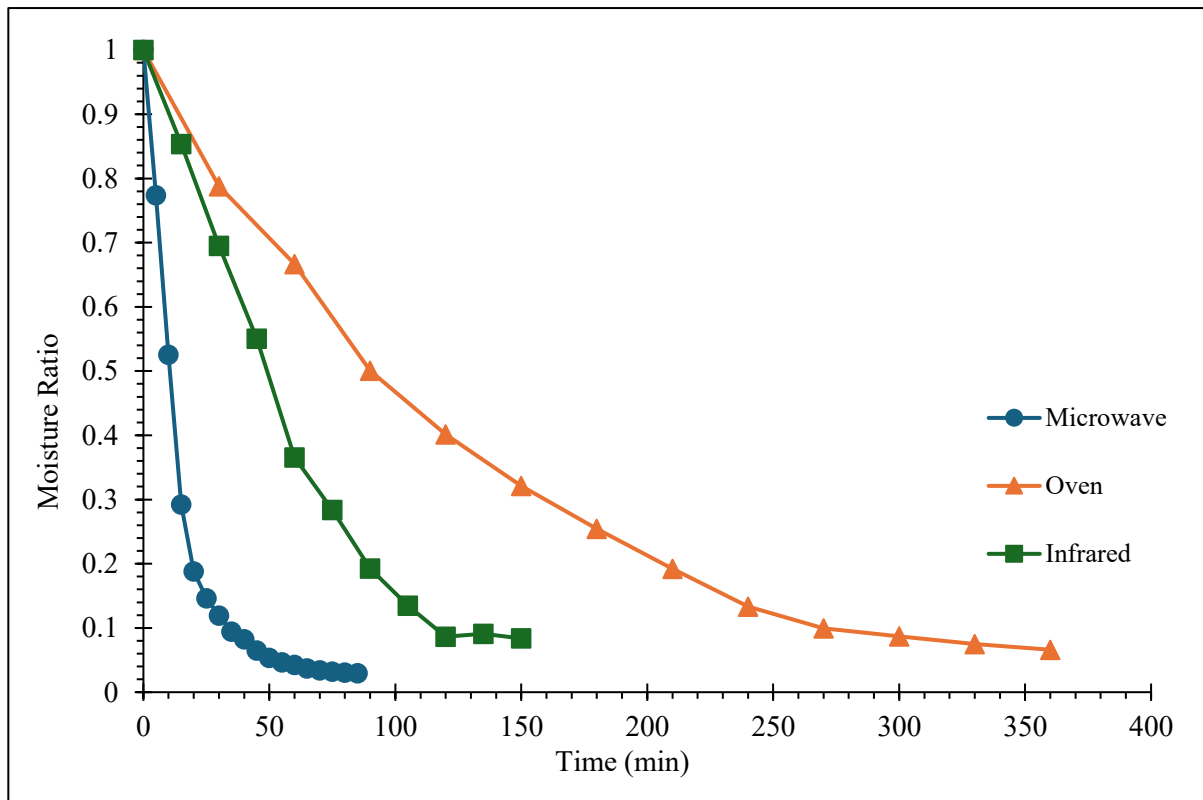


Figure 3. Effect of Different Drying Method on Moisture Ratio Over Time

In contrast to oven drying that took the longest time. According to (El-Mesery & Elabd, 2021) oven drying relies on convective heat transfer in which the heat is supplied from the hot air to the surface of the material. Then, the moisture must diffuse from the interior to the surface before evaporating resulting in a relatively slow process as the internal moisture must travel through the material by diffusion. It was the slowest as the moisture ratio gradually decreased over more than 350 minutes where it indicates slower moisture removal due to its reliability on external convective heat transfer.

The drying rate profiles of pineapple slices subjected to microwave, infrared, and hot-air oven drying are presented in Figure 4. All three

drying techniques operated entirely within the falling rate regime without displaying a constant rate phase, confirming that moisture removal was governed primarily by internal mass transfer resistance. However, the magnitude and kinetics of moisture evaporation differed substantially among the methods. Microwave drying exhibited an exceptionally high initial drying rate, reaching a maximum peak of 29.8 g H₂O/ g dry matter . min within the first 5 minutes of processing. This dramatic initial surge reflects rapid internal dielectric energy conversion, which induces prompt phase change and vigorous vaporization of unbound water molecules. Following this peak, the microwave drying rate declined abruptly as

free moisture was depleted, shifting the process toward the removal of bound water. This rapid drop further demonstrates the efficiency of volumetric heating in accelerating moisture migration and hastening the transition through the falling rate period (Ma et al., 2023).

In contrast, infrared drying exhibited a more moderate and steady drying rate profile. As illustrated in Figure 4, infrared drying lacked the pronounced initial spike observed in microwave drying, starting at 10.0 g H₂O/ g dry matter . min and reaching a secondary peak of 7.8 g H₂O/ g dry matter . min at 30 minutes while maintaining consistent drying

momentum over a longer duration. According to Ismail et al. (2018), the absence of a sharp initial surge during infrared drying reflects surface-dominated heat absorption and steadier moisture evaporation over time. Wang and Sheng (2006) noted that because infrared energy is primarily absorbed at the material surface and conducted inward, it facilitates controlled water removal rather than rapid internal moisture expulsion. The gradual decline in the infrared drying rate indicates a smooth transition from unbound to bound water removal (Delfiya et al., 2022), which can minimize structural collapse in the fruit matrix.

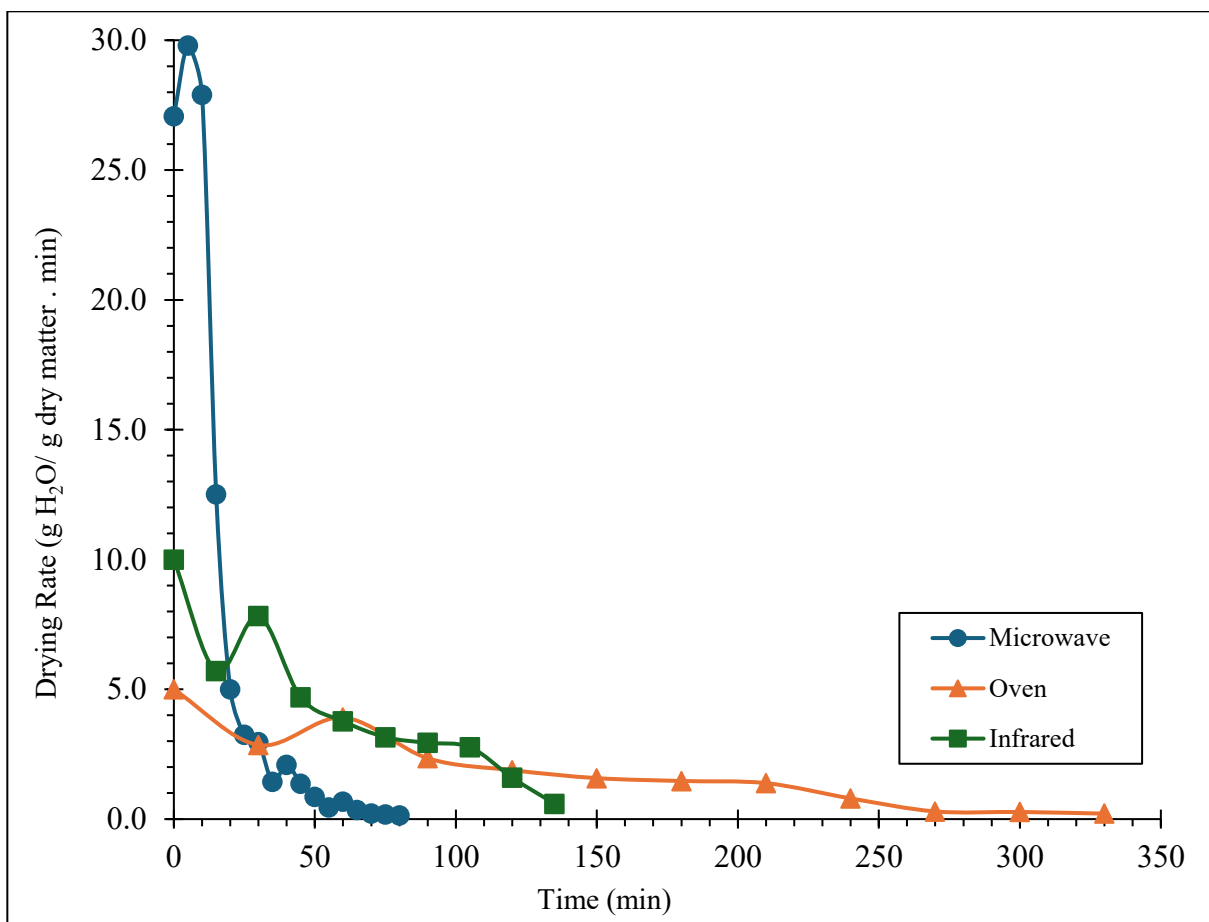


Figure 4. Drying Rate Profiles of Pineapple Slices Using Different Drying Methods

Meanwhile, hot-air oven drying exhibited the lowest initial drying rate of 5.0 g H₂O/ g dry matter . min and the most prolonged drying trajectory overall. The rate in oven drying declined steadily without any notable peaks, indicating slow heat penetration due to reliance on external convective air circulation (Norhadi et al., 2020). Consequently, internal moisture migration to the fruit surface was less

efficient, leading to extended drying times. Overall, the drying rate profiles demonstrate that microwave drying is the most energy-intensive and rapid technique, infrared drying offers a balanced and moderately efficient alternative, and oven drying is the least effective method for promoting moisture transfer.

Across all evaluated drying techniques, no constant rate period was observed in the drying curves (Figure 3 and Figure 4). According to Chokngamvong and Suvanjumrat (2023), the absence of a constant rate phase indicates that the rate of moisture supply to the sample surface was insufficient to maintain steady surface evaporation. Consequently, the drying process was governed entirely by the falling rate period, where mass transfer is dictated by internal moisture diffusion rather than surface evaporation. This behavior is characteristic of biological tissue like pineapple, where surface moisture evaporates rapidly during initial heating, leaving bound internal water that migrates slowly toward the surface via diffusion. Unlike materials with free surface water that sustain constant evaporation rates until surface moisture is depleted (He et al., 2021), the cellular matrix and sugar-rich structure of pineapple restrict internal moisture mobility, thereby prolonging the falling rate phase (Muyonga et al., 2021). Ultimately, the exclusive presence of a falling rate period confirmed that internal moisture diffusion was

the primary rate limiting mechanism controlling moisture removal across all three drying methods.

3.2. Antioxidant Activity

The antioxidant properties of the dried pineapple samples were evaluated through Total Phenolic Content (TPC) and DPPH radical scavenging capacity assays. These analyses provide critical insights into the retention of bioactive compounds particularly phenolics which contribute significantly to antioxidant potential but are highly susceptible to thermal degradation. Among the three drying techniques evaluated, microwave drying resulted in the highest retention of both TPC and DPPH radical scavenging activity, followed by infrared drying, while hot-air oven drying yielded the lowest values across both assays. One-way ANOVA confirmed that all three drying methods differed significantly from one another ($p < 0.05$) in both TPC and DPPH values, as shown in Table 1 and illustrated in Figure 5.

Table 1. Effect of drying methods on Total Phenolic Content (TPC) and DPPH radical scavenging activity of pineapple slices.

Drying Method	Total Phenolic Content (mg GAE/100g)	DPPH Radical Scavenging (% Inhibition)
Microwave	729.41±12.35	56.03±1.12
Infrared	516.28±9.84	44.03± 0.95
Oven	331.37±8.15	40.20± 0.88

The superior antioxidant preservation in microwave-dried samples aligns with findings by Joseph Bassey et al. (2024) and Wang and Sheng (2006), who reported enhanced bioactive retention in microwave-treated fruits compared to conventional thermal methods despite variations in sample matrices or process scale. Similarly, Zulkipli et al. (2024) observed significantly higher phenolic preservation and radical scavenging capacity in microwave-dried plant materials relative to

oven drying. The moderate retention observed during infrared drying and the severe losses in oven drying further demonstrate that bioactive preservation is inversely related to the duration of thermal and oxidative stress during drying (Athira et al., 2022). These observations agree with Si et al. (2015), who found that infrared drying retained significantly higher phenolic content (123.22 g GAE/kg dry weight) at 70 °C than conventional convective drying.

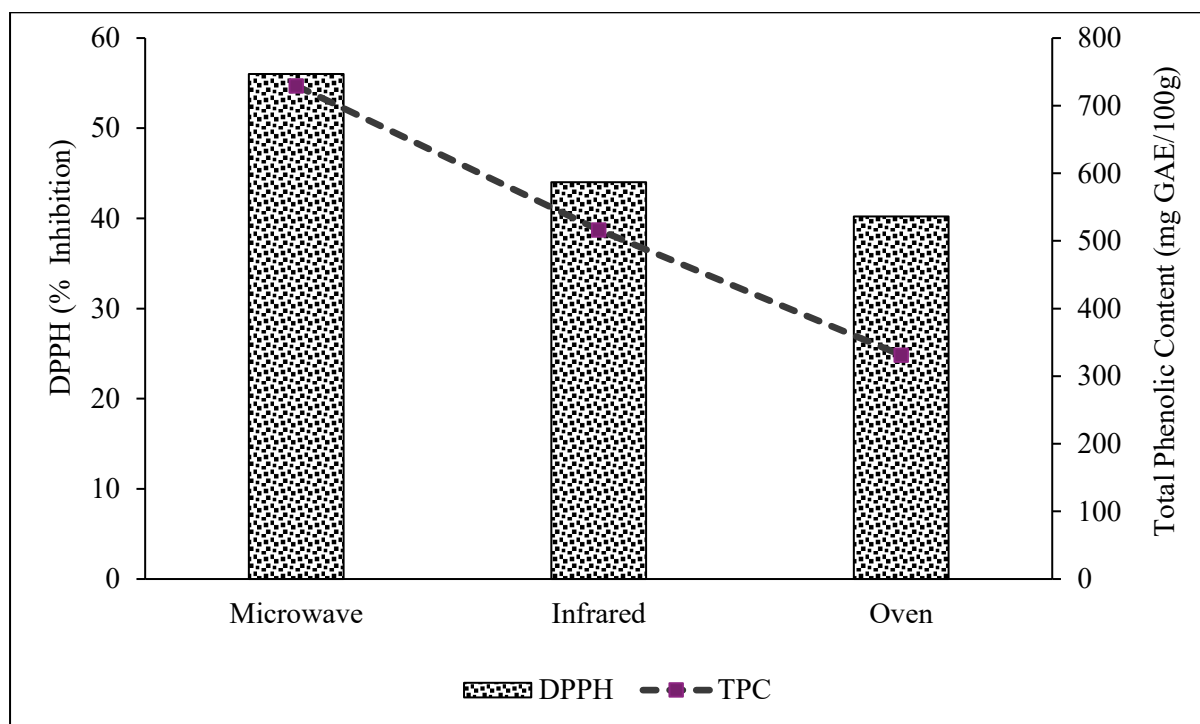


Figure 5. Effect of Different Drying Methods on Antioxidant Activity and Total Phenolic Content

Although drying kinetics dictate physical moisture removal rates, bioactive degradation is governed by chemical breakdown pathways influenced by heat intensity and oxygen exposure (Toydemir, 2016). The enhanced retention in microwave dried pineapple stems directly from its volumetric dielectric heating mechanism. Microwave radiation directly excites dipolar water molecules throughout the fruit matrix simultaneously, generating a steep internal vapor pressure gradient that rapidly drives moisture outward. This substantial reduction in total drying duration minimizes heat accumulation at the surface and limits exposure to atmospheric oxygen, thereby suppressing the thermal oxidation and cleavage of phenolic structures (Toydemir, 2016). Furthermore, rapid dielectric heating rapidly inactivates endogenous oxidative enzymes, such as polyphenol oxidase (PPO) and peroxidase (POD), halting enzyme catalyzed phenolic degradation early in the process. In contrast, infrared and convective oven drying rely on surface heat absorption followed by inward thermal conduction. This creates prolonged thermal stress and sustained exposure to elevated temperatures and atmospheric oxygen at the surface layer, accelerating the oxidative breakdown of heat-sensitive flavonoids and phenolics.

Whereas TPC reflects the overall concentration of phenolic compounds, the DPPH assay measures their functional radical-scavenging activity. The lower DPPH inhibition observed in infrared and oven dried samples indicates that prolonged thermal exposure not only reduces phenolic quantity but also compromises their functional radical-neutralizing efficiency due to structural alterations (Toydemir, 2016). The consistent parallel trend between TPC and DPPH across all treatments (Figure 5) supports the interpretation that drying method plays an important role not only in the retention of antioxidants but also in maintaining the chemical integrity of bioactive phenolic compounds.

4. Conclusion

This study evaluated the impact of microwave, infrared, and hot-air oven drying on the moisture removal kinetics and antioxidant retention of pineapple slices under standardized thermal conditions. Across all three drying techniques, moisture removal occurred entirely within the falling rate period without exhibiting a constant rate phase, confirming that the drying process was governed by internal moisture diffusion. Among the evaluated methods, microwave

drying achieved the fastest moisture removal kinetics and shortest overall drying duration, whereas convective oven drying was the slowest and least efficient. Antioxidant evaluation through Total Phenolic Content (TPC) and DPPH radical scavenging activity demonstrated that microwave drying preserved significantly higher ($p < 0.05$) levels of bioactive compounds compared to infrared and oven drying. This superior retention is directly attributed to the volumetric dielectric heating mechanism of microwave energy, which generates rapid internal vapor pressure gradients and shortens drying time. By substantially reducing total thermal exposure and limiting contact with atmospheric oxygen, microwave drying minimizes thermal degradation, cleavage, and oxidative breakdown of heat sensitive phenolics. These results demonstrate that microwave drying is the most effective technique for preserving antioxidant quality in pineapple under the evaluated conditions. However, future investigations evaluating multi-factor operating parameters (e.g., variable temperatures and power levels) are recommended to establish fully optimized processing conditions for industrial scaling. However, a limitation of this study is the investigation of a single drying temperature (80 °C) and fixed microwave power setting (300 W). Future investigations evaluating multi-factor operating parameters (e.g., variable temperatures, power densities, and wavelengths) are recommended to establish fully optimized processing conditions for industrial scaling.

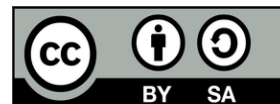
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Research article

ADVANCED QUALITY CHARACTERIZATION OF COMMERCIAL THAI ARABICA GREEN COFFEE BEANS USING SYNCHROTRON FTIR MICROSCPECTROSCOPY AND X-RAY TOMOGRAPHY

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ABSTRACT

Thai Arabica coffee has gained increasing recognition in the specialty coffee market due to its distinctive quality attributes and continuing development in post-harvest handling. This study applied synchrotron-based techniques for advanced quality characterization of commercially obtained Thai Arabica green coffee beans representing four post-harvest processing types: dry, washed, honey, and low-temperature/low-humidity (LTLH). Moisture content was determined in triplicate for each commercial sample. Synchrotron Fourier transform infrared (SR-FTIR) microspectroscopy, combined with second-derivative spectral preprocessing and principal component analysis (PCA), was applied to evaluate biochemical fingerprints associated with lipid-, protein-, and carbohydrate-related constituents, which are important quality-related components and potential contributors to aroma and flavor development during roasting. Approximately 50 spectra were initially acquired across three separately prepared subsamples for each processing type. Moisture contents differed significantly ($p < 0.05$) among the commercial samples, with LTLH beans showing the lowest value ($10.48 \pm 0.37\%$) and honey-processed beans showing the highest value ($12.64 \pm 0.09\%$). PCA of the SR-FTIR spectra showed clustering trends among the four commercial sample groups, with PC1 and PC2 explaining 15% and 12% of the total spectral variance, respectively. Dry-processed beans exhibited relatively stronger lipid- and ester-associated spectral features, whereas the LTLH sample showed more pronounced carbohydrate/polysaccharide-related features. Complementary synchrotron X-ray tomographic microscopy (XTM), performed on three representative beans per processing type, provided non-destructive three-dimensional visualization of representative internal air-channel organization and dense bean matrix structure. The LTLH sample showed a more continuous and well-defined air-channel pattern in the representative XTM images. Since

the coffee beans were commercially obtained and were not experimentally processed under controlled conditions by the authors, the observed differences should be interpreted as comparative quality-related characteristics rather than direct causal effects of specific processing parameters. Overall, the integration of SR-FTIR microspectroscopy and XTM provides complementary biochemical and structural information that may support advanced quality assessment of commercial Thai Arabica green coffee beans.

1. Introduction

Green coffee bean quality is determined by a complex combination of biochemical composition and internal physical structure. Major constituents such as lipids, polysaccharides, proteins, chlorogenic acids, caffeine, trigonelline, and other cell-wall-associated compounds contribute to the formation of aroma precursors, roasting behavior, extraction characteristics, and overall beverage quality (Wang & Lim, 2012; Aung Moon et al., 2024). In green coffee beans, lipids are associated with aroma retention and mouthfeel, while polysaccharides and cell-wall materials influence bean hardness, porosity development, heat transfer, and the release of volatile compounds during roasting (Schenker et al., 2000; Li et al., 2021; Al-Shemmeri et al., 2024). Therefore, understanding both the chemical composition and internal microstructure of green coffee beans is essential for evaluating coffee quality beyond conventional grading and sensory assessment.

Post-harvest processing is one of the key factors influencing the biochemical and structural characteristics of coffee beans. Processing types such as dry, washed, honey, and controlled drying differ in the degree of mucilage retention, microbial activity, drying rate, moisture removal, and exposure of the bean to fruit-derived compounds (Zhang et al., 2019; Jakkaew et al., 2024; Coelho et al., 2024; Aung Moon et al., 2024). These differences can modify the chemical matrix of green coffee beans, particularly lipid-associated, protein-associated, phenolic-associated, and carbohydrate-related components (Aung Moon et al., 2024). They may also influence internal structural organization by affecting

dehydration stress, cell-wall integrity, and air-channel formation within the bean (Schenker et al., 2000; Dong et al., 2018). Such chemical and structural differences are especially relevant for specialty coffee, where consistency in roasting performance and quality attributes is highly valued.

Thailand's Arabica coffee sector has developed rapidly in recent years, particularly in highland areas such as Chiang Rai and Chiang Mai, where agro-climatic conditions are suitable for specialty Arabica production (Noppakoonwong et al., 2015; Aung Moon et al., 2024). Commercial Thai Arabica coffee is increasingly available in several post-harvest processing types, including washed, dry, honey, and controlled low-temperature drying. However, integrated studies combining SR-FTIR microspectroscopy and synchrotron XTM to jointly evaluate the biochemical fingerprints and internal microstructure of commercially processed Thai Arabica green coffee beans remain scarce. Most routine quality evaluations rely on moisture content, physical grading, and sensory assessment, which are useful but provide incomplete information on the molecular and structural basis of bean quality (Castillejos-Mijangos et al., 2022; Aung Moon et al., 2024).

Fourier transform infrared (FTIR) spectroscopy has been widely applied as a rapid vibrational spectroscopic technique for characterizing coffee composition, including lipid-associated bands, ester carbonyl groups, protein-related amide bands, and carbohydrate fingerprint regions (Wang & Lim, 2012; Weldegebreal et al., 2017; Castillejos-Mijangos et al., 2022). When combined with multivariate analysis such as principal component analysis (PCA), FTIR can

differentiate coffee samples according to origin, processing, storage condition, and chemical composition (Castillejos-Mijangos et al., 2022; Aung Moon et al., 2024). Synchrotron-based FTIR microspectroscopy offers additional advantages because the high brightness of synchrotron radiation enables spectra to be collected from small sampling areas with improved signal-to-noise characteristics. This is particularly useful for studying heterogeneous biological materials such as coffee beans, in which biochemical composition may vary at the microscale.

In addition to chemical fingerprinting, internal microstructural information is important for understanding coffee quality. X-ray tomographic microscopy (XTM) provides non-destructive three-dimensional visualization of internal features such as air channels, dense bean matrix, and structural continuity (Schoeman et al., 2016). These microstructural characteristics may affect heat and mass transfer during roasting and may therefore contribute to differences in roasting uniformity and quality consistency (Schenker et al., 2000; Al-Shemmeri et al., 2024). The integration of SR-FTIR and XTM can provide complementary biochemical and structural observation.

In the present study, commercially obtained Thai Arabica green coffee beans representing four post-harvest processing types—dry, washed, honey, and low-temperature/low-humidity (LTLH)—were characterized using synchrotron FTIR microspectroscopy and synchrotron X-ray tomographic microscopy. The samples were not experimentally processed by the authors; rather, they were analyzed as commercial green coffee beans representing different processing types. The objectives of this study were to (i) compare the biochemical fingerprints of commercial Thai Arabica green coffee beans using SR-FTIR microspectroscopy and PCA, (ii) visualize representative internal microstructural features using XTM, and (iii) evaluate how combined chemical and structural information may

support advanced quality assessment of Thai specialty coffee.

2. Materials and Methods

2.1. Sample collection

Commercial Thai Arabica green coffee beans (*Coffea arabica* L.) representing four post-harvest processing types, namely dry, washed, honey and low-temperature/low-humidity (LTLH), were used in this study. The dry-, washed-, and honey-processed green coffee beans were obtained from a local commercial coffee producer in Doi Chang, Chiang Rai Province, northern Thailand. The LTLH green coffee bean samples were obtained from Preda Roasting House, Lampang Province, Thailand. All samples were purchased as finished green coffee beans after commercial post-harvest processing. Therefore, the authors did not experimentally perform, control, or modify the post-harvest processing conditions. The processing types used in this study were based on information provided by the producer and the commercial classification of the samples.

The dry-processed beans represented naturally processed coffee, in which whole coffee cherries were dried before removing the outer fruit layers. The washed beans represented coffee processed by pulp removal and mucilage removal prior to drying. The honey-processed beans represented coffee in which partial mucilage was retained during drying. The LTLH beans represented coffee dried under a controlled low-temperature and low-humidity condition, as described by the producer. Since all samples were commercially obtained, detailed commercial processing parameters, including exact drying duration, airflow rate, drying-layer thickness, turning frequency, and batch-to-batch environmental variation, were not controlled by the authors. This limitation was considered when interpreting the results. After purchase, the green coffee bean samples were kept in sealed plastic bags and stored in a refrigerator at 4 °C. This storage condition was used to minimize moisture uptake and reduce potential

compositional changes before analysis. Prior to analysis, beans with visible physical defects were removed. Representative beans from each commercial processing category were used for moisture content determination, synchrotron FTIR microspectroscopy, and synchrotron X-ray tomographic microscopy.

2.2. Moisture Content Determination

Moisture content of green coffee beans was determined using the oven-drying method based on the gravimetric loss in mass at 105 °C, following the principle described in ISO 6673:2003 (International Organization for Standardization, 2003) for green coffee moisture analysis. Approximately 10 g of whole coffee beans were placed in pre-weighed aluminum moisture cans and dried in a hot-air oven at 105 °C until constant weight was achieved. Moisture content (%) was calculated from the difference between the initial and final sample mass using the following equation:

Moisture content (%)

$$= \frac{(\text{Initial weight} - \text{Dried weight})}{\text{Initial weight}} \times 100$$

(1)

Moisture content was determined in triplicate for each commercial sample ($n = 3$). The results were expressed as mean $\pm$ standard deviation. Statistical differences among the commercial samples were evaluated using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at the 95% confidence level ($p < 0.05$), using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA).

2.3. Synchrotron FTIR Microspectroscopy (SR-FTIR)

Synchrotron-based Fourier transform infrared (SR-FTIR) microspectroscopy was performed at the BL4.1 beamline of the Synchrotron Light Research Institute (SLRI), Nakhon Ratchasima, Thailand. For SR-FTIR

analysis, three separate subsamples, each weighing approximately 50 g, were randomly collected from each commercial processing type. Each subsample was ground separately and thoroughly homogenized. A small amount of each ground sample was compressed between diamond windows of a diamond anvil cell to form a thin film suitable for the transmission-mode FTIR measurement. FTIR spectra were collected in transmission mode over the mid-infrared range of 4000-800 cm^{-1} using synchrotron radiation as the infrared source. Spectra were acquired at a spectral resolution of 6 cm^{-1} , with 32 scans accumulated per spectrum. The aperture size was $30 \times 30 \mu\text{m}^2$. Background spectra were collected under the same instrumental conditions before sample measurement and were used for spectral correction. Approximately 50 spectra in total were initially acquired from multiple representative positions across the three prepared subsamples for each processing type. Following spectral quality screening, spectra with poor signal quality, excessive scattering, or strong atmospheric interference were excluded, and the remaining valid spectra were used for spectral preprocessing. Second-derivative transformation was applied to enhance overlapping bands and resolve subtle spectral differences. Extended multiplicative signal correction (EMSC) was used to reduce baseline variation and scattering effects. Principal component analysis (PCA) was subsequently performed using The Unscrambler X software (version 10.1) to explore biochemical variation among green coffee bean samples representing different commercial processing types. Major absorption regions used for interpretation included lipid-associated CH_2 stretching vibrations around 2924 and 2854 cm^{-1} , ester carbonyl absorption around 1745 cm^{-1} , protein-associated amide bands at approximately 1642, 1548, and 1531 cm^{-1} and carbohydrate/polysaccharide fingerprint regions around 1200-1000 cm^{-1} . Band assignments were interpreted based on

previous FTIR studies of coffee and food matrices.

2.4. Synchrotron X-ray Tomographic Microscopy (XTM)

Synchrotron X-ray tomographic microscopy (XTM) was performed at Beamline 1.2W of the Synchrotron Light Research Institute (SLRI), Thailand. For XTM analysis, three representative green coffee beans from each commercial processing type were selected (based on available synchrotron beamtime) for internal microstructural visualization. Each bean was secured on a metal rod using adhesive and mounted on the rotation stage for tomographic scanning. The synchrotron storage ring operated at 1.2 GeV and 150 mA. A 350- μm alumina foil was used as an attenuator to reduce imaging artifacts and adjust the X-ray intensity to a suitable level for coffee bean imaging. The mean X-ray energy was approximately 11.5 keV. The detection system consisted of a 100- μm -thick YAG:Ce scintillator (Crytur, Czech Republic), a lens-coupled X-ray microscope (Optique Peter, France), and an sCMOS camera (Andor NEO 5.5; 2560×2160 pixels, 16-bit), providing an effective optical pixel size of 3.61 μm . Due to the limited field of view, each bean was scanned at three consecutive sample positions to cover the entire bean. Therefore, nine tomographic scans were acquired for each commercial processing type (three scans per bean across three representative beans). For each tomographic scan, X-ray projection images were collected over 180° rotation with an angular step of 0.3° . Projection images were corrected using background normalization before reconstruction. Tomographic reconstruction was performed using Octopus Reconstruction software (TESCAN, Gent, Belgium). For each bean, the three reconstructed volumes corresponding to the three scan positions were subsequently stitched to generate a complete three-

dimensional dataset. The reconstructed image volumes were further analyzed using Octopus Analysis software to visualize internal air-channel structures and dense bean matrix. Three-dimensional volume renderings were generated with Drishti software.

The reconstructed XTM images were used for qualitative visualization and descriptive comparison of internal microstructural features among the commercial green coffee bean samples. Since three representative beans from each processing category were analyzed based on the available synchrotron beamtime, the XTM results were interpreted as representative microstructural observations rather than as a fully replicated quantitative pore-network analysis.

3. Results and Discussion

3.1. Moisture content of green coffee beans

The moisture content of commercial Thai Arabica green coffee beans differed among the four post-harvest processing types (Table 1). The honey-processed beans showed the highest moisture content ($12.64 \pm 0.09\%$), followed by washed beans ($11.30 \pm 0.13\%$) and dry-processed beans ($11.19 \pm 0.25\%$). In contrast, the LTLH beans exhibited the lowest moisture content ($10.48 \pm 0.37\%$). These differences indicate that the commercial coffee samples varied in their residual moisture levels at the time of analysis.

Although the beans were commercially obtained and post-harvest processing conditions were not controlled by the authors, the moisture results provide useful supporting information for interpretation of the subsequent spectroscopic and microstructural observations. The relatively lower moisture content of the LTLH beans may be associated with more controlled dehydration, whereas the higher moisture content of the honey-processed beans may reflect the influence of mucilage retention and drying process.

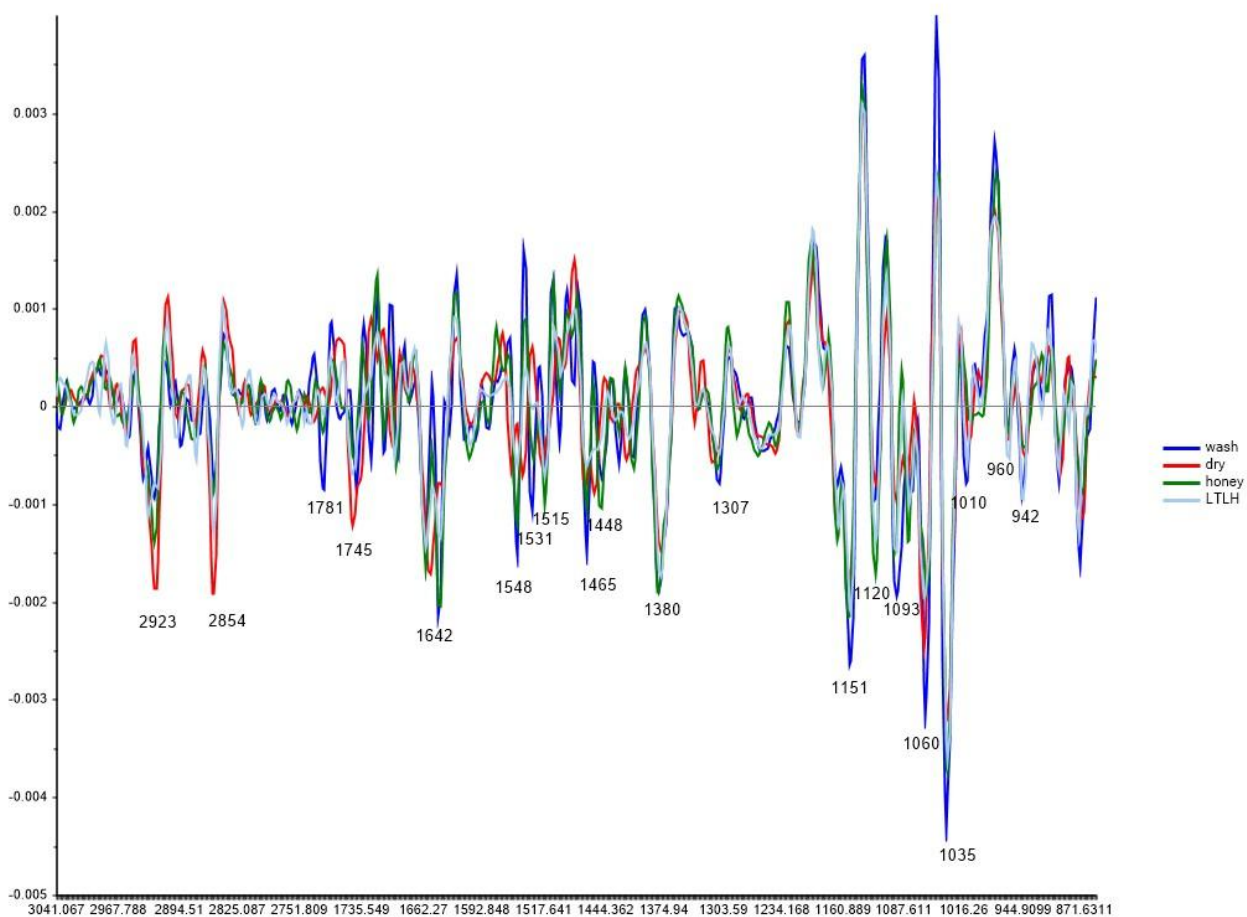
Table 1. Moisture content (%MC) of Thai Arabica green coffee beans classified by post-harvest processing type

Post-harvest preprocessing type	% MC
Washed	11.30±0.13 ^b
Dry	11.19±0.25 ^b
Honey	12.64±0.09 ^c
LTLH	10.48±0.37 ^a

Previous studies have shown that drying methods can affect the final moisture and physicochemical characteristics of Arabica coffee beans (Coelho et al., 2024; Aung Moon et al., 2024). However, these interpretations should be considered comparative observations among commercial samples

rather than direct causal effects of controlled processing parameters.

Data are presented as Mean ± Standard Deviation. Values followed by different lowercase letters in the same column are significantly different at the 95% confidence level ($p < 0.05$).

**Figure 1.** Average second-derivative FTIR spectra of commercial Thai Arabica green coffee beans. Differences were observed in lipid-, protein-, and polysaccharide-related spectral regions, indicating biochemical variation among the commercial processing types.

3.2. Synchrotron FTIR spectral characteristics

Average second-derivative SR-FTIR spectra of the green coffee bean samples are shown in Figure 1. The spectra revealed characteristic absorption bands associated with major biochemical constituents of coffee beans. Prominent differences were observed in lipid-associated CH_2 stretching bands at approximately 2924 and 2854 cm^{-1} , ester carbonyl absorption band around 1745 cm^{-1} , amide/protein-associated bands at approximately 1642 , 1548 , and 1531 cm^{-1} , and carbohydrate/polysaccharide-related fingerprint regions between 1200 and 1000 cm^{-1} . These spectral regions are commonly associated with lipids, proteins, polysaccharides, and other compounds associated with the cell wall in coffee matrices (Wang & Lim, 2012; Castillejos-Mijangos et al., 2022).

Dry-processed beans showed relatively stronger lipid-associated spectral features, particularly in the CH_2 stretching region at 2924 and 2854 cm^{-1} and ester carbonyl region around 1745 cm^{-1} . This suggests a relatively higher contribution of lipid-related molecular

features in the analyzed micro-regions of the dry-processed beans. In contrast, the LTLH beans exhibited more pronounced spectral features in the carbohydrate/polysaccharide fingerprint region, suggesting a stronger contribution of cell-wall-associated polysaccharide structures. Meanwhile, washed and honey-processed beans showed intermediate spectral patterns. The major FTIR bands used for interpretation are summarized in Table 2.

The amide-related region also exhibited differences among the sample groups, particularly around bands 1642 , 1548 , and 1531 cm^{-1} . These bands are mainly associated with protein-related amide I and amide II vibrations, although overlapping contributions from aromatic or phenolic compounds may also be present. Previous studies have shown that post-harvest processing can affect protein modification and phenolic-associated changes in green coffee beans (Figuerola Campos et al., 2022). Therefore, the observed spectral variation suggests differences in the relative contributions of lipid-, protein-, and carbohydrate-associated biochemical features among the commercial samples.

Table 2. Major FTIR band assignments and observed spectral trend among commercial processing types.

Wavenumber region (cm^{-1})	Band assignment	Observed trend in this study
2924 and 2854	Lipid-related CH_2 asymmetric and symmetric stretching vibrations	Relatively more pronounced in dry-processed beans
1745	Ester $\text{C}=\text{O}$ stretching associated with lipid ester/carbonyl containing compounds	Relatively more pronounced in dry-processed beans
1642, 1548, and 1531	Protein-associated amide I and II bands, with possible overlapping contributions from aromatic/phenolic compounds	Minor spectral differences observed among commercial processing types
1200-1000	Carbohydrate/polysaccharide fingerprint region, mainly $\text{C}-\text{O}$ and $\text{C}-\text{O}-\text{C}$ vibrations of cell-wall-associated compounds	Relatively more pronounced in LTLH beans

3.3. PCA analysis of SR-FTIR spectra

Principal component analysis (PCA) was applied to the second-derivative FTIR spectra to explore biochemical variation among the washed, dry, honey, and LTLH commercial coffee samples. As shown in Figure 2, the PCA score plot revealed clustering trends among the four commercial sample groups, indicating variation in their biochemical fingerprints. FTIR spectroscopy combined with multivariate analysis has been widely reported as a useful tool for coffee quality evaluation, authentication, and discrimination based on chemical fingerprints (Wang & Lim, 2012; Castillejos-Mijangos et al., 2022).

PC1 explained 15% of the total spectral variance. Along this component, the dry and LTLH samples were located mainly on the negative side, whereas the washed and honey-processed samples were located on the positive side. PC2 explained a further 12% of the spectral variance and described additional variation in the sample positioning, particularly between the LTLH and dry-processed samples. Together, PC1 and PC2 accounted for 27% of the total spectral variance. The honey-processed sample group showed an intermediate clustering pattern.

Because the coffee beans were commercially obtained and were not

experimentally processed under controlled conditions by the authors, PCA was used as an exploratory multivariate analysis. Accordingly, the clustering trends should be interpreted as comparative biochemical variation among the commercial sample groups rather than as inferential statistical evidence or direct evidence of processing effects. Nevertheless, these trends may be considered in the context of previous reports showing that post-harvest processing and drying conditions can influence the physicochemical properties and metabolite profiles of Arabica coffee beans (Zhang et al., 2019; Aung Moon et al., 2024).

To aid interpretation of the biochemical variation observed in the PCA score plot, the average second-derivative spectra (Figure 1) and FTIR band assignments (Table 2) were considered together. Descriptive comparison of the average spectra showed apparent variation in lipid-related bands, ester carbonyl absorption, amide/protein-associated bands, and carbohydrate/polysaccharide fingerprint regions. Taken together, the results demonstrate the potential of SR-FTIR microspectroscopy combined with PCA as a rapid biochemical fingerprinting approach for the quality characterization of commercial Thai Arabica green coffee beans.

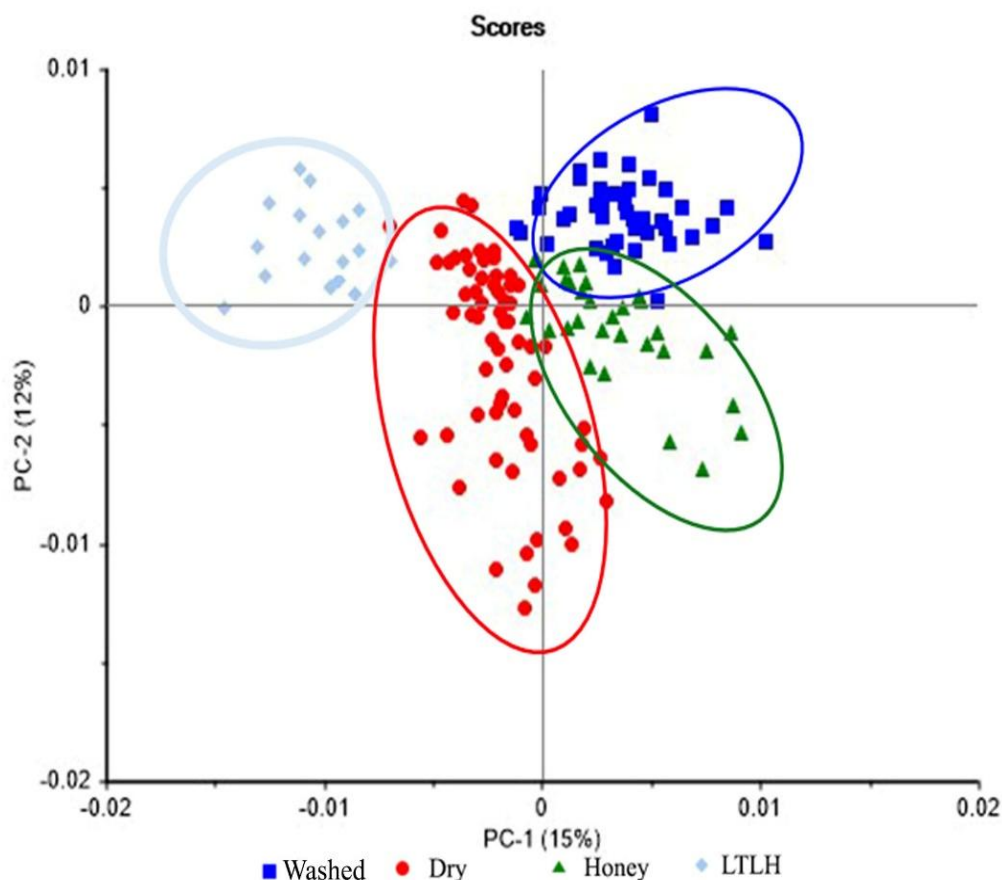


Figure 2. PCA score plot of second-derivative FTIR spectra of commercial Thai Arabica green coffee beans. The score plot shows clustering trends among the washed, dry, honey, and LTLH commercial sample groups, indicating variation in their biochemical fingerprints. The ellipses are included as visual guides to illustrate clustering patterns and do not represent statistical confidence regions.

3.4. Microstructural Characterization by XTM

Synchrotron X-ray tomographic microscopy (XTM) was applied to visualize the internal microstructural features of representative green coffee beans from each commercial processing type (Figure 3). XTM provides non-destructive three-dimensional visualization of internal structures in food materials, allowing observation of internal air spaces and matrix organization without physical sectioning (Schoeman et al., 2016). In the present study, the reconstructed

tomographic images visualized the internal air-channel network and dense bean matrix of the Arabica green coffee beans. Visual comparison of the representative XTM images indicated apparent differences in air-channel distribution, continuity, and structural organization among the commercial samples.

The representative washed and honey-processed beans appeared to exhibit relatively uniform air-channel distributions, whereas the dry-processed beans appeared to have more heterogeneous internal air-channel arrangements. The representative LTLH

sample showed a more continuous and well-defined air-channel pattern than the other commercial samples. One possible explanation is that differences in dehydration rate and moisture-removal history may influence the internal structural organization of green coffee beans. However, because the samples were commercially obtained and were not processed under controlled conditions by the authors, these interpretations should be considered possible mechanisms rather than confirmed causal relationships. In addition, because the LTLH sample was obtained from a different commercial source, potential source- and lot-related variation could not be separated from variation associated with the commercial processing classifications. Nevertheless, these observations suggest that XTM can provide

useful complementary information on the internal structure of green coffee beans, particularly in relation to air-channel organization and matrix continuity.

Previous studies have reported that drying or thermal processing conditions can influence the microstructure and pore characteristics of coffee beans and other food materials. Dong et al. (2018) showed that microwave-vacuum drying affected the drying characteristics, color, microstructure, and antioxidant activity of green coffee beans. Schenker et al. (2000) also demonstrated that roasting conditions affected coffee bean pore structure, which is relevant to heat and mass transfer during roasting. These studies provide useful context for interpreting the apparent structural variation observed in the present XTM images.

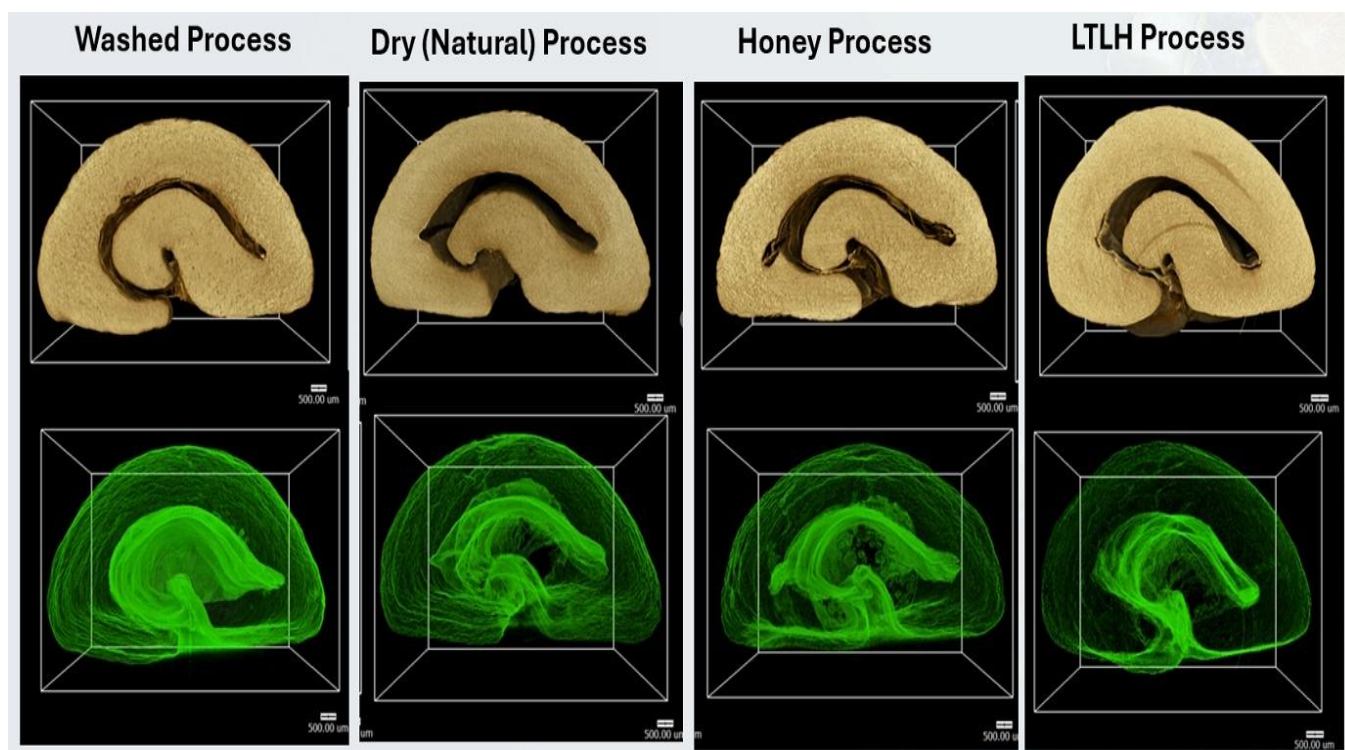


Figure 3. Representative X-ray tomographic microscopy (XTM) images of commercial Thai Arabica green coffee beans representing washed, dry, honey, and LTLH processing types. Green regions represent internal air channels, whereas black regions correspond to dense coffee bean matrix. Apparent differences in air-channel continuity and structural organization were observed among the commercial samples. The XTM images are representative and were used for qualitative visualization and descriptive comparison rather than quantitative pore-network analysis.

In this study, the XTM analysis was intended for qualitative visualization and descriptive comparison of representative internal structures. Due to the limited synchrotron beamtime, three representative beans from each commercial processing type were analyzed. Quantitative image segmentation and morphometric analysis were not performed; consequently, no statistical comparison of pore-network parameters was conducted. Further studies using larger sample numbers and validated quantitative morphometric analysis, including porosity, pore-size distribution, connectivity, and tortuosity, are required to confirm these structural trends.

3.5. Combined Interpretation of Biochemical and Microstructural Features

The integration of SR-FTIR and XTM observations provided complementary information on the biochemical and structural characteristics of the commercial Thai Arabica green coffee bean samples. SR-FTIR microspectroscopy showed variation in lipid-, protein and carbohydrate-associated spectral features, whereas XTM provided representative visualization of internal air-channel organization and dense bean matrix structure.

Dry-processed beans revealed relatively stronger lipid- and ester-associated spectral features, while their representative XTM images appeared to show more heterogeneous internal air-channel arrangements. In contrast, the LTLH sample exhibited more pronounced carbohydrate/polysaccharide-related spectral features, while its representative XTM images appeared to show a more continuous and well-defined internal air-channel network.

The combined SR-FTIR and XTM observations suggest that variation in biochemical fingerprints occurred alongside apparent variation in internal structural organization among the commercial coffee samples. Cell wall polysaccharides,

particularly galactomannans and arabinogalactans, are major structural components of coffee beans and contribute to their physical and quality-related properties (Li et al., 2021). However, the present study did not perform correlative chemical and structural mapping or statistical correlation between the SR-FTIR and XTM results. Therefore, the more pronounced carbohydrate-associated spectral features and the more continuous air-channel pattern observed in the LTLH sample should be interpreted as parallel observations rather than as evidence of a direct relationship.

Previous studies have shown that drying and thermal processing can affect the microstructure, pore characteristics, and heat and mass transfer behavior of coffee beans (Schenker et al., 2000; Dong et al., 2018; Al-Shemmeri et al., 2024). In the present study, however, the coffee beans were commercially obtained and were not experimentally processed under controlled conditions. In addition, because the LTLH sample was obtained from a different commercial source, potential source- and lot-related variation could not be separated from variation associated with the commercial processing classifications. Therefore, the relationship between SR-FTIR spectral variation and XTM structural features should be interpreted as a comparative association among the commercial samples rather than a confirmed causal relationship.

Overall, the combined SR-FTIR and XTM results indicate that synchrotron-based chemical fingerprinting and three-dimensional microstructural visualization can provide complementary information for the advanced quality characterization of commercial Thai Arabica green coffee beans. This integrated approach may support a deeper understanding of quality-related variation among commercial coffee samples. Future studies using controlled processing conditions, larger sample numbers, quantitative pore-network analysis, roasting trials, and sensory evaluation are required to

confirm the relationships among biochemical fingerprints, microstructural features, roasting performance, and sensory quality.

4. Conclusion

This study demonstrated the potential of synchrotron-based techniques for advanced quality characterization of commercial Thai Arabica green coffee beans. SR-FTIR microspectroscopy combined with PCA revealed clustering trends among commercial green coffee bean samples representing washed, dry, honey, and LTLH processing types, based on biochemical fingerprints associated with lipid-, protein-, and carbohydrate-related spectral features. Complementary XTM analysis provided non-destructive, representative qualitative visualization of internal air-channel organization and dense bean matrix structure.

Among the commercial samples, dry-processed beans showed relatively stronger lipid- and ester-associated spectral features, whereas the LTLH sample exhibited more pronounced carbohydrate/polysaccharide-related spectral characteristics. The representative XTM images of the LTLH sample appeared to show a more continuous air-channel pattern. These observations indicate that the commercial samples varied in both biochemical fingerprints and representative microstructural appearance. However, because the coffee beans were commercially obtained and were not experimentally processed under controlled conditions by the authors, the observed variation should be interpreted as comparative quality-related characteristics rather than as direct causal effects of specific processing parameters. Potential source- and lot-related variation should also be considered because the LTLH sample was obtained from a different commercial source.

Overall, the combined use of SR-FTIR microspectroscopy and XTM provides complementary biochemical and structural information that is not readily obtained from conventional visual grading alone. This

integrated synchrotron-based approach may provide a foundation for further investigation of potential quality indicators for commercial Thai specialty coffee. Future studies should include controlled post-harvest processing conditions, larger sample numbers, quantitative pore-network analysis, roasting trials, and sensory evaluation to validate the relationships among biochemical fingerprints, internal microstructure, roasting performance, and final cup quality.

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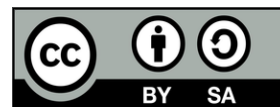
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Declaration of Interest Statement

The authors declare that they have no conflict of interests.

**Research article****PERFORMANCE ENHANCEMENT OF CITRIC ACID–CROSSLINKED BAMBOO–STARCH FILMS: EFFECT OF BAMBOO POWDER DOSAGE**

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ABSTRACT

The performance limitations of starch-based biodegradable films remain a major barrier to their application in food packaging, particularly in terms of mechanical strength, moisture sensitivity, and antimicrobial functionality. In this study, citric acid–crosslinked bamboo–starch composite films were developed and systematically evaluated to elucidate the effect of bamboo powder dosage on the film's structure and performance. To the best of our knowledge, this study is the first to systematically evaluate the effect of bamboo powder dosage (3–6 g) in citric acid–crosslinked bamboo–starch films and correlate dosage-dependent changes with mechanical, barrier, morphological, and antibacterial performance. Bamboo powder loadings of 3, 4, 5, and 6 g were incorporated into a gelatinized potato starch matrix using citric acid as a food-grade crosslinker, with a neat starch film serving as the control. The films were characterized for mechanical, chemical, morphological, barrier, and antibacterial properties. Tensile strength increased markedly with the incorporation of bamboo, reaching a maximum of 9.65 MPa at 6 g of bamboo, representing a 170% improvement over the control (3.57 MPa). However, elongation at break decreased due to restricted polymer chain mobility. FTIR analysis confirmed the formation of an ester bond between starch, bamboo cellulose, and citric acid, indicating enhanced crosslinking efficiency with increasing bamboo content. SEM observations revealed optimal filler dispersion at moderate bamboo loading, whereas higher dosages led to fiber agglomeration and structural heterogeneity. Moisture content decreased progressively from 21.57% in the control to 9.96% at 6 g of bamboo, indicating reduced moisture retention as bamboo incorporation increased. Antibacterial evaluation against *Escherichia coli* showed a dose-dependent increase in inhibition activity, with the largest antibacterial ring radius (3 mm) observed for the highest bamboo loading. Overall, citric acid–crosslinked bamboo–starch films exhibit improvements in mechanical strength, moisture-related properties, and antimicrobial activity, highlighting their strong potential as sustainable active food packaging materials, with the bamboo dosage serving as a critical design parameter.

1.Introduction

The environmental burden caused by persistent petroleum-based plastics has created an urgent need for cleaner packaging solutions. Materials such as polyethylene (PE) continue to dominate the industry due to their mechanical reliability, barrier strength, and low cost. However, their non-biodegradable nature has made them a significant contributor to long-term pollution (Khandeparkar et al., 2024). This has spurred global interest in biodegradable packaging films derived from renewable sources. Although biobased polymers offer clear sustainability advantages, many still fall short in performance, particularly in terms of moisture resistance and mechanical robustness, limiting their direct replacement of conventional plastics (Abdul Khalil et al., 2018). This gap has driven the development of composite systems, in which natural fillers reinforce biopolymer matrices to create stronger, more stable, and functionally improved materials suitable for food packaging applications.

Starch has long been recognized as one of the most promising biodegradable film-forming materials due to its abundance, low cost, and ease of processing. However, its high hydrophilicity, susceptibility to deformation in humid conditions, and generally weak mechanical strength restrict its standalone use (Dirpan et al., 2023; Mohammad et al., 2022). To overcome these limitations, reinforcement from natural fibers or particulate fillers is often required. Bamboo offers distinct advantages in this regard. Its composition, which is rich in cellulose, hemicellulose, and lignin, provides a unique balance of rigidity, thermal stability, and biodegradability (Kumar et al., 2022). Bamboo's microfibrillar structure contributes to better hydrophobicity and barrier behavior, while its rapid growth and natural antimicrobial attributes present additional benefits (Abdul Khalil et al., 2018; F. Song et al., 2015). Compared with other agricultural residues such as banana pseudostem, coconut husk fiber, and bagasse, bamboo provides more consistent strength, durability, and water resistance, making it an attractive candidate to improve starch-based films (Nyerere et al., 2024; Sangeetha et al., 2024; Vinay et al., 2024).

Broad research has explored the potential of

bamboo for film and composite fabrication. Previous studies have demonstrated that bamboo veneer films strengthened by hydrogen bonding (X. Liu et al., 2024), active films incorporating bamboo leaf antioxidants with ZnO nanoparticles (J. Liu et al., 2021) and bamboo powder reinforced matrices modified using chitosan, ZnO or TiO₂ (Guan et al., 2019). Bamboo cellulose blended with polyphenols and polyvinyl alcohol (PVA) has also shown potential for extending food shelf life (D. Song et al., 2023), while bamboo chips have been converted into antimicrobial films effective against multiple pathogens (Sobri et al., 2021). Although these findings consistently highlight bamboo's versatility and functional improvements, few studies have specifically examined bamboo–starch composite films, and fewer still have investigated how different bamboo powder dosages, in combination with a food-safe crosslinker, affect film strength, structure, and antimicrobial performance.

Alongside the choice of reinforcement, the crosslinking strategy used during film formation plays a crucial role in determining the stability and behavior of bamboo-based composites. Various crosslinkers, including maleic anhydride (MAH), zinc oxide nanoparticles (ZnO NPs), propanediol diglycidyl ether (PTGE), and deep eutectic solvents (DES) have been applied to improve adhesion, reduce water uptake, and enhance thermal or antibacterial properties (J. Liu et al., 2021; Qian et al., 2015; Su et al., 2023). Among these, citric acid is particularly notable for its food-grade safety, low toxicity, and strong reactivity with hydroxyl-containing polymers, allowing esterification under mild processing conditions (Jin et al., 2017; Widyorini et al., 2017). Citric acid has been shown to strengthen film networks, improve water resistance, and contribute antibacterial effects. While considerable research has explored citric acid-mediated crosslinking in starch-based films, the effect of bamboo powder dosage on the resulting crosslinked network has not been systematically investigated. There is limited understanding of how increasing bamboo content affects crosslinking efficiency, microstructural development, and the mechanical, barrier, and antibacterial properties of bamboo–starch

composite films.

Evaluating these interactions requires close attention to performance indicators repeatedly highlighted in earlier research. Past studies show that morphology, mechanical strength, chemical bonding, barrier performance, and antimicrobial activity are the most critical parameters for determining the suitability of bamboo-reinforced films in food packaging (C. Liu et al., 2024; Sobri et al., 2021; K. Song et al., 2017). SEM findings generally reveal that moderate bamboo incorporation produces a denser, more uniform microstructure, whereas excessive filler leads to fiber agglomeration, voids, and structural defects that compromise strength (Guan et al., 2019; Sobri et al., 2021). FTIR analyses often confirm ester formation in citric acid-treated systems through intensified carbonyl and C–O–C peaks, indicating the development of a more tightly crosslinked network (Jin et al., 2017; Widyorini et al., 2017). Mechanically, bamboo has been shown to increase tensile strength and stiffness when well dispersed, though excessive loading tends to restrict polymer mobility and reduce elongation-at-break (Abdul Khalil et al., 2018; Kumar et al., 2022). Barrier studies similarly report reduced moisture uptake in bamboo-reinforced or citric acid-crosslinked films due to the presence of hydrophobic lignocellulosic components and a denser internal matrix (Chen et al., 2019; F. Song et al., 2015). In biological assessments, films containing bamboo extracts, metal oxides or chemically modified fibers often exhibit enhanced antibacterial behavior, particularly against *E. coli* and other foodborne pathogens (J. Liu et al., 2021; Sobri et al., 2021). These consistent patterns highlight the need for a structured investigation into how different bamboo powder dosages affect key film properties in citric acid-crosslinked starch matrices.

Although bamboo-based films have been widely explored for food packaging applications, existing studies have primarily focused on different bamboo forms, crosslinking agents, and film formulations, with limited attention given to the effect of bamboo powder dosage in citric acid-crosslinked systems. Consequently, the influence of bamboo loading on the morphological, chemical, mechanical,

barrier, and antibacterial properties of bamboo–starch composite films remains insufficiently understood. Therefore, this study aimed to investigate the effect of different bamboo powder dosages (3–6 g) on citric acid-crosslinked bamboo–starch films using SEM, FTIR, tensile, moisture content, and antibacterial analyses. The findings provide new insights into dosage-dependent structure–property relationships and lay the groundwork for optimizing bamboo-reinforced starch films as sustainable food packaging materials.

2. Methodology

2.1. Material

The materials used in this research were raw bamboo (Semantan) purchased from Kelantan, sodium chlorite (NaClO_2 , 80%) brand SIGALD from Sigma-Aldrich, glacial acetic acid (CH_3COOH , 99.5%) brand SIGALD from Sigma-Aldrich, sodium hydroxide (NaOH pellets, 95%) from Thermofisher Scientific, citric acid brand Merck from Sigma-Aldrich (MW: 192.12 g/mol), and ethanol from Thermofisher Scientific provided by Universiti Teknologi MARA.

2.2. Preparation of bamboo powder

The raw Semantan bamboo was first cut into smaller sections using a band saw and oven-dried at 70 °C for 24 h to remove free moisture, which is essential for preventing microbial degradation and improving milling efficiency (Su et al., 2023). The dried sections were then processed with a light-duty cutter to obtain coarse sawdust, after which a second drying step at 60 °C for 24 h was carried out to eliminate bound moisture that could otherwise interfere with subsequent chemical treatments and reduce powder stability (Qian et al., 2015). The fully dried material was further ground using a high-speed laboratory blender to produce fine particles, increasing the surface area and enhancing reactivity during pre-treatment and crosslinking. The powder was then passed through a 100-mesh sieve to obtain a uniform particle size distribution, a critical factor for ensuring consistent dispersion within the starch matrix and reproducible film properties (Huang et al., 2024). The sieved bamboo powder was stored in airtight containers at ambient

temperature to prevent moisture uptake and preserve its physicochemical integrity prior to further processing.

2.3.Preparation of delignified and crosslinked bamboo powder

The bamboo powder (100-mesh fraction) was first subjected to an alkaline pre-treatment, following the approach of Huang et al., (2024), to selectively remove non-cellulosic components and increase the accessibility of reactive functional groups. In this step, the powder was dispersed in 250 mL of distilled water containing 2.5 g of NaOH (1% w/v) and heated at 80 °C for 1 h under continuous agitation. The alkaline environment promotes the swelling of lignocellulosic fibres and cleaves ester and ether linkages associated with hemicellulose and residual waxes, thereby improving subsequent chemical reactivity. The suspension was filtered and rinsed repeatedly with hot distilled water until neutral pH was achieved to ensure complete removal of solubilised hemicellulose and extractives, preventing alkaline residues from interfering with later reactions.

A delignification treatment was then performed by immersing the pre-treated powder in 250 mL of distilled water containing 2.5 g of NaClO₂. The pH was adjusted to ~4.6 using glacial acetic acid, and the mixture was heated at 80 °C for 1 h, in accordance with Liu et al., (2024). Under acidic conditions, NaClO₂ acts as a strong oxidising agent, selectively degrading lignin's aromatic structure while leaving cellulose chains intact. The removal of lignin is crucial for increasing fibre–matrix compatibility and exposing additional hydroxyl groups that participate in crosslinking. The characteristic whitening of the material signified the completion of lignin oxidation. The solid fraction was filtered and washed multiple times with hot distilled water and ethanol to eliminate chlorite by-products and residual organic impurities, preventing unwanted side reactions during crosslinking.

For the crosslinking step, the delignified bamboo powder was immersed in a citric-acid-based solution comprising distilled water, ethanol, and NaOH, as adapted from Liu et al., (2024). 50 mL of 50% (w/v) citric acid solution

prepared from anhydrous citric acid was mixed with 80 mL of distilled water and 80 mL of ethanol, then 20 mL of 6.5 M NaOH was added. The delignified bamboo powder was then immersed in the prepared solution. NaOH partially neutralises citric acid, forming citrate salts that enhance solubility and promote ester formation during heating. The reaction mixture containing the bamboo powder was heated to 90 °C and stirred gently for 1 h. At this temperature, citric acid undergoes dehydration to form an anhydride intermediate, which readily reacts with cellulose hydroxyl groups to form covalent ester bonds. This step is essential for improving the dimensional stability, water resistance, and interfacial adhesion of the bamboo filler when incorporated into the starch film. After crosslinking, the powder was washed repeatedly with deionized water at 80 °C until neutral pH was reached to remove unreacted citric acid and sodium citrate, ensuring chemical purity and stable functional performance. The crosslinked material was then dried and kept at ambient temperature before use.

2.4.Preparation of Bamboo-starch-based film

For the preparation of the bamboo–starch film-forming matrix, the procedure described by Nadia et al., (2022) was adapted. Initially, 3 g of potato starch was dispersed in 100 mL of distilled water and heated to 70 °C for 30 min under continuous stirring. This temperature range is critical for starch gelatinisation, during which granules swell, lose crystallinity, and release amylose, forming a homogeneous hydrocolloid matrix capable of embedding solid fillers. After gelatinisation, glycerol was incorporated at 25% (w/w) relative to the dry starch content. Glycerol functions as a plasticiser, reducing intermolecular hydrogen bonding within the starch network and thereby improving film flexibility and preventing brittleness during drying. The crosslinked bamboo powder was then introduced into the gelatinised starch matrix. Continuous high-speed stirring for 1 h was employed to ensure uniform dispersion of the filler, a necessary condition for achieving consistent mechanical reinforcement and preventing aggregation, which could otherwise create weak points in the final film. A fixed volume of 25 mL of the

resulting film-forming mixture was cast into each Petri dish (90 mm diameter) and dried at 70 °C for 24 h. This drying protocol promotes gradual solvent evaporation, allowing the polymer network to reorganize without cracking and facilitating strong interfacial adhesion between starch and the citric-acid-modified bamboo particles. The same procedure was performed for all formulations containing 3 g, 4 g, 5 g, and 6 g of bamboo powder to maintain process consistency. A control film was also prepared using only starch and glycerol, without the addition of crosslinked bamboo, to allow direct assessment of the reinforcement and barrier contributions introduced by the modified bamboo filler.

2.5. Bamboo-starch based film characterization

2.5.1. Mechanical Properties

Mechanical performance was evaluated using a universal tensile testing machine, operated at 25 °C and 50% relative humidity to ensure standardised testing conditions. A 5 kN load cell and a strain rate of 10 mm/min were applied. Prior to tensile testing, the thickness of each film specimen was measured and entered the tensile-testing software together with the specimen width. Tensile strength was calculated based on the maximum force divided by the initial cross-sectional area of each specimen. Tensile strength and elongation at break were measured to assess the influence of crosslinked bamboo powder on the films' mechanical performance.

2.5.2. Chemical Properties

Chemical structure and functional group interactions were examined using Fourier Transform Infrared Spectroscopy (FTIR). Small sections of the films were placed in the sample holder, and spectra were recorded across 4000–400 cm⁻¹ to detect characteristic absorbance peaks associated with starch, bamboo cellulose, and citric-acid-induced esterification. Changes in peak position or intensity were used to confirm chemical bonding and structural modifications resulting from crosslinking.

2.5.3. Morphological Properties

Surface morphologies were analysed using SEM operated at 5 kV, allowing high-resolution imaging of fibre distribution, filler–matrix

compatibility, and structural defects. The electron beam scans the film surface and generates detailed microstructural information critical for evaluating dispersion quality, interfacial adhesion, and the impact of increasing bamboo content on film uniformity.

2.5.4. Barrier Properties

Moisture content was determined using an oven-drying method (Murillo & López, 2015), where samples were weighed (W_1), dried at 80 °C for 24 h, cooled, and reweighed (W_2). The moisture content was calculated using Equation (1):

$$\text{Moisture content (\%)} = \frac{(W_1 - W_2)}{W_1} \times 100 \quad (1)$$

where W_1 is the initial weight of the film before drying (g), and W_2 is the final weight of the film after drying (g). This test was used to evaluate the moisture-retention characteristics of the films, where a lower moisture content indicates reduced moisture retention associated with crosslinking and the incorporation of bamboo.

2.6. Antimicrobial Properties

The antibacterial activity of the films was assessed using an agar diffusion assay (Guan et al., 2019). First, 5.6 g of agar was dissolved in 200 mL of distilled water for 5–10 minutes to obtain a homogeneous medium. The prepared medium was then sterilized by autoclaving at 121 °C for 15 minutes to eliminate microbial contaminants. The sterilized agar was cooled to 40–50 °C, an essential temperature range that prevents thermal degradation of bacterial cells while maintaining the agar in a pourable state, and 25 mL of the medium was dispensed into sterile petri dishes and allowed to solidify at room temperature. The agar surface was uniformly inoculated with an *Escherichia coli* suspension using a sterile cotton swab to obtain a uniform bacterial lawn. Film samples were cut into 15-mm discs and placed on the inoculated agar. The control starch film without bamboo powder was used as the negative control. No separate positive antimicrobial control was included in the original experimental design. The plates were incubated at 37 °C for 24 h, after

which the inhibition zones were measured, and the antibacterial ring radius was calculated to quantify the films' antibacterial activity. The antibacterial ring radius was calculated using Equation (2):

$$\text{Antibacterial ring radius (mm)} = (d_1 - d_2)/2$$

→ Equation 2

where d_1 is the outer diameter of the inhibition zone (mm), and d_2 is the diameter of the film disc (mm).

2.7. Statistical analysis

Statistical analysis of mechanical performance was performed in Microsoft Excel using one-way analysis of variance (ANOVA).

Post hoc Tukey's tests were performed using IBM® SPSS® Statistics to detect significant differences at $p < 0.05$.

3. Results and discussion

Figure 3.1 shows the visual appearance of bamboo-starch-based films crosslinked with citric acid, Figure 3.1(a) represents the control film containing only potato starch and glycerol, 3.1(b) - (e) show films incorporating increasing amounts of bamboo powder, which are 3g (b), 4g (c), 5g (d), and 6g (e). The films were obtained by peeling them from the petri dish after the drying process was completed during the bamboo-starch-based film formation.

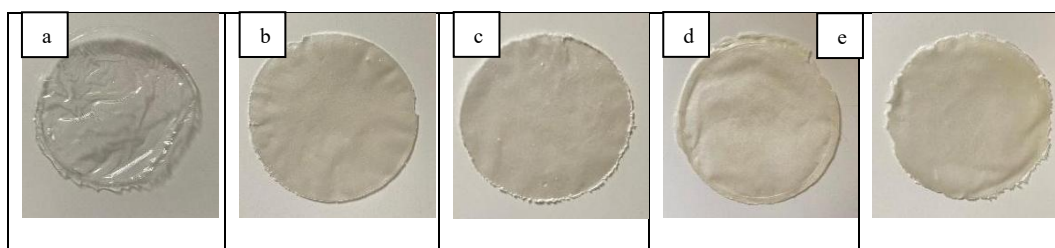


Figure 3.1. Bamboo-starch based film crosslinked with citric acid, a) control sample, b) 3g of bamboo, c) 4g of bamboo, d) 5g of bamboo, e) 6g of bamboo

3.1. Mechanical performance

Figure 3.2 presents the influence of bamboo powder loading on the tensile strength of citric acid-crosslinked bamboo-starch films. A pronounced increase in tensile strength was observed with increasing bamboo content, reaching a maximum of 9.65 ± 1.18 MPa at 6 g bamboo, corresponding to approximately a 170% increase relative to the control film (3.57 ± 0.45 MPa). According to Tukey's HSD test ($p < 0.05$), the control and 4 g films belonged to the same statistical group (a), while the 3 g and 6 g films formed a higher tensile strength group (b). The 5 g film exhibited intermediate behavior (ab) and was not significantly different from either group. This improvement is attributed to the reinforcing effect of bamboo-derived cellulose, which increases load-bearing capacity by enabling efficient stress transfer and enhancing intermolecular interactions within the polysaccharide network. At lower filler loading, the incorporation of 3 g bamboo significantly increased tensile strength (7.68 ± 0.52 MPa), indicating favorable dispersion and effective filler-matrix adhesion. In contrast, a marked

reduction in tensile strength at 4 g of bamboo (3.66 ± 1.65 MPa) suggests disruption of network continuity, which is likely caused by the local aggregation of bamboo particles acting as stress concentrators. Such non-monotonic behavior is characteristic of polysaccharide-based composites in which mechanical performance is governed by a balance between filler content and dispersion state (C. Liu et al., 2024). The subsequent recovery in tensile strength at higher bamboo loadings (5–6 g) implies that increased availability of hydroxyl functionalities from bamboo cellulose promotes more extensive citric acid-mediated ester crosslinking and hydrogen bonding. This results in a denser and more interconnected polymer network, thereby compensating for dispersion-related defects and improving mechanical integrity (Abdul Khalil et al., 2018). Overall, these results underscore that the tensile performance of citric acid-crosslinked starch composites is controlled by the interplay between filler-induced reinforcement and network homogeneity, rather than filler content alone.

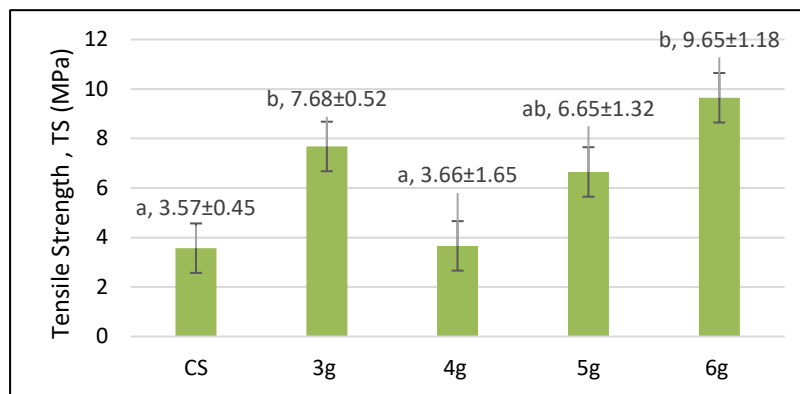


Figure 3.2. Effect of bamboo powder dosage on the tensile strength of citric acid-crosslinked starch film. Data presented as mean $\pm$ S.D. ($n = 3$). Different alphabets between columns represent significance at $p < 0.05$ after applying post hoc Tukey's test.

Figure 3.3 illustrates the effect of bamboo powder dosage on the elongation at break of citric acid-crosslinked bamboo-starch films. The elongation at break of all bamboo-containing films was significantly lower than that of the control film ($p < 0.05$), whereas no significant differences were observed among the bamboo-containing formulations. The control film exhibited the highest elongation at break ($31.40 \pm 5.52\%$), reflecting the high flexibility and unrestricted chain mobility of the starch matrix in the absence of rigid lignocellulosic fillers. Upon incorporation of bamboo powder, a pronounced reduction in elongation was observed, indicating a progressive loss of film ductility. At 3 g bamboo, the elongation at break decreased sharply to $6.70 \pm 0.18\%$, corresponding to a reduction of approximately 78.7% relative to the control. Further increases in bamboo loading resulted in continued decreases in elongation, with the lowest value

recorded at 6 g bamboo ($4.44 \pm 2.15\%$, ~85.9% reduction). This monotonic decline suggests that bamboo incorporation significantly restricts polymer chain mobility, primarily due to the rigid and fibrous nature of bamboo cellulose, which limits segmental motion within the starch matrix. In addition, the formation of citric acid-mediated ester crosslinks and hydrogen bonding further increases network density, thereby enhancing stiffness at the expense of extensibility. Similar reductions in elongation at break have been reported for starch- and cellulose-reinforced polysaccharide composites, where increased filler content and crosslink density promote brittleness and suppress ductile deformation (Abdul Khalil et al., 2018; Rashid et al., 2023). Overall, the results highlight a clear strength-ductility trade-off in citric acid-crosslinked starch films, in which improved mechanical reinforcement is accompanied by reduced flexibility as bamboo dosage increases.

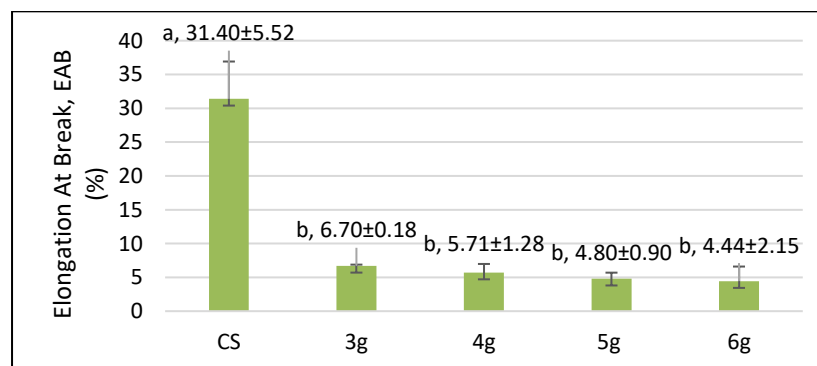


Figure 3.3. Effect of bamboo powder dosage on the elongation at break of citric acid-crosslinked starch films. Data presented as mean $\pm$ S.D. ($n = 3$). Different alphabets between columns represent significance at $p < 0.05$ after applying post hoc Tukey's test.

3.2. Chemical Properties

Figure 3.4 presents the FTIR spectra of bamboo–starch–based films with varying bamboo loadings (3–6 g) alongside the control sample. Clear compositional and structural modifications were observed as a function of bamboo content in films crosslinked with citric acid. In Region I ($3700\text{--}3000\text{ cm}^{-1}$), all samples exhibited a broad O–H stretching band. The control film exhibited an intense and broad peak centered at approximately 3300 cm^{-1} , reflecting the high density of hydroxyl groups inherent to potato starch. With increasing bamboo incorporation (3–6 g), this band became broader and exhibited slight peak shifts, indicating enhanced hydrogen bonding and partial esterification between hydroxyl groups of starch and bamboo cellulose with the carboxyl groups of citric acid (Omar et al., 2022). As the citric acid concentration was maintained constant, these spectral changes are attributed to the increased availability of hydroxyl groups introduced by bamboo, which promoted more effective crosslinking within the polymer matrix.

In Region II ($3000\text{--}2800\text{ cm}^{-1}$), C–H stretching vibrations displayed minor peak sharpening and shifts with increasing bamboo content, suggesting interactions between the aliphatic components of bamboo and the starch matrix, consistent with previous reports on bamboo-reinforced biopolymer films (Abdul Khalil et al., 2018). Region III ($1750\text{--}1600\text{ cm}^{-1}$) exhibited a pronounced intensification of carbonyl (C=O) stretching bands in the range of $1720\text{--}1745\text{ cm}^{-1}$, characteristic of ester linkages formed between the carboxyl groups of citric acid and the hydroxyl groups of lignocellulosic constituents. It is suggested that the addition of bamboo enhanced the esterification degree, as the greater availability of hydroxyl groups in bamboo cellulose provides more active sites for reaction with citric acid, promotes the formation of ester bonds, and thereby increases the crosslinking density. The observed peak shift from approximately 1640 to 1646 cm^{-1} further confirms the successful formation of the ester bond, with bamboo cellulose contributing additional reactive sites for crosslinking

(Widyorini et al., 2017).

In Region IV ($1200\text{--}900\text{ cm}^{-1}$), a progressive increase in the intensity of C–O–C stretching vibrations was detected with higher bamboo loading, indicating enhanced polysaccharide interactions and the formation of a more organized and interconnected polymer network (Huang et al., 2024). This structural densification was further supported by a consistent decrease in transmittance intensity with increasing bamboo content. Specifically, transmittance at 3315 cm^{-1} decreased from 74.02% in the control film to 53.08% in the 6 g bamboo film, while in Region IV ($\approx 1000\text{ cm}^{-1}$) it declined from 51.45% to 13.08%. These reductions in light transmission reflect increased molecular packing density and reduced free volume arising from bamboo-enhanced crosslinking.

Overall, the FTIR results confirm that, despite a constant citric acid concentration, increasing bamboo content significantly enhances crosslinking efficiency, leading to pronounced modifications in chemical bonding, matrix organization, and optical behavior of the bamboo–starch–based films.

3.3. Morphological structure

Figure 3.5 presents the surface morphology of bamboo–starch–based films containing different bamboo powder loadings and crosslinked with citric acid, as observed by SEM at $100\times$ magnification. The control film, composed solely of gelatinized potato starch, exhibited a relatively smooth and continuous surface with limited surface defects. Minor microcracks and faint outlines of starch granules were observed, which are commonly associated with the brittle nature of uncrosslinked starch films due to weak intermolecular cohesion, as previously reported by Hirpara et al., (2021). The control film, composed of gelatinized potato starch and glycerol without crosslinked bamboo powder, exhibited a relatively smooth, continuous surface with few surface defects. Minor microcracks and faint outlines of starch granules were observed, which may be associated with the inherent structural characteristics of starch-based films.

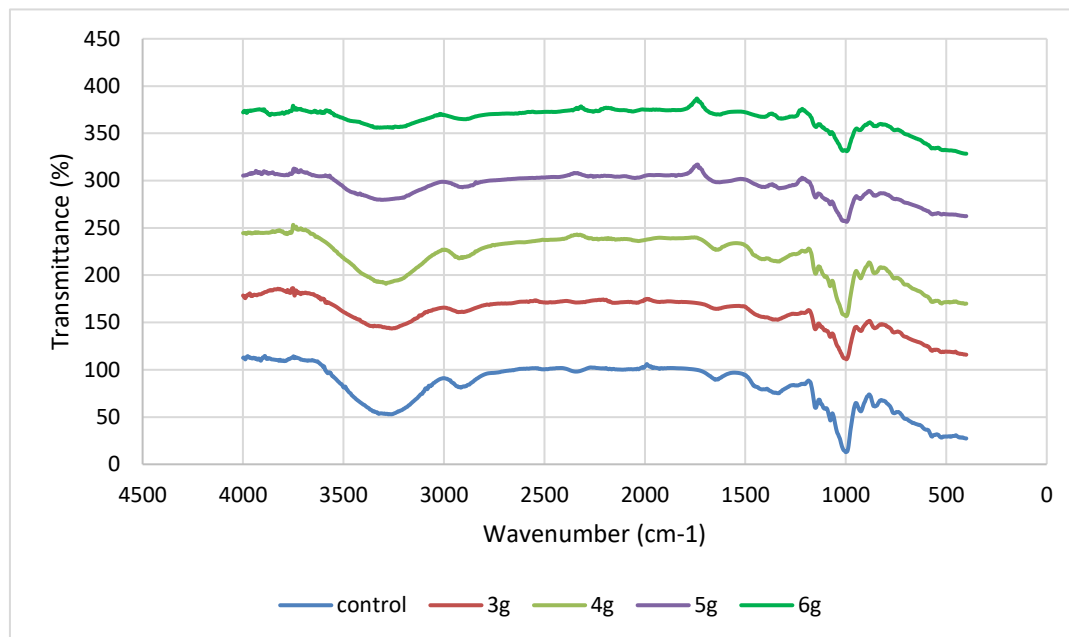


Figure 3.4. FTIR spectra of different bamboo powder dosages on citric acid-crosslinked starch films

Upon incorporation of 3 g of bamboo powder, the film surface became noticeably rougher, with dispersed fibrous features embedded within the starch matrix. This morphology suggests an effective interfacial interaction between bamboo cellulose and starch, facilitated by citric acid acting as a compatibilizing cross-linker between the hydroxyl groups of both components. Increasing the bamboo content to 4 g further intensified surface roughness; however, the bamboo fibers remained uniformly distributed without visible agglomeration. This sample exhibited the most homogeneous and compact morphology, indicating optimal filler dispersion and effective stress transfer within the polymer network, consistent with observations reported by Abdul Khalil et al., (2018).

In contrast, films containing 5 g of bamboo powder exhibited pronounced surface irregularities, characterized by fiber clustering and voids within the matrix. These features indicate the onset of filler agglomeration and reduced matrix continuity, suggesting that the bamboo content exceeded the dispersion capacity of the starch-citric acid system. Such agglomerated regions can act as stress

concentration points during tensile loading, promoting localized crack initiation and reducing the efficiency of stress transfer between the starch matrix and bamboo filler. This effect became more severe at 6 g bamboo loading, where extensive fiber agglomeration, surface fractures, and poorly bonded regions were evident. The formation of large fiber bundles and voids reflects inadequate interfacial adhesion and disrupted polymer continuity, which may hinder uniform stress distribution throughout the composite and ultimately compromise film structural integrity (Omar et al., 2022).

Overall, the SEM analysis confirms that bamboo loading has a strong influence on the morphological characteristics of citric acid-crosslinked starch films. Moderate bamboo incorporation, particularly at 4 g, promotes uniform dispersion and strong interfacial bonding, resulting in a well-structured composite. Conversely, excessive bamboo addition leads to filler agglomeration and matrix disruption, indicating that the effectiveness of citric acid as a crosslinker diminishes when filler content surpasses the matrix's dispersion threshold.

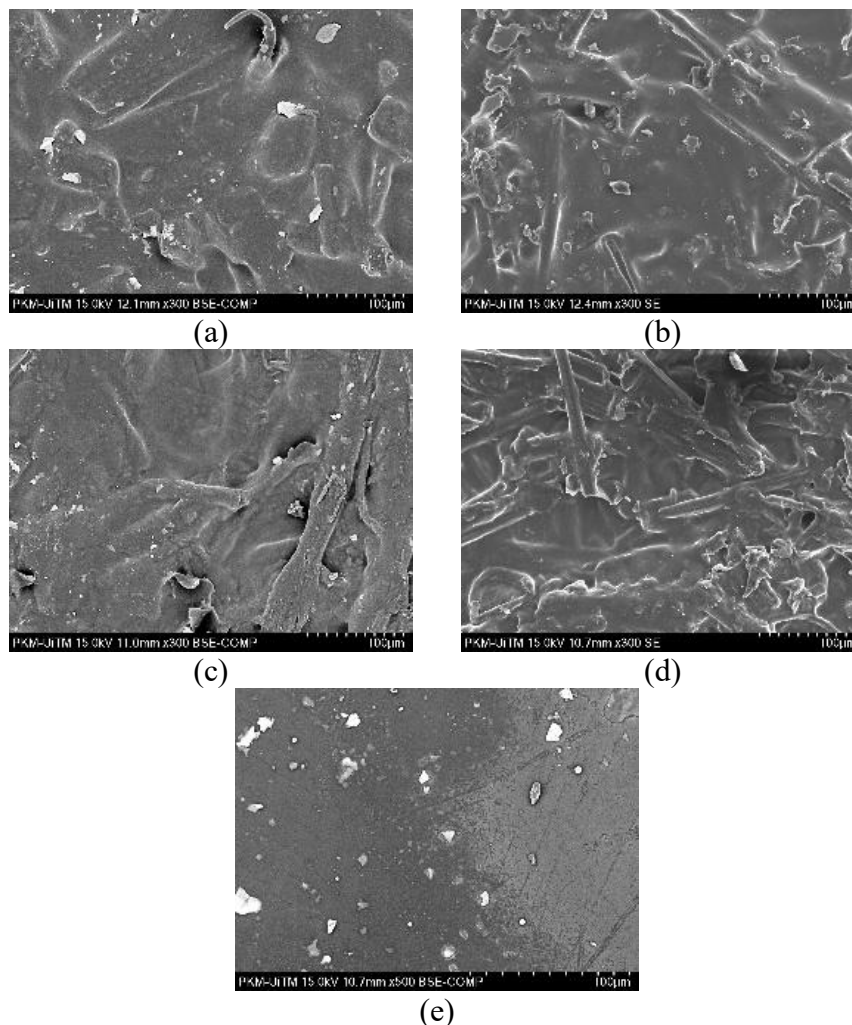


Figure 3.5. Surface morphology of different bamboo powder dosages on citric acid-crosslinked starch films, a) 3g of bamboo dosages, b) 4g of bamboo dosages, c) 5g of bamboo dosages, d) 6g of bamboo dosages, e) control sample

3.4. Moisture Content

Figure 3.6 presents the moisture content of bamboo-starch-based films as an indicator of their moisture-retention behaviour. A clear and monotonic decrease in moisture content was observed with increasing bamboo loading, indicating a progressive reduction in moisture retention. The control starch film exhibited the highest moisture content (21.57%), consistent with the inherently hydrophilic character of starch arising from its high density of hydroxyl (–OH) groups that readily interact with water molecules (Wu et al., 2019). Upon citric-acid crosslinking, partial esterification of these hydroxyl groups reduced the availability of hydrophilic sites and promoted the formation of a more compact polymer network, thereby enhancing water insolubility. The incorporation

of bamboo further reduced moisture retention, with moisture content decreasing from 20.06% at 3 g bamboo to 18.65% at 4 g, 14.62% at 5 g, and reaching a minimum of 9.96% at 6 g bamboo. This substantial reduction reflects increased matrix densification and stronger filler–matrix interactions, which limit free volume and restrict water diffusion pathways within the film. The nearly 54% reduction in moisture content at 6 g of bamboo relative to the control demonstrates a substantial reduction in moisture retention with increasing bamboo incorporation. However, moisture content alone does not directly represent water-vapor barrier performance; therefore, further evaluation using water-vapor permeability or water-absorption measurements is required to establish the barrier properties of these films.

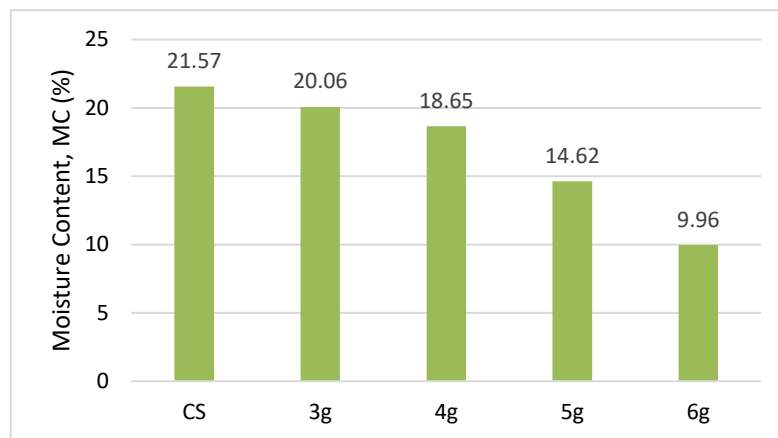


Figure 3.6. Effect of bamboo powder dosage on the moisture content of citric acid-crosslinked starch films.

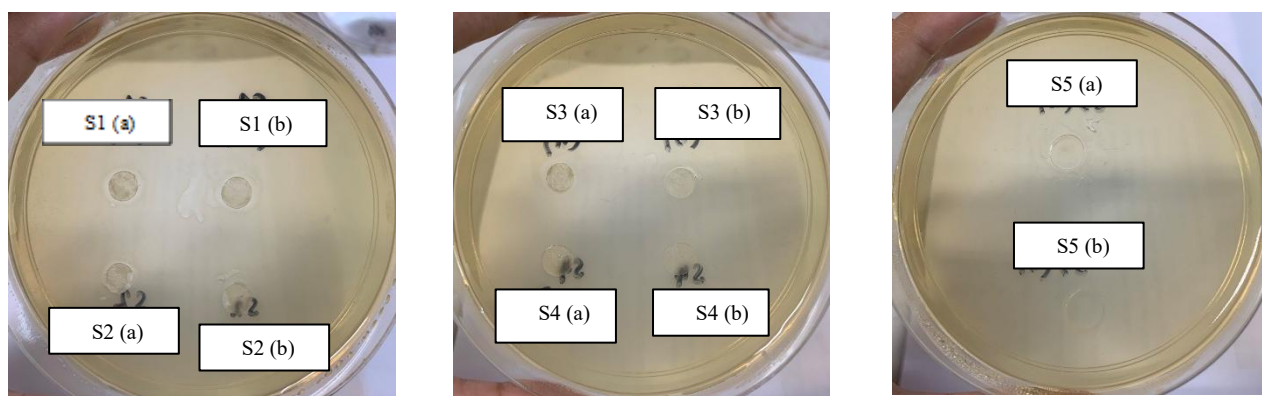


Figure 3.7. Effect of bamboo powder dosage on the antibacterial performance (antibacterial ring radius) of citric acid-crosslinked starch films, S1) 3g of bamboo dosages, S2) 4g of bamboo dosages, S3) 5g of bamboo dosages, S4) 6g of bamboo dosages, S5) control sample.

3.5. Antibacterial Performance

The antibacterial performance of the films was evaluated against *E. coli* using the agar diffusion method, with activity quantified as the antibacterial ring radius. The control film, consisting solely of potato starch, exhibited no detectable inhibition zone (0 mm), confirming the absence of intrinsic antimicrobial activity in native starch matrices (Dudeja et al., 2023). In contrast, all bamboo-reinforced films demonstrated measurable antibacterial activity, with the antibacterial ring radius increasing with bamboo loading. The films containing 3, 4, and 5 g of bamboo exhibited antibacterial ring radii of 1.0, 1.5, and 2.0 mm, respectively. The 6 g bamboo film exhibited the highest antibacterial activity, with an antibacterial ring radius of 3.0 mm. This dose-dependent enhancement is attributed primarily to the presence of bioactive constituents in bamboo, including phenolic

compounds, lignin, and flavonoids. Phenolic compounds can interact with bacterial cell membranes, increasing membrane permeability and causing leakage of intracellular components, while lignin-derived phenolic structures may inhibit microbial enzyme activity and induce oxidative stress within bacterial cells. As bamboo loading increased, a greater concentration of these bioactive compounds became available within the film matrix, thereby enhancing antibacterial efficacy. Additionally, citric acid, used as a crosslinking agent in all bamboo-containing films, may exert a synergistic antimicrobial effect through residual acidic groups within the film network, creating a localized acidic microenvironment that suppresses bacterial growth. This acidic environment can disrupt membrane transport processes, reduce intracellular pH, and further compromise bacterial membrane integrity,

thereby enhancing growth inhibition against *E. coli* (Zhu et al., 2023). From an antimicrobial performance benchmarking perspective, the threefold increase in antibacterial ring radius observed at the highest bamboo loading compared to the lowest reinforced sample highlights the effectiveness of incorporating bamboo in imparting functional antimicrobial activity. These findings are consistent with

4. Conclusion

This study demonstrated that the incorporation of citric acid–crosslinked bamboo powder significantly affects the structural and functional properties of bamboo–starch-based films. Increasing bamboo dosage improved tensile strength, reduced moisture content, and enhanced antibacterial activity, indicating enhanced intermolecular interactions and effective crosslinking within the polymer matrix. The film containing 6 g of bamboo exhibited the highest tensile strength (9.65 MPa), the lowest moisture content (9.96%), and the greatest antibacterial activity against *E. coli*, confirming the role of bamboo as an effective reinforcing and bioactive component. However, higher bamboo loadings resulted in reduced elongation at break and increased surface irregularities, as observed in SEM analysis, suggesting limitations associated with filler agglomeration and restricted polymer chain mobility. These results indicate a trade-off between mechanical strength and flexibility that must be considered when designing starch-based composite films for food packaging applications. Overall, citric acid proved to be an effective food-grade green crosslinker, promoting ester bond formation and improved interfacial adhesion between starch and bamboo components. The findings highlight the potential of citric acid–crosslinked bamboo–starch films as environmentally friendly, biodegradable materials for food packaging. The improved mechanical strength, reduced moisture content, and antibacterial performance demonstrate the potential of these films for further development as sustainable food-packaging materials. The reduced moisture content may help minimize moisture transfer during storage, while the observed antibacterial activity against *E. coli* warrants further evaluation of the films in actual

previous reports demonstrating improved *E. coli* inhibition in starch-based films containing plant-derived bioactive fillers (Dudeja et al., 2023). Overall, the bamboo-reinforced, citric-acid-crosslinked films developed in this study exhibit promising antimicrobial functionality, supporting their potential application in sustainable active food packaging systems.

food systems. In addition, the biodegradable nature of the bamboo–starch matrix offers a sustainable alternative to conventional petroleum-based packaging materials, supporting current efforts toward environmentally responsible food packaging solutions. Further studies focusing on dispersion optimization and application-specific performance requirements are recommended to enhance film flexibility while maintaining functional properties.

5. References

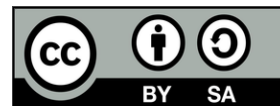
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Research article

CHARACTERISATION OF CHITOSAN-STARCH FILM CONTAINING BANANA PEEL EXTRACT FOR POTENTIAL ACTIVE FOOD PACKAGING APPLICATIONS

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ABSTRACT

This study investigates the development of biodegradable composite films composed of chitosan-starch (CS) and banana peel extract (BPE) for food packaging applications. Films with varying BPE concentrations (0-2%) were analysed for physicochemical, mechanical and antimicrobial properties. Fourier Transform Spectroscopy (FTIR) confirmed enhanced intermolecular interactions due to polyphenolic compounds in the extract. Tensile strength increased from 28 MPa to 39 MPa, while elongation at break increased from 45% to 87%. Additionally, antibacterial activity against *E. coli* improved with increasing BPE concentration, with inhibition zones increasing from 1.00 cm to 1.70 cm showed increased *E. coli* inhibition with higher BPE levels. This study demonstrates the valorisation of banana peel waste as a multifunctional bioactive additive in chitosan-starch films, simultaneously enhancing mechanical, barrier and antimicrobial properties.

1.Introduction

Food preservation plays a critical role in extending the shelf life of food products, preventing spoilage, and ensuring food safety. With an estimated one-third of the world's food being wasted annually, effective preservation methods are crucial to reducing food loss and enhancing food security. However, conventional preservation techniques, such as refrigeration, freezing, and canning, while effective, often result in non-sustainable packaging materials that contribute to environmental pollution. The

growing demand for food packaging has led to an increase in the use of synthetic plastics, whose non-biodegradable nature poses long-term ecological risks (Mia, 2023).

To address these concerns, researchers have explored biodegradable packaging materials derived from renewable sources as a sustainable alternative to petroleum-based plastics. These biodegradable films not only reduce plastic waste but also offer functional properties suitable for food packaging applications. Among these, chitosan and starch emerged as

widely studied biopolymers due to their film-forming ability and biodegradability for composite films. More recently, BPE has gained attention for its richness in bioactive compounds, particularly phenolics, which can further enhance the functional and protective performance of biodegradable films (Evyang et al., 2022; Oyekunle et al., 2024; Razak et al., 2020).

Despite the potential of CS films infused with BPE for sustainable food packaging, further research is needed to fully understand their synergistic effects. While previous studies have highlighted the individual benefits of each material, very few studies report a comprehensive analysis of the structural, mechanical, optical, and antibacterial effects of varying BPE concentrations in biopolymer matrices. In particular, the combined influence of BPE on intermolecular interactions, water barrier behaviour, and microbial inhibition in CS-based films remains underexplored. Therefore, this study aims to investigate the antibacterial properties of CS films with BPE, exploring their potential as effective and environmentally friendly solutions for food preservation (Luchese et al., 2018; Priyadarshi & Rhim, 2020).

Recent studies have demonstrated the potential of natural plant extracts as active additives in biodegradable packaging films owing to their antimicrobial activities (Cazon et al., 2025). Although banana peel-derived components have been investigated in starch-based films, the concentration-dependent effects of banana peel extract within a chitosan–starch matrix remain insufficiently characterised, particularly regarding the relationship between spectral changes, mechanical response and antibacterial activity. Existing studies have generally focused on selected film properties or different polymer matrices, making direct comparison of these responses within a chitosan–starch system difficult (Ahmed et al., 2025).

Most previous studies focused on starch-based films or investigated only selected film properties. Comprehensive evaluation of the simultaneous effects of BPE on molecular

interactions, mechanical performance, water resistance and antimicrobial activity within chitosan–starch matrices is still limited. Therefore, this study investigates the effect of BPE concentration (0–2%) on the FTIR characteristics, tensile properties and antibacterial activity of chitosan–starch films against *E. coli*. The study seeks to determine how increasing BPE concentration is associated with changes in the physicochemical and functional properties of the composite films.

2. Material and Methods

2.1. Material

Dwarf Cavendish bananas from Pasar Borong Selangor were cleaned, dried and used for extraction. Chitosan powder served as the primary biopolymer, while corn starch formed the film matrix. Glycerol improved film flexibility, acetic acid solubilised chitosan, and ethanol extracted bioactive components from banana peel. Materials were chosen based on availability, cost-effectiveness, and suitability for food preservation. All measurements were conducted in triplicate ($n = 3$), and the results are reported as mean $\pm$ standard deviation.

2.2 Methods

2.2.1. Preparation of Banana Peel Powder

The ripe banana peel was cleaned, dried with absorbent paper and chopped into small pieces. About 100 g of peel was boiled in 500 mL of distilled water at 90°C for 1 hour to extract bioactive compounds. The peels were dried with tissue and oven-dried at (40–50) °C for 12–24 hours, then ground into a fine powder and stored in a sealed container.

2.2.2. Preparation of Banana Peel Extract

BPE of 0.5%, 1.0%, 1.5% and 2% were prepared by mixing the corresponding amounts of banana peel powder (0.5g, 1.0 g, 1.5 g and 2 g) with ethanol (100 mL). The mixtures were stirred for 24 hours at room temperature, then filtered using vacuum filtration. The liquid extracts were collected and stored in sealed containers for further use.

2.2.3. Preparation of Starch Solution

2 g of cornstarch were mixed with 100 mL of distilled water, followed by the addition of 0.5

mL of glycerol to the mixture. The solution was heated to 75°C and stirred at 750 rpm until the cornstarch was fully dissolved and the solution became clear. Once clear, stirring continued for an additional 30 minutes to ensure uniformity and consistency throughout the solution.

2.2.4. Preparation of Chitosan Solution

1.5 g of chitosan was added to 100 mL of distilled water, followed by 0.5 mL of acetic acid to facilitate the dissolution of the chitosan. The solution was stirred at 350 rpm at room temperature until the chitosan fully dissolved and the solution became clear. After dissolution, the solution was left undisturbed for 24 hours to allow complete settling.

2.2.5. Preparation of Biodegradable Film

Chitosan and starch solutions were mixed in a clean beaker and stirred for several minutes to ensure a uniform blend. Twenty millilitres of the mixed solution were transferred into a separate beaker, and BPE at varying concentrations (0%, 0.5%, 1.0%, 1.5% and 2%) was added. The mixture was stirred for several minutes to distribute the extract a thin layer of approximately 10 mL was poured into a clean Petri dish and spread evenly to ensure consistent film thickness. The Petri dish was placed in an oven set to 50°C and allowed to dry for 24 hours.

2.3. Preparation of Agar Plates

14 g of nutrient agar powder was weighed and mixed with 500 mL of distilled water in a clean container. The mixture was stirred thoroughly until the agar powder was fully dissolved. The solution was then transferred into a reagent bottle, sealed tightly and autoclaved at 121°C and 15 psi for 15–20 min and the medium was allowed to cool. In a sterile environment, 20 mL of the agar solution was poured into a sterile petri dish. The Petri dishes were sealed with parafilm to prevent contamination and stored in a refrigerator for at least 24 hours before use.

2.4. Preparation of Antimicrobial Analysis

The *Escherichia coli* (*E. coli*) culture tube was gently swirled to ensure the bacteria were transferred onto the prepared agar plate, and a spreader was used to evenly distribute the bacteria across the surface. Paper discs were

dipped into a solution of chitosan and starch with varying concentrations of BPE (0%, 0.5%, 1.0% and 1.5%). Paper discs were soaked in the film solution were placed carefully onto the inoculated agar plates, ensuring they were evenly spaced. The Petri dish was covered with a lid, sealed with parafilm to prevent contamination and placed in an incubator for 24 hours to allow for bacterial growth and observe any antimicrobial effects. They were incubated at 37°C and inhibition zones were measured using a digital caliper.

2.5. Fourier Transform Spectroscopy Analysis (FTIR)

Fourier Transform Spectroscopy Analysis (FTIR) was used to identify and analyse the chemical composition of a material by detecting its molecular vibrations. It provided information about the functional groups present in the chitosan, starch and BPE samples. The films were analysed using FTIR with a range of 400–4000 cm⁻¹ and a resolution of 4 cm⁻¹ (Medeiros Silva et al., 2020).

2.6. Tensile Strength Analysis

The tensile strength (TS), elongation at break (EB), and elastic modulus (EM) tests were conducted on all films using an Instron H50KT Tinius Olsen universal testing machine equipped with a tensile group probe. The film samples were cut into rectangular grips (3 cm x 7 cm) and positioned in the film-extension grips of the texture testing machine, following the standard test method outlined in ASTM B882-10: Standard Test Method for Tensile Properties of Thin Plastic Sheeting. The test was conducted at a crosshead speed of 10 mm/s. (Chandrasekar et al., 2023). The film samples were stretched until breaking. A microcomputer was used to log the stress-strain curves. Some studies have used amylases to measure tensile strength, with values ranging from approximately 4.43 to 31.20 MPa (Taweecat et al., 2021). The recommended range for tensile strength is 5 to 25 MPa, as this balance of strength and flexibility is required for biodegradable films (Tan et al., 2022). For each film, a minimum of three replicate tests were performed. The results

of tensile strength were expressed in MPa, and elongation at break was expressed in % (Saber et al., 2017).

2.7. Statistical Analysis

All experiments were performed in triplicate and results are presented as mean $\pm$ standard deviation. Statistical analysis was conducted using one-way analysis of variance (ANOVA) to evaluate the effect of banana peel extract concentration on film properties. Significant differences among means were determined using Tukey's post hoc test at a confidence level

of 95% ($p < 0.05$). Statistical analyses were performed using IBM SPSS Statistics software.

3. Results and Discussion

3.1. Fourier Transform Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to evaluate the molecular interactions between CS with various concentrations of BPE extract. The spectra for pure CS films and composite films with 0.5%, 1.0%, 1.5% and 2.0% BP extract are shown in Fig. 1.

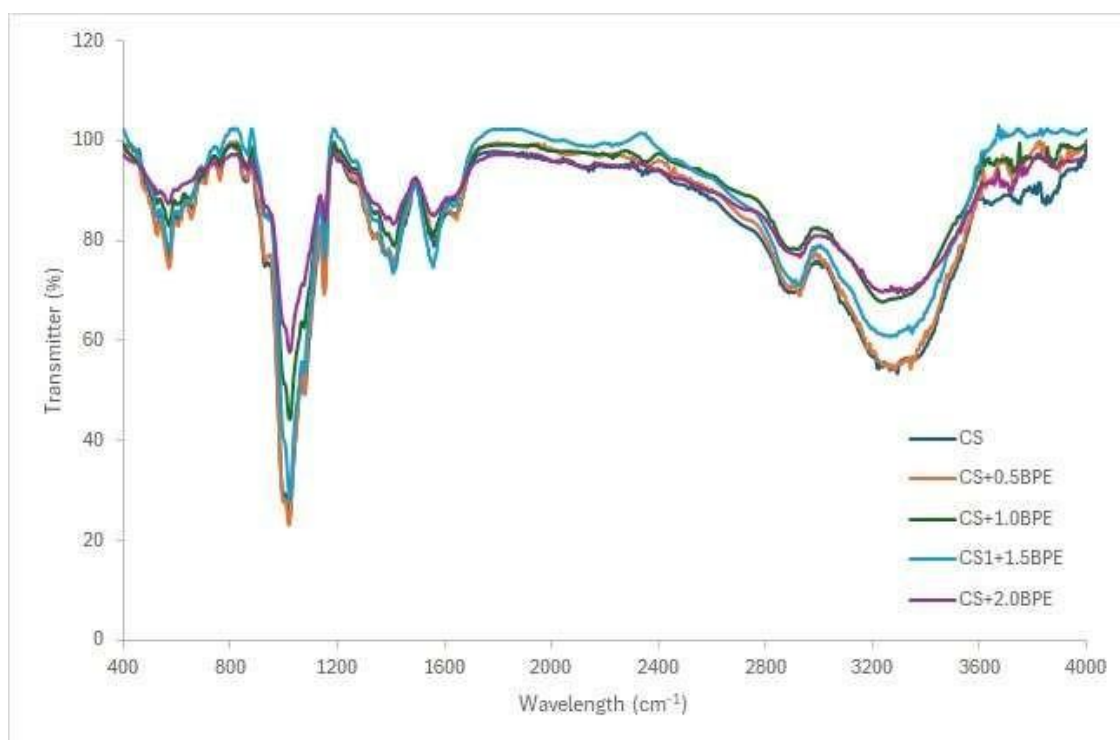


Figure 1. FTIR spectra of CS films with different BPE concentrations

The broad absorption band observed in the region of 3200-3400 cm^{-1} corresponded to the stretching vibrations of O-H and N-H functional groups. This band became broader and more intense with increasing BPE concentration, particularly at 1.5% and 2.0%, suggesting enhanced hydrogen bonding. Resulting from interactions between hydroxyl-rich phenolic compounds in the BPE and the CS matrix (Medeiros Silva et al., 2020).

The peak near 1640-1650 cm^{-1} , corresponding to C=O stretching (amide I) and possibly aromatic C=C stretching, also

increased in intensity with BPE addition. This indicates the presence and incorporation of polyphenolic and aromatic compounds from the BPE extract into the film matrix (Medeiros Silva et al., 2020). These observations are consistent with the findings of Medeiros Silva et al. (2020), who reported similar peak shifts and intensifications when incorporating plant extract into polysaccharide films.

In the region between 100-1150 cm^{-1} , associated with C-O and C-O-C stretching vibrations of polysaccharides, the spectra exhibited stronger absorbance with increasing

BPE concentration. This indicated that the natural polysaccharides in banana peel contribute to the film's structure, complementing the existing CS backbone. Minor but noticeable shifts were also observed in the fingerprint region ($600\text{-}900\text{ cm}^{-1}$), indicating subtle changes in the molecular structure resulting from BPE incorporation.

Overall, increasing the concentration of BPE from 0.5% to 2.0% resulted in more pronounced structural changes in the FTIR spectra. These changes indicate alterations in the vibrational environment of the composite matrix following

BPE incorporation, although the specific molecular interactions responsible cannot be conclusively identified from FTIR alone.

3.2. Tensile Strength

The mechanical performance of the BPE composite film was significantly influenced by the incorporation of BPE. As shown in Fig. 2, both tensile stress and strain exhibited non-linear trends across different BPE concentrations, indicating complex interactions between the extract and the biopolymer matrix.

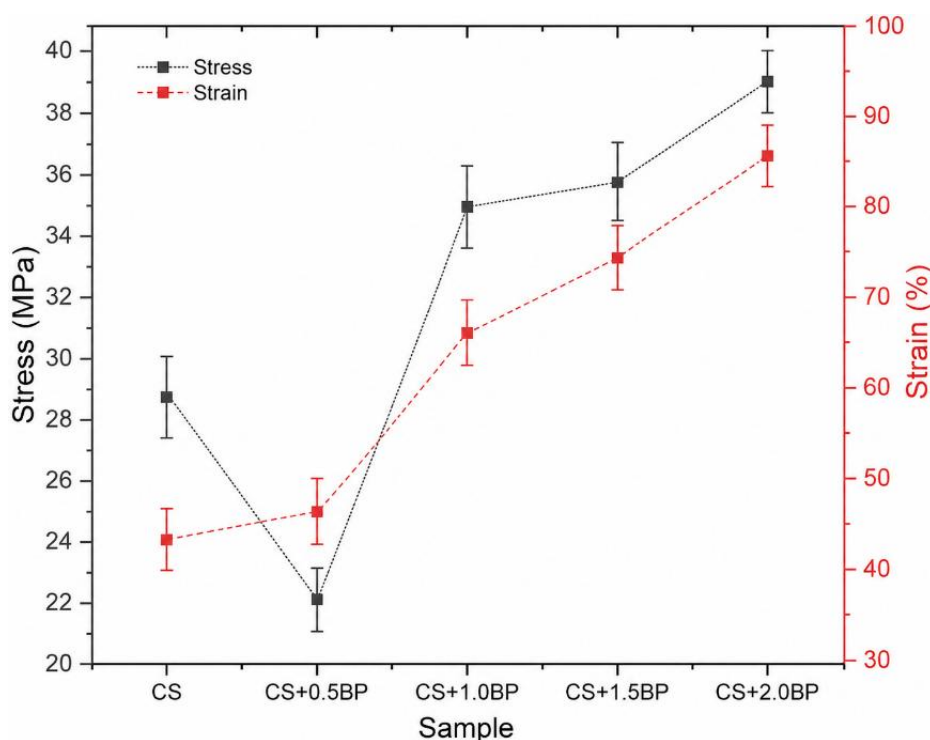


Figure 2. Tensile stress and strain of CS films with varying BPE concentrations.

At 0.5% BPE, a decrease in tensile stress was observed compared to the control film, likely due to the initial disruption of hydrogen bonding between chitosan by small molecules present in the extract. However, with increasing BPE concentration (1.0%-2.0 %), a consistent enhancement in both tensile strength and elongation at break was observed. The sample containing 2.0% BPE showed the highest mechanical performance, with a tensile stress of 39MPa and a strain of 87%, compared to 28MPa and 45% in control sample.

This dual improvement may be attributed to the polyphenolic and lignocellulosic composition of banana peel. Polyphenols such as tannins and flavonoids can form intermolecular hydrogen bonds or even weak cross-links with hydroxyl and amine groups in chitosan and starch, thereby reinforcing the matrix structure. Moreover, BPE contains plasticising components, such as sugars and organic acids, which may enhance chain mobility and flexibility at higher extract concentrations. Polyphenolic compounds

present in BPE may interact with hydroxyl groups of starch and amino groups of chitosan through hydrogen bonding, producing a denser polymer network that improves stress transfer and mechanical strength (Santhosh et al., 2024).

Additionally, the presence of insoluble lignocellulosic particles at higher extract levels may contribute to matrix reinforcement through a stress transfer mechanism, as seen in other natural fiber-reinforced biopolymers. These combined effects suggest that BPE serves both as a plasticizer and a reinforcing agent, depending on its concentration. Cellulose and hemicellulose particles present in banana peel may function as natural reinforcing fillers, improving load distribution within the matrix (Azriena et al., 2025).

These findings are consistent with earlier reports on the integration of natural plant-based extracts into biopolymer films, where improvements in mechanical strength and ductility were achieved due to enhanced intermolecular interactions and filler effects (Chandrasekar et al., 2023; Tan et al., 2022). The results highlight the potential of banana peel as a functional additive in sustainable packaging materials.

The incorporation of BPE affected both tensile strength and elongation at break, although the response was not linear across the tested concentrations. Tensile strength increased from 28 MPa for the control film to 39 MPa at 2% BPE. One-way ANOVA followed by Tukey's post hoc test indicated that the tensile strength differed significantly among BPE concentrations ($p < 0.05$), with the 2% BPE film exhibiting a significantly higher value than the control. Similar changes in mechanical properties have been reported for polysaccharide-based films containing plant-derived components. The observed improvement may be associated with changes in polymer-extract interactions and the presence of structural components in banana peel. However, because the present study did not independently quantify the phenolic, lignocellulosic or other extract constituents, these mechanisms should be considered possible explanations rather than directly demonstrated mechanisms.

The tensile strength obtained at 2% BPE was higher than values reported within the comparative range cited in previous studies, indicating that BPE incorporation can substantially modify the mechanical response of the chitosan-starch matrix. Nevertheless, suitability for a particular packaging application would require evaluation of additional properties and application-specific performance criteria.

3.3. Antibacterial Analysis

The antimicrobial activity increased with increasing BPE concentration, although the response was concentration-dependent (see Table 2 and Fig. 3). The inhibition zones of the control, 0.5% BPE and 1.0% BPE films were statistically comparable, as indicated by the same Tukey grouping ($p > 0.05$). In contrast, the 1.5% and 2.0% BPE films showed greater inhibition zones than the lower-concentration films ($p < 0.05$). The 2.0% BPE film produced the largest inhibition zone (1.70 ± 0.08 cm), indicating that a higher BPE concentration was associated with enhanced inhibition of *E. coli*.

The increasing inhibition-zone diameter with BPE concentration indicates that incorporation of BPE was associated with greater inhibitory activity against *E. coli* under the conditions tested. This effect may be related to antimicrobial constituents present in the extract, including phenolic compounds; however, the specific compounds responsible were not identified in the present study. Similar trends were reported by Taweechat et al. (2021).

Phenolic compounds alter membrane permeability, induce leakage of intracellular components and interfere with enzyme activity. Previous studies have proposed that phenolic and flavonoid compounds can inhibit bacterial growth through mechanisms involving membrane disruption and interference with cellular processes. These mechanisms may contribute to the observed inhibition in the present study, although they were not directly investigated. Flavonoids may additionally inhibit nucleic acid synthesis and energy metabolism, resulting in reduced bacterial growth.

This finding supports the potential use of CSBPE films in active food packaging to extend shelf life and improve food safety. However, the plateau at 0.5-1.0% suggests a threshold concentration below which the extract may not

be sufficiently active to inhibit microbial growth. The incorporation of BPE altered the functional properties of the CS films, with statistically significant differences observed for selected measured properties.

Table 2. Effect of BPE concentration on antimicrobial activity

Sample	Diameter of inhibition zone (cm)	Related FTIR observation
CS	1.00 ± 0.05^c	Baseline O-H and N-H stretch; no BPE functional groups
CS+0.5BPE	1.10 ± 0.04^c	Slight increase in O-H and C=O peaks
CS+1.0BPE	1.10 ± 0.06^c	Moderate increase in phenolic-
CS+1.5BPE	1.30 ± 0.07^b	Enhanced O-H/N-H and C=O stretch; signs of polyphenol presence
CS+2.0BPE	1.70 ± 0.08^a	Strongest O-H and C=O intensities; highest phenolic interaction

* Means with similar letters (a, b and c) are not significantly different among samples according to Tukey's test ($p < 0.05$) with 3 repetitions.

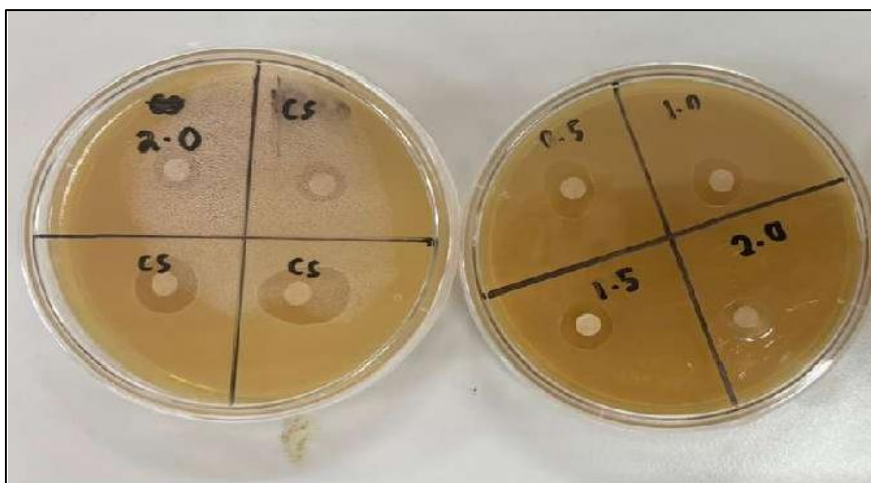


Figure 3. Antimicrobial Activity of CSBPE Films

4. Conclusion

The incorporation of banana peel extract altered the FTIR characteristics, mechanical properties and antibacterial response of chitosan–starch films. Increasing BPE concentration produced progressively more pronounced spectral changes, while the 2% BPE film exhibited the highest tensile stress and elongation at break among the formulations tested. Antibacterial activity against *E. coli* also increased at higher BPE concentrations, with the

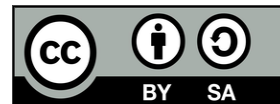
largest inhibition zone observed for the 2% BPE film. These findings indicate that BPE incorporation can modify the physicochemical and antibacterial characteristics of chitosan–starch films. However, the specific molecular mechanisms responsible for these changes cannot be established solely from FTIR and tensile measurements. Further studies involving chemical profiling of BPE, barrier-property measurements and direct food-storage experiments are required before claims

regarding antioxidant functionality or food shelf-life extension can be established.

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Research article

DEVELOPMENT OF INSECT-BASED TRADITIONAL CRACKERS: NUTRITIONAL PROFILE, PHYSICAL CHARACTERISTICS, AND SENSORY ACCEPTANCE

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ABSTRACT

Entomophagy, or the consumption of insects, has been an integral part of food culture in several regions of Indonesia, where crickets (Gryllidae) and grasshoppers (Acrididae) are typically fried and eaten as snacks or protein-rich side dishes. While these insects are usually served in simple fried forms incorporating them into widely recognized local foods could enhance consumer acceptance. *Kerupuk*, a culturally-familiar and widely-consumed popular Indonesian traditional cracker made from a starch–protein blend that is steamed dried, and deep-fried to achieve its airy crunch, is traditionally prepared with shrimp, fish, or plant-based ingredients, but rarely with insects. This study presents a novel approach by incorporating insect flour into Indonesian *kerupuk* as a strategy to improve the nutritional value of the product while reducing potential consumer resistance toward visible insect-based foods. Traditional crackers were produced using cricket flour (CC) and grasshopper flour (GC), comparing them with conventional shrimp crackers (SC) in terms of nutritional profile, physical characteristics, and sensory acceptance. Proximate analysis showed that fried insect crackers contained higher protein (CC: 24.93%, GC: 23.72%) than SC (19.65%), despite showing a slightly lower protein and lipid digestibility. Color analysis indicated insect crackers were darker than SC, yet expansion ratios (~375-379%) and crispness metrics were higher compared to SC (~313%). Sensory evaluation with 186 untrained panelists found no significant differences ($p>0.05$) among CC, GC, and SC in overall liking, but a higher preference related to crispness and hardness towards CC and GC compared to SC. These findings demonstrate that Indonesian *kerupuk* can serve as a practical and culturally acceptable food vehicle for edible insect incorporation, producing a protein-enriched snack with desirable crispness and sensory acceptance while supporting protein diversification and sustainable entomophagy in modern diets.

1. Introduction

The global population is projected to reach 9.8 billion by 2050, which is expected to cause a concurrent rise in food demand of up to 98% (Omuse *et al.*, 2024). Meeting this demand would put an unprecedented strain on natural resources, threatening the long-term viability of our current global food systems. Protein production is a major component of this challenge, as meat demand has already tripled over the last 50 years, reaching approximately 340 million metric tons per year (Ritchie & Poser, 2019). This immense scale of livestock farming is a primary driver of environmental pressure and raises significant questions about future food security (Imathiu, 2020). This unsustainable trajectory creates an urgent need for innovative and alternative ways to ensure that nutritious foods are available and accessible to all.

The human consumption of insects, known as entomophagy, has been a common practice throughout history in many cultures, particularly in parts of Africa, Asia, and Latin America (La Barbera *et al.*, 2018; Tedjakusuma *et al.*, 2022). It is estimated that close to 2,000 species are consumed globally, with the most common orders being beetles (31%), caterpillars (18%), and bees, wasps, and ants (14%), locusts, crickets and grasshoppers (13%) (Van Huis, 2019). In recent years, interest in entomophagy has surged as a potential solution to global food security challenges. This growing attention stems from the exceptional nutritional profile and sustainability of insects as a food source. Their crude protein content, which ranges from 40-75%, often surpasses that of traditional livestock and many plant proteins (Pereira & Vicente, 2013; Tang *et al.*, 2019; Tanga & Ekesi, 2024). Furthermore, insects are highly efficient, with about 80% of their body weight being consumable, compared to 55% for chicken and 40% for cattle. Beyond protein, they also offer diverse essential nutrients, including healthy fats, vitamins, antimicrobials, and chitin (van Huis & Rumpold, 2023).

Despite the potential benefits of insects as food, psychological factors such as food neophobia (reluctance to ingest unknown foods)

and disgust represent a significant barrier to the widespread adoption of insects persists, especially in Western cultures and urbanized society (Sogari *et al.*, 2019; Dagevos, 2021). A key strategy to overcome this psychological hurdle is to incorporate insects into familiar and well-liked food products in an ingredients such as powder, which are then incorporated into familiar foods such as pasta, snack, biscuits, or crackers (Boukid *et al.*, 2022; Montanari *et al.*, 2021).

Recent food product development studies have increasingly focused on improving the quality of familiar food matrices through the incorporation of alternative, sustainable, or underutilized ingredients (Petsong *et al.*, 2025; Karnjanapratum *et al.*, 2025). More specifically, several studies have demonstrated the feasibility of incorporating insect flour into familiar food products such as cookies, biscuits, pasta, bread, and other snack products, where insect powder is used to improve protein content while reducing the visual presence of whole insects (Colamatteo *et al.*, 2025; Adeniji *et al.*, 2025). However, many of these products are based on relatively dense bakery or cereal matrices, whereas expandable crackers such as *kerupuk* depend strongly on starch gelatinization, moisture vaporization, and the formation of a porous structure during frying. The incorporation of insect flour into this type of product may therefore affect not only nutritional value, but also expansion behavior, color development, oil absorption, microstructure, and crispness. Since insect flour contains high levels of protein, lipid, and chitin, its interaction with tapioca starch during steaming, drying, and frying may produce different physicochemical and sensory characteristics compared with conventional shrimp-based crackers. Therefore, traditional *kerupuk* provides a distinct and relevant food matrix for evaluating the application of insect flour in a culturally familiar snack product.

Kerupuk is a traditional expandable cracker widely consumed in Southeast Asia, particularly in Indonesia (Kusumaningrum *et al.*, 2019). It is typically prepared from tapioca starch combined with a protein source such as fish or shrimp,

followed by steaming, drying, and deep-frying to produce its characteristic airy and crispy texture (Mohamed *et al.*, 1989). Their expandable, crispy texture and savory flavor have made them a staple snack for all age groups. By substituting the traditional protein source with a sustainable insect-based alternative, there is a significant opportunity to enhance the nutritional value of a beloved food product while simultaneously increasing consumer acceptance of entomophagy.

While research has explored the fortification of various foods like bread and pasta with insect powder, there remains a specific gap in the literature concerning the development of traditional, deep-fried, expandable crackers. The unique physicochemical properties of these crackers, particularly their nutrition value and physical properties, present a novel challenge when incorporating a new protein ingredient. Therefore, this study aims to develop a traditional crackers made from cricket flour (CC) and grasshopper flour (GC), comparing them with conventional shrimp crackers (SC) in terms of nutritional profile, physical characteristics, and sensory acceptance.

2. Materials and methods

2.1. Materials

Live house crickets (*Acheta domesticus*) and live Javanese grasshoppers (*Valanga nigricornis*) were obtained from a local supplier in Gunung Kidul, Yogyakarta, Indonesia. The insects were selected based on visual quality, including intact body structure, absence of visible defects, and apparent healthy condition. Fresh whiteleg shrimp (*Litopenaeus vannamei*) was purchased from a local market in Jakarta, Indonesia, and used as the conventional animal protein source for the control cracker formulation. Tapioca starch, salt, sugar, and palm oil for frying were also purchased from a local market in Jakarta, Indonesia. All raw materials were transported to the laboratory under hygienic conditions and processed immediately or stored appropriately before cracker preparation.

2.2. Methods

2.2.1. Preparation of insect flour and traditional crackers

The preparation of insect-based traditional crackers was performed following the traditional method communicated by a local cracker producer in Jakarta, Indonesia, with minor adjustments for hygienic and laboratory-scale handling. Insect handling until euthanization was performed as previously described by Surya *et al.* (2025). Wingless live house crickets and Javanese grasshoppers in the juvenile stage (approximately 3 weeks and 4 weeks post-hatch respectively, sex-mixed), clinically healthy and free of visible defects, were obtained from a local commercial farm. Insects were held off-feed for 48 h with access to moisture only to purge gut contents. Live insects were then humanely euthanized by rapid cryo-immersion in liquid nitrogen (-196°C ; 60–120 s) using a perforated stainless-steel cup, thawed on ice, rinsed with potable water, and blanched in boiling water for 5 min. Blanched insects were drained and dried in a hot-air oven at 60°C until constant weight, ground with a high-speed blender, and sieved through a 60-mesh screen to obtain uniform cricket flour (CF) and grasshopper flour (GF). Commercial shrimp raw material was similarly processed to produce shrimp flour (SF) as the conventional control protein source.

Kerupuk dough was prepared based on a traditional formulation communicated by a local cracker producer in Jakarta, Indonesia, with minor adjustments for hygienic and laboratory-scale handling. The same base formulation was used for all treatments, consisting of 45% tapioca starch, 50% protein source, 2% salt, 3% sugar, and 60 mL water per 100 g dry mix. The protein source consisted of shrimp flour, cricket flour, or grasshopper flour for the preparation of shrimp crackers (SC), cricket crackers (CC), and grasshopper crackers (GC), respectively. Water was added gradually at approximately 60 mL per 100 g of dry mixture to obtain a thick and cohesive dough suitable for steaming and slicing. The protein source was substituted at the same proportion across treatments to ensure that differences in nutritional composition, physical

characteristics, microstructure, and sensory acceptance were mainly attributable to the type of protein flour used. Dry ingredients were homogenized, water was gradually added to form a thick cohesive dough, and the dough was kneaded until uniform. The dough was then shaped into cylindrical logs (approximately 3–4 cm diameter), wrapped in heat-resistant plastic, and steamed at 100 °C for 60 min. After steaming, the logs were cooled to room temperature and stored at 4 °C overnight (about 12–16 h) to stabilize the gel structure. The chilled logs were sliced into discs of uniform thickness (2 mm) using a stainless-steel slicer and sun-dried for approximately 48 h under hygienic conditions until a moisture content below about 10% was reached, yielding dried raw crackers. For final preparation, dried crackers were deep-fried in palm oil at 180 °C for 30–45 s until fully expanded and light golden, producing shrimp crackers (SC), cricket crackers (CC), and grasshopper crackers (GC), which were drained of excess oil, cooled to room temperature, and stored in airtight containers prior to analysis.

2.2.2. Proximate composition and oil uptake analysis

The proximate composition of the samples was determined using standard methods from the Association of Official Analytical Chemists (AOAC), adapted from the procedure described by Surya *et al.* (2024) with minor modifications. Specifically, moisture content was measured gravimetrically after oven-drying at 135°C for 2 hours, and ash content was determined by incineration at 550°C. Crude fat was quantified using a Soxhlet apparatus with petroleum ether, while crude protein was analyzed via the Kjeldahl method. Total carbohydrate content was then calculated by difference, with all final values expressed on a wet weight basis.

Frying oil uptake was estimated based on the increase in crude fat content after frying. The calculation was performed by subtracting the crude fat content of the corresponding dried raw cracker from that of the fried cracker, as follows: oil uptake (%) = fat content of fried cracker (%) – fat content of dried raw cracker (%). Using this approach, the estimated oil uptake values were

calculated for each treatment based on the proximate composition data.

2.2.3. *In vitro* nutrient digestibility

Aqueous extract of crackers was prepared by homogenized it with distilled water in a 1:10 (w/v) ratio. The resulting puree was filtered through cheesecloth, and the collected filtrate was used for subsequent analysis.

2.2.3.1. Protein digestibility

The *in vitro* protein digestibility was carried out based on a previously described method (Surya *et al.*, 2024). In brief, a 5 mL aliquot of samples was subjected to enzymatic hydrolysis with a protease mixture containing trypsin, chymotrypsin, and pancreatin (1 mg each). The digestion was carried out at 37°C for 30 minutes. A blank sample (extract without enzymes) and a control sample (casein) were run concurrently. Following incubation, undigested proteins were precipitated by adding 2 mL of 0.5 M trichloroacetic acid and separated via centrifugation. The quantity of soluble, digested peptides in the resulting supernatant was then quantified using the Folin-Ciocalteu reagent. The absorbance of the final mixture was measured spectrophotometrically at 750 nm.

2.2.3.2. Lipid digestibility

In vitro lipid digestibility was evaluated by measuring the release of free fatty acids (FFAs) using a modified titration method (Lie & Surya, 2024). Briefly, 25 mL of samples were subjected to enzymatic hydrolysis with a mixture of lipase (10 mg), pancreatin (10 mg), and bile salt (20 mg) during a 15-minute incubation at 37°C. Following digestion, a 5 mL aliquot of the digestate was prepared for Formol titration by adding 1.35 mL of 37.5% formaldehyde and phenolphthalein indicator. The free fatty acids were then quantified by titrating the mixture against 0.1 N sodium hydroxide (NaOH). Lipid digestibility was calculated based on the FFA content and expressed as a percentage of oleic acid equivalents.

2.2.4. Physical properties analysis

The surface color of the fried crackers was measured using a HunterLab colorimeter, recording the CIE L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) values. The crispness

parameter of the crackers were evaluated using a texture analyzer equipped with an acoustic sensor according to Sanz *et al.*, (2007). To simulate the action of biting, a wedge-shaped aluminum probe (30° angle, 15 mm wide) was used to fracture the cracker transversally at a speed of 40 mm/s. During penetration, a force-deformation curve was generated. Simultaneously, the sound emitted during the fracture event was recorded by a microphone placed at a fixed distance of 5 cm from the sample. To ensure the accuracy of the acoustic data, all tests were conducted inside an isolated acoustic chamber to eliminate external noise interference. The linear expansion ratio was calculated by measuring the average diameter of the crackers before and after frying, expressed as a percentage increase.

2.2.5. Microstructure analysis

The internal microstructure of the insect-based crackers was examined using Scanning Electron Microscopy (SEM). Cracker samples were cut into rectangular pieces ($\sim 5 \times 5$ mm) using a stainless-steel blade to expose the internal fracture surface without compression. Samples were subsequently frozen at -80 °C for 24 h and freeze-dried (-55 °C, <0.05 mbar; 48 h) to remove structural water and prevent collapse of the porous matrix. Dried specimens were mounted onto standard aluminum stubs using conductive carbon adhesive tabs, ensuring that the fractured surface was oriented upward and remained uncompressed. Mounted samples were sputter-coated with a gold layer (~ 10 nm thickness) using a vacuum sputter coater (60 s coating time; 20 mA current) to improve surface conductivity and image quality. SEM observations were conducted using a high-resolution field-emission scanning electron microscope under high-vacuum mode. Images were captured at an acceleration voltage of 10–15 kV, a working distance of 8–12 mm, and chamber pressure of 5–10 Pa.

2.2.6. Sensory evaluation

All crackers were evaluated by 186 untrained adult panelists aged approximately 18–45 years, comprising both male and female participants. Panelists were recruited from the university community, including students and

staff, and participated voluntarily after providing written informed consent. Before participation, panelists were informed that the products contained shrimp and/or edible insects, and individuals reporting known allergies to crustaceans, shellfish, or edible insects were excluded from the sensory evaluation.

All crackers were evaluated for sensory quality attributes with 186 untrained panelists. Each cracker sample was assigned a randomly generated three-digit code and presented to the panelists in a randomized and balanced serving order to minimize potential order and carry-over effects. Approximately two pieces of each cracker sample were served at room temperature on separate plates. Drinking water was provided, and panelists were instructed to cleanse their palate between samples. For the hedonic rating test, panelists were asked to rate the samples for appearance, aroma, hardness, crispness, mouthfeel, taste, and overall liking using a 9-point hedonic scale, where 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. In addition, panelists were also asked to perform an overall preference ranking of the three cracker samples. For the ranking test, panelists ranked the samples from 1 to 3, where 1 indicated the most preferred sample and 3 indicated the least preferred sample. The mean ranking score was then calculated for each sample, with a lower mean rank indicating greater overall preference. Written informed consent was obtained from all participants before testing. All procedures related to sensory evaluation were approved by the BINUS University Research Ethics Committee, followed institutional guidelines for human subject research, and complied with the principles of the Declaration of Helsinki.

2.3. Data analysis

All data were organized and analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Nutritional composition, *in vitro* digestibility, color parameters, texture/acoustic parameters, and expansion ratio data were obtained from at least three independent measurements ($n \geq 3$) and are presented as mean $\pm$ standard deviation.

Differences among samples were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test to determine significant differences between means. Sensory hedonic rating data obtained from 186 untrained panelists were also expressed as mean $\pm$ standard deviation and analyzed using one-way ANOVA followed by Tukey's HSD test. For the overall preference ranking test, rank data were analyzed using the Friedman test, followed by pairwise comparison using the Wilcoxon signed-rank test with Bonferroni adjustment when significant differences were observed. For the ranking test, lower mean rank values indicated higher overall preference. Statistical significance was declared at $p < 0.05$.

3. Results and discussions

3.1. Effect of cricket and grasshopper flour on the nutritional profile of fried crackers

Incorporating insect flour into crackers was shown to increase protein content (Table 1).

Both CF and GF had the highest crude protein level, at 75.26% and 72.58%, respectively, which was significantly higher than SF's 63.77%. This pattern was observed in the final product, where fried CF and GF had a significantly higher protein content than fried SF. In contrast, after deep-frying, the fat content in all crackers increased significantly, going from 4-7% in raw forms to more than 25% in fried crackers. Based on the difference in crude fat content between the dried raw and fried crackers, the estimated oil uptake was 21.38% for CC, 20.53% for GC, and 20.82% for SC, with no significant statistical difference ($p > 0.05$). These values indicated that the incorporation of cricket or grasshopper flour did not markedly increase oil uptake compared with the shrimp-based control, suggesting that the higher fat content of the fried crackers was mainly associated with the deep-frying process rather than the type of protein source used.

Table 1. Nutritional proximate composition expressed in wet basis (%) and oil uptake (%) of fried crackers (%)

Sample	Water	Ash (minerals)	Fat	Protein	Carbohydrate	Oil uptake
CF	6.69 $\pm$ 0.72 ^{ab}	2.56 $\pm$ 0.16 ^d	11.85 $\pm$ 1.01 ^c	75.26 $\pm$ 4.94 ^a	3.64 $\pm$ 0.35 ^d	-
GF	7.18 $\pm$ 0.63 ^a	3.82 $\pm$ 0.27 ^b	12.32 $\pm$ 0.84 ^c	72.58 $\pm$ 4.88 ^a	4.10 $\pm$ 0.31 ^d	-
SF	5.87 $\pm$ 0.55 ^b	5.85 $\pm$ 0.42 ^a	17.75 $\pm$ 1.15 ^b	63.77 $\pm$ 4.18 ^b	6.76 $\pm$ 0.45 ^c	-
CC (raw)	4.39 $\pm$ 0.36 ^c	2.28 $\pm$ 0.11 ^d	4.24 $\pm$ 0.35 ^e	38.81 $\pm$ 2.31 ^c	50.28 $\pm$ 3.21 ^b	-
GC (raw)	4.68 $\pm$ 0.54 ^c	2.67 $\pm$ 0.20 ^d	4.58 $\pm$ 0.36 ^e	35.25 $\pm$ 2.03 ^d	52.82 $\pm$ 4.72 ^{ab}	-
SC (raw)	4.72 $\pm$ 0.58 ^c	3.08 $\pm$ 0.23 ^c	6.91 $\pm$ 0.55 ^d	27.55 $\pm$ 1.97 ^e	57.74 $\pm$ 3.83 ^a	-
CC (fried)	2.15 $\pm$ 0.16 ^d	0.14 $\pm$ 0.01 ^e	25.62 $\pm$ 2.03 ^a	24.93 $\pm$ 1.78 ^f	47.16 $\pm$ 3.36 ^b	21.38 $\pm$ 1.48 ^a
GC (fried)	1.94 $\pm$ 0.18 ^d	0.16 $\pm$ 0.01 ^e	25.11 $\pm$ 1.84 ^a	23.72 $\pm$ 1.73 ^f	49.07 $\pm$ 4.05 ^b	20.53 $\pm$ 1.26 ^a
SC (fried)	1.99 $\pm$ 0.15 ^d	0.19 $\pm$ 0.01 ^e	27.73 $\pm$ 2.29 ^a	19.65 $\pm$ 1.28 ^g	50.44 $\pm$ 3.91 ^b	20.82 $\pm$ 1.53 ^a

Data (n=3) are reported as mean $\pm$ SD. Different letters in a column indicate significant difference among samples ($p < 0.05$). Abbreviations: CC: cricket cracker, CF: cricket flour, GC: grasshopper cracker, GF: grasshopper flour, SC: shrimp cracker, SF: shrimp flour.

The increasing in protein content was accompanied by a decrease in both protein and lipid digestibility (Table 2). Among the fried crackers, fried SC showed a significantly higher protein digestibility (75.35%) than CC (68.02%) or GC (60.68%). A similar pattern was observed for lipid digestibility, with the fried SC (59.41%) being significantly more digestible

than CC (52.26%) and GC (51.04%). The phenomenon is due to the presence of chitin, a structural N-acetylglucosamine polymer found in the insect exoskeleton (Merzendorfer & Zimoch, 2003). Chitin, which is indigestible by human enzymes, forms a fibrous and robust matrix that can entrap other nutrients (Kouřimská & Adámková, 2016). This

phenomenon, known as steric hindrance, can limit the ability of digestive enzymes, such as trypsin and lipase, to access their target protein

and lipid substrates, thereby reducing the overall rate and extent of digestion (Wang *et al.*, 2025).

Table 2. *In vitro* nutrient digestibility (%)

Sample	Lipid digestibility	Protein digestibility
CF	36.86±3.52 ^d	58.31±5.21 ^d
GF	32.65±3.17 ^e	52.27±4.82 ^e
SF	42.81±4.25 ^{cd}	68.75±5.43 ^{bc}
CC (raw)	46.15±4.19 ^{bc}	62.15±4.51 ^d
GC (raw)	40.35±4.27 ^d	56.67±5.09 ^{de}
SC (raw)	54.15±4.33 ^{ab}	71.26±5.73 ^{ab}
CC (fried)	52.26±4.55 ^b	68.02±5.33 ^{bc}
GC (fried)	51.04±5.58 ^b	60.68±5.25 ^d
SC (fried)	59.41±5.40 ^a	75.35±5.83 ^a

Data (n=3) are reported as mean±SD. Different letters in a column indicate significant difference among samples (p<0.05). Abbreviations: CC: cricket cracker, CF: cricket flour, GC: grasshopper cracker, GF: grasshopper flour, SC: shrimp cracker, SF: shrimp flour

3.2. Effect of cricket and grasshopper flour on the physical characteristic of fried crackers

The physical appearance and internal microstructure of the final fried crackers are shown in Figure 1 and Figure 2, respectively. Visually, SC appeared lighter and more uniform in color (Figure 1A), while CC (Figure 1B) and GC (Figure 1C) exhibited a noticeably darker,

brownish hue. This finding was confirmed in Table 3 where SC had a significantly higher L* value (68.25) compared to CC (42.38) and GC (54.27). The increased in browning may be from melanin that are present in insect and a more intense Maillard reaction during the high heat of deep-frying because of insect high concentration of amino acids (Hamza & Zahran, 2023; Hasnan *et al.*, 2023; El Hosry *et al.*, 2025).

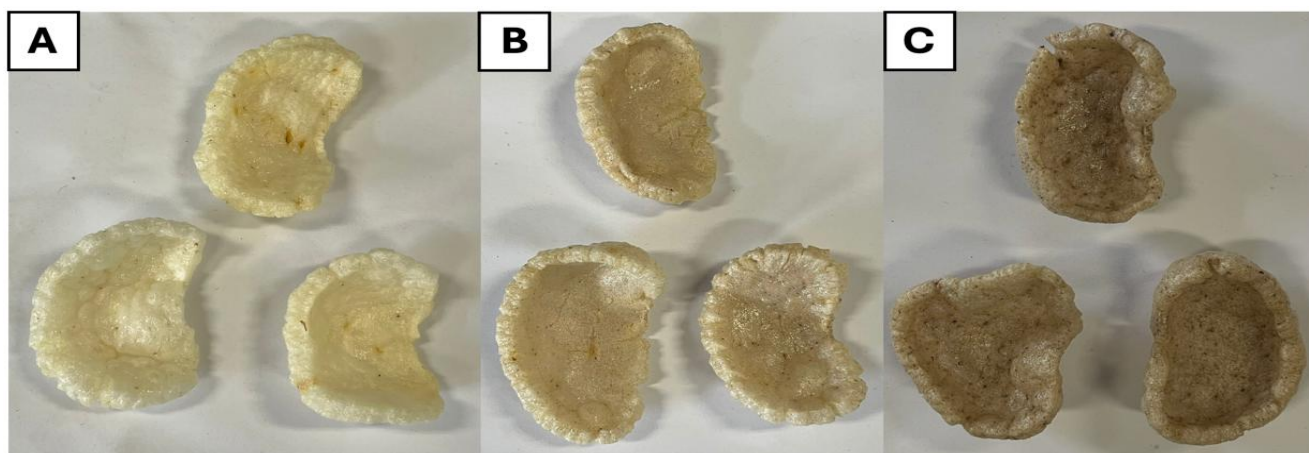


Figure 1. Physical appearance of fried crackers: A) shrimp cracker (SC), B) cricket-flour cracker (CC), and C) grasshopper-flour cracker (GC)

Scanning electron microscopy of the internal structure showed significant changes (Figure 2). SC (Figure 2A) showed a dense,

compact matrix including fewer and smaller air spaces. In contrast, CC (Figure 2B) and GC (Figure 2C) exhibited a highly porous, sponge-

like structure with numerous big spaces. This structural difference was confirmed by the expansion ratio data in Table 3, which showed that both CC and GC had larger expansion ratios than SC. It is hypothesised that insect proteins interact with gelatinized starch to generate a protein-starch matrix, increasing starch swelling (Leach *et al.*, 1959). This swelling may trap water molecules inside the granule, resulting in increased puffing and the creation of bigger voids (Cheow *et al.*, 2004). This difference in internal structure also affected the crispness of

crackers. The dense matrix of SC required a significantly higher breaking force to break, indicating a superior hardness of SC compared to CC and GC (Table 3). Meanwhile, the porous, air-filled structure of both CC and GC resulted in a crispier product, requiring significantly less force to break and higher number of number of peaks and acoustic amplitude as compared to SC (Table 3). Overall, the instrumental texture data indicate that CC exhibits greater crispness than GC.

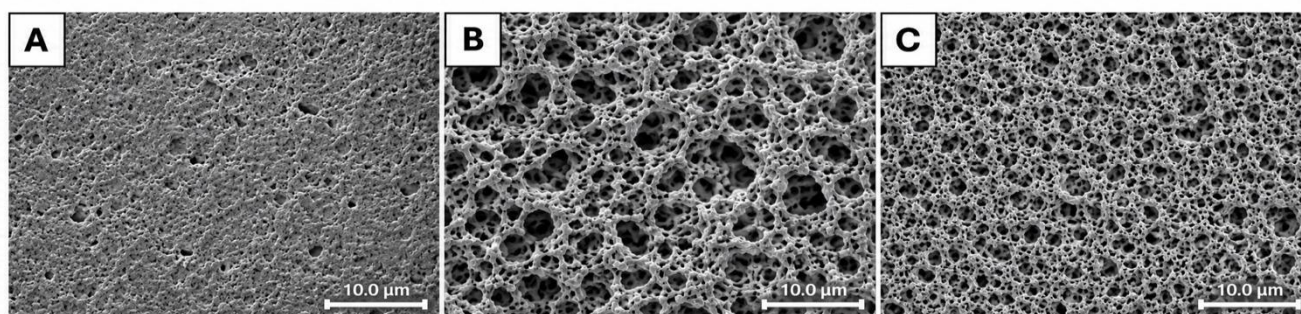


Figure 2. Scanning electron microscope (SEM) imaging of fried crackers: A) shrimp cracker (SC), B) cricket-flour cracker (CC), and C) grasshopper-flour cracker (GC)

3.3. Effect of cricket and grasshopper flour on the sensory evaluation of fried crackers

The results of the sensory evaluation involving 186 panelists are shown in Table 4. There were no significant differences ($p > 0.05$) found among CC, GC, and SC for the key attributes of appearance, aroma, mouthfeel, taste, and overall rate. These findings indicate that incorporating insect flour into *kerupuk* did not negatively affect sensory acceptance. The only significant difference was observed in texture-related attributes where panelists rated the shrimp cracker as significantly harder (6.73) and less crispy (7.17) than the insect-based crackers. This sensory finding aligns with the instrumental texture analysis where both CC and GC required a significantly lower breaking force to fracture because of its airy texture.

From a market acceptance perspective, the comparable overall liking scores among SC, CC, and GC suggest that incorporating insects in flour form into a familiar traditional product may be a promising strategy to reduce consumer

resistance toward entomophagy. Unlike whole insects, which may trigger visual rejection or disgust among some consumers, powdered insect ingredients can be integrated into recognizable food matrices while maintaining desirable sensory attributes. *Kerupuk* is also a suitable product for this purpose because it is already widely consumed as a snack or meal accompaniment in Indonesia, has a familiar crispy texture, and can be positioned as a protein-enriched traditional snack. Such a potential has been shown in our previous study (Widjaja & Surya, 2025). The higher protein content and favorable crispness of CC and GC may therefore provide useful selling points for commercialization. However, successful market introduction would require careful consumer education, clear communication of nutritional and sustainability benefits, and transparent labeling (Alhujaili *et al.*, 2023). Safety and regulatory aspects should also be considered, including the use of food-grade insect raw materials, hygienic processing, microbiological

quality control, and allergen labeling, particularly because edible insects may present allergenic risks or cross-reactivity with crustacean allergens (Aguilar-Toalá *et al.*, 2022). Future commercialization studies should therefore evaluate consumer perception, willingness-to-purchase, willingness-to-pay, shelf-life stability, packaging, and scale-up feasibility to determine whether insect-based kerupuk can be developed into a commercially viable alternative protein snack (Siddiqui *et al.*, 2023).

3.4. Limitations of the study

Several limitations of the present study should be considered when interpreting the findings. First, the *in vitro* protein and lipid digestibility assays were performed using aqueous extracts of the crackers rather than the intact food matrix. Therefore, the resulting values primarily reflect the digestibility of water-extractable components and may not fully account for the influence of the starch–protein matrix, insoluble components such as chitin, and the porous structure of the fried crackers during gastrointestinal digestion. Future studies employing standardized simulated gastrointestinal digestion of the whole product would provide a more physiologically representative assessment.

Second, crude protein content was determined using the Kjeldahl method, which estimates protein indirectly from total nitrogen. In insect-based ingredients, part of the measured nitrogen may originate from non-protein nitrogenous compounds, including chitin. Consequently, the use of a conventional nitrogen-to-protein conversion factor may result in some overestimation of the actual protein content. Amino acid analysis and the use of insect-specific nitrogen conversion factors would allow a more accurate evaluation of protein content and quality.

Third, although sensory evaluation involved a relatively large group of untrained panelists, the study assessed immediate sensory liking and preference rather than broader consumer perceptions toward edible insects. Factors such as food neophobia, willingness to purchase, willingness to pay, and responses to information regarding nutritional or sustainability benefits were not directly evaluated. Therefore, comparable sensory acceptance of the insect-based and shrimp-based crackers should not be interpreted as evidence that incorporation of insect flour overcomes food neophobia.

Fourth, the study evaluated only one formulation level, in which the type of protein source was varied while the proportion of the protein ingredient was kept constant. Consequently, the effects of different levels of cricket or grasshopper flour on nutritional composition, expansion, texture, oil uptake, and sensory acceptance remain unknown. Further formulation optimization may identify lower or higher incorporation levels that provide a more favourable balance between nutritional enhancement and product quality.

Finally, the present study focused primarily on nutritional composition, physical properties, digestibility, microstructure, and sensory acceptance. Microbiological safety, allergenicity, oxidative stability, and shelf-life were not directly investigated. These aspects are particularly important for insect-based foods because of potential allergenic cross-reactivity and their relevance to storage, regulatory compliance, and commercial application. Further studies addressing these parameters are therefore required before the practical commercialization of insect-based kerupuk can be fully evaluated.

Table 3. Physical parameters (color, crispness, and expansion rate) of fried crackers

Sample	Color parameters			Crispness parameters			Expansion ratio (%)
	L*	a*	b*	Breaking force (N)	Number of peaks	Acoustic amplitude (dB)	
CC (fried)	42.38±1.33 ^c	34.19±1.22 ^c	48.39±2.76 ^c	4.35±0.15 ^b	30.91±3.85 ^c	15.67±2.52 ^b	378.23±8.74 ^b
GC (fried)	54.27±1.81 ^b	10.82±1.03 ^b	40.42±2.08 ^b	4.73±0.18 ^b	25.97±1.33 ^b	16.67±1.15 ^b	375.60±9.65 ^b
SC (fried)	68.25±2.36 ^a	5.62±0.64 ^a	34.70±2.12 ^a	5.68±0.37 ^a	18.68±0.37 ^a	12.67±1.15 ^a	313.27±9.18 ^a

Data (n=3) are reported as mean±SD. Different letters in a column show significant difference among samples (p<0.05). Abbreviations: CC: cricket cracker, GC: grasshopper cracker, SC: shrimp cracker.

Table 4. Sensory acceptance of fried crackers measuring panelists' liking expressed in hedonic rate (scale 1-9) and rank

Sample	Appearance	Aroma	Hardness	Crispness	Mouthfeel	Taste	Overall rate	Overall rank
CC (fried)	7.44±1.16 ^a	7.48±1.06 ^a	7.38±0.95 ^a	7.75±0.92 ^a	6.95±0.88 ^a	7.87±1.25 ^a	7.53±1.15 ^a	1.97±0.35 ^a
GC (fried)	7.36±0.92 ^a	7.72±1.15 ^a	7.62±1.07 ^a	7.52±0.93 ^a	7.21±0.93 ^a	7.63±1.08 ^a	7.68±0.92 ^a	2.09±0.33 ^a
SC (fried)	7.67±1.14 ^a	7.91±1.16 ^a	6.73±0.82 ^b	7.17±1.03 ^b	6.91±0.87 ^a	7.80±1.22 ^a	7.39±1.03 ^a	2.14±0.36 ^a

Data (n=186) are reported as mean±SD. Different letters in a column show significant difference among samples (p<0.05). Abbreviations: CC: cricket cracker, GC: grasshopper cracker, SC: shrimp cracker

4. Conclusions

The current study demonstrated that cricket and grasshopper flour are highly efficient ingredients for nutritional fortification of traditional crackers. In comparison to the shrimp-based control, the addition of insect flour enhanced the protein content of the final product while also resulting in a greater expansion ratio and a crispier, less hard texture. Critically, these insect crackers were well received by customers, with sensory evaluations showing no significant change in overall liking, taste, or aroma when compared to the traditional prawn cracker.

The novelty of this study lies in demonstrating that cricket and grasshopper flour can be successfully incorporated into Indonesian *kerupuk*, a traditional and familiar snack matrix, without reducing overall sensory acceptance. This approach may help bridge the gap between the nutritional and sustainability benefits of edible insects and consumer reluctance toward direct insect consumption. Therefore, insect-based *kerupuk* has practical potential as a culturally acceptable, protein-enriched snack for promoting sustainable protein diversification.

Future studies should further evaluate the nutritional quality and safety implications of insect-based crackers. In particular, amino acid composition, protein quality indices, and protein bioavailability should be assessed to determine whether the higher protein content of cricket- and grasshopper-based crackers translates into improved nutritional value. Further work should also include consumer perception analysis, including food neophobia, willingness-to-purchase, willingness-to-pay, and the influence of product information or sustainability claims on acceptance. In addition, allergenicity should be carefully investigated, especially because edible insects may show cross-reactivity with crustacean and mite allergens, which is relevant for labeling and consumer safety. Other promising directions include optimization of processing conditions to balance expansion, crispness, oil uptake, and nutrient retention, as well as shelf-life, oxidative stability, microbiological safety, and scale-up studies to support the potential commercialization of insect-based traditional crackers.

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Data availability

All data related to this work are presented in the manuscript.

Authors' contribution

Conceptualization: R.S. and D.N.; methodology: R.S. and D.N.; validation: R.S. and D.N.; formal analysis: F.W. and F.T.; investigation: F.W. and F.T.; resources: R.S. and D.N.; data curation: R.S. and D.N.; writing—original draft preparation: R.S. and F.T.; writing—review and editing: R.S. and D.N.; visualization: R.S. and F.T.; supervision: R.S.; project administration: R.S.; funding acquisition: R.S.. All authors have read and agreed to the published version of the manuscript.

Declaration of conflicts of interest

All authors declared no conflicts of interest.



Review article

FOOD-WASTE-DERIVED BIOACTIVE, GREEN EXTRACTION TECHNOLOGIES AND GEL-BASED WOUND HEALING FORMULATIONS: A PRISMA-GUIDED SYSTEMATIC REVIEW

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ABSTRACT

The valorisation of food-waste-derived bioactive materials offers a potential route for developing wound-care products while supporting more sustainable resource utilisation. This PRISMA-guided systematic review synthesised 33 Q1/Q2 original research articles published between 2019 and 2024 that investigated natural waste- and related plant-derived bioactive sources relevant to extraction technologies, formulation strategies, and wound-healing applications. Maceration was reported in nine studies, followed by ultrasound-assisted extraction in eight, Soxhlet extraction in five, microwave-assisted extraction in four, pressurised or accelerated solvent extraction in two, and supercritical fluid extraction in one. These categories were not mutually exclusive because some studies employed multiple extraction techniques. Nineteen studies were classified as *in vitro*, seven as *in vivo*, and seven had insufficiently reported experimental designs and were therefore classified as unclear. Wound-healing or regenerative outcomes were reported in 23 studies, anti-inflammatory activity in 21, and antimicrobial and antioxidant activities in 20 studies each. Extracts and natural bioactive materials were incorporated into gels, hydrogels, films, nanofibrous dressings, and related delivery systems. However, direct comparisons were limited by substantial heterogeneity in feedstock characteristics, extraction conditions, analytical procedures, formulation composition, bioactive concentration, experimental models, and outcome measures. Evidence concerning stability, safety, scale-up, environmental performance, and clinical effectiveness was also limited. Overall, the findings demonstrate preclinical therapeutic potential but do not establish the superiority or clinical effectiveness of the reviewed extraction technologies and formulations. Standardised extraction and chemical-characterisation procedures, complete formulation testing, stability and safety evaluation, and well-designed clinical investigations are required before reliable translation into wound-care applications.

1.Introduction

Wound healing is a complex and tightly regulated process involving four overlapping phases: haemostasis, inflammation, proliferation, and remodelling, as illustrated in Figure 1.1. Although acute wounds generally progress through these phases without major complications, wounds associated with diabetes, vascular disease, infection, surgery, or trauma may remain unresolved for weeks or months,

resulting in persistent inflammation, impaired tissue regeneration, fibrotic scarring, and loss of function (Fernández-Guarino et al., 2023). Chronic wounds impose substantial healthcare expenditure, reduce patients' quality of life, and increase the demand for wound-care strategies that are effective, safe, affordable, and environmentally sustainable.

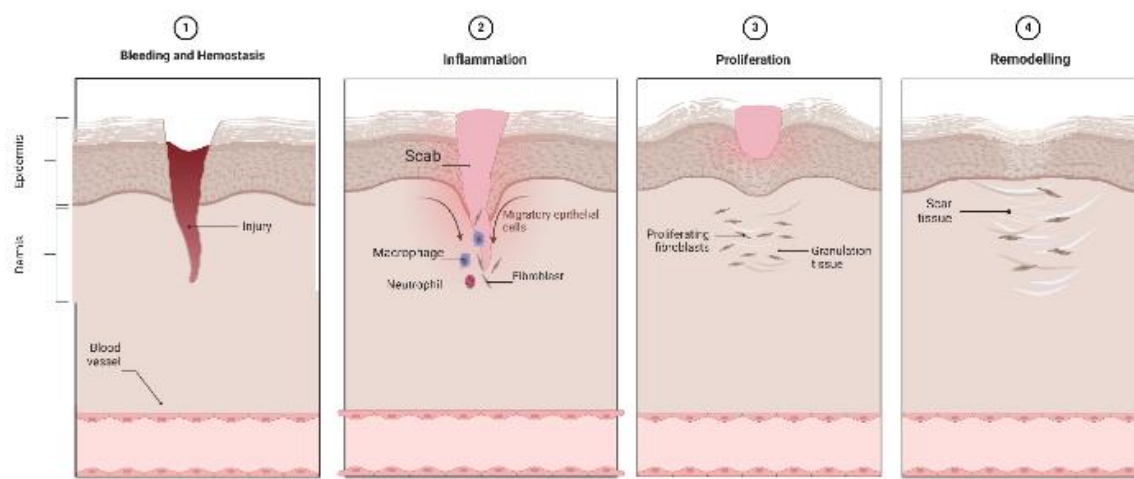


Figure 1.1. The four overlapping phases of wound healing: haemostasis, inflammation, proliferation, and remodelling. (<https://www.biorender.com/>). Accessed on December 12, 2024

Conventional wound management includes synthetic or semi-synthetic dressings, such as polyurethane films, foams, and hydrocolloids, as well as antiseptics, antibiotics, and anti-inflammatory agents (Rezvani Ghomi et al., 2019; Yousefian et al., 2023). These products perform important functions, including exudate management, physical protection, moisture regulation, and infection control. However, inappropriate or prolonged exposure to certain antimicrobial or antiseptic agents may cause cytotoxicity, delay tissue repair, or contribute to antimicrobial resistance (Yousefian et al., 2023). These limitations have increased interest in plant-derived bioactive compounds with antioxidant, antimicrobial, and anti-inflammatory activities that may support the wound-healing environment (Rezvani Ghomi et al., 2019; Abdelsattar et al., 2022).

Agro-industrial residues and food-processing by-products represent potentially

valuable sources of bioactive compounds. Fruit peels, seeds, pomace, and cereal brans are frequently discarded or directed towards low-value applications despite containing phytochemicals with potential biomedical value. Banana peel has been reported to contain phenolic acids, flavonoids, and tannins associated with antioxidant and wound-healing-related activities (Kumar et al., 2023). Citrus peel contains flavanones such as hesperidin and naringin, together with other phenolic and limonoid constituents associated with antioxidant, antimicrobial, and anti-inflammatory activities (Kaur et al., 2023). Pomegranate peel contains ellagitannins, ellagic acid, and related polyphenols that have been investigated for inflammation-modulating and tissue-regenerative applications (Costa et al., 2020). Recovering these compounds for potential wound-care applications may create value from underutilised residues while

supporting waste-reduction and circular-bioeconomy objectives.

The potential performance of food-waste-derived ingredients is strongly influenced by the extraction method used to recover and preserve their bioactive constituents. Conventional techniques such as maceration and Soxhlet extraction may require extended processing periods, substantial solvent volumes, and considerable energy input, depending on the operating conditions employed (Rafińska et al., 2019; Araujo et al., 2021). Intensified technologies, including ultrasound-assisted extraction, microwave-assisted extraction, pressurised or accelerated solvent extraction, and supercritical fluid extraction, have therefore been investigated as alternatives or complementary approaches (Hassim et al., 2019; Araujo et al., 2021). Under appropriately selected conditions, these technologies may enhance mass transfer, shorten extraction time, or reduce solvent consumption. Nevertheless, extraction performance depends on the feedstock, targeted compound class, solvent system, equipment configuration, and operating parameters. No single extraction technology can therefore be regarded as universally optimal.

Following extraction, bioactive fractions must be incorporated into delivery systems that protect their activity and enable application at the wound site. Hydrogels and related gel-based formulations are attractive because they can maintain a moist wound environment, conform to irregular wound surfaces, and incorporate bioactive compounds within biocompatible polymer networks (Witkowska et al., 2023; Yadav et al., 2024). Other delivery systems, including films and nanofibrous dressings, have also been investigated to provide physical protection and controlled or sustained release of bioactive constituents. Plant- and food-waste-derived extracts have been incorporated into matrices containing chitosan, poly(vinyl alcohol), and other natural or synthetic polymers, with reported antioxidant, antimicrobial, anti-inflammatory, and wound-regenerative activities (Rezvani Ghomi et al., 2019; Ferreira et al., 2022; Witkowska et al., 2023). However, variability in extract

composition, degradation of sensitive bioactive compounds, interactions between complex extracts and polymer matrices, and limited long-term stability, safety, and efficacy evidence continue to restrict reproducibility and clinical translation (Gościński et al., 2025; Sharifi & Bahrami, 2024).

Despite increasing research interest, the available evidence remains fragmented across several related fields. Previous reviews have generally examined natural products for wound healing, green extraction technologies, or the valorisation of food-processing by-products for functional ingredients and biomaterials as separate topics. Comparatively limited attention has been given to integrating natural-waste feedstocks, extraction technologies, bioactive composition, formulation strategies, and wound-healing-related biological activities within a single systematic synthesis. This review therefore focuses on studies published between 2019 and 2024 that investigated the extraction of bioactive compounds from natural waste- and related plant-derived materials and their application in hydrogels, gels, films, nanofibrous dressings, and other wound-care delivery systems.

Guided by the PRISMA framework for study identification, screening, eligibility assessment, and reporting (Sarkis-Onofre et al., 2021), this review aimed to: (i) map the natural-waste and related plant-derived bioactive sources, extraction technologies, and wound-care formulations investigated in the included studies; (ii) quantitatively summarise the reported extraction methods, experimental study designs, and wound-healing-related biological activities; (iii) examine the reported efficacy, stability, and safety of the investigated formulations, including any available comparisons with conventional wound-care products; (iv) evaluate the completeness of methodological reporting across the included studies; and (v) identify technological, reporting, sustainability, scale-up, and translational gaps requiring further investigation.

2..Materials & Methods

2.1. Review Design

This systematic review was conducted and reported with reference to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 framework (Sarkis-Onofre et al., 2021). The review process comprised the formulation of research questions, development of the database-search

strategy, removal of duplicate records, title and abstract screening, full-text eligibility assessment, data extraction, and qualitative synthesis, as summarised in Figure 2.1. Owing to substantial heterogeneity in the included extraction methods, formulations, biological models, and reported outcomes, a meta-analysis was not considered appropriate.



Figure 2.1. Overview of the systematic-review process employed in this study

2.2. Research Questions

The review was guided by five research questions addressing the technological, biological, methodological, and translational dimensions of natural waste-derived wound-healing products:

RQ1: Which extraction technologies have been used to recover bioactive compounds from natural waste- and related plant-derived materials for potential wound-healing applications?

RQ2: What formulation strategies have been employed to incorporate the recovered bioactive compounds into wound-care products, and what advantages and challenges have been reported?

RQ3: What evidence is available regarding the efficacy, stability, and safety of natural waste-derived wound-healing formulations, including any reported comparisons with synthetic or conventional wound-care products?

RQ4: What technological advances have been reported in the development of natural waste-derived wound-healing products, and

what evidence supports their potential environmental sustainability?

RQ5: What methodological limitations, evidence gaps, and future research priorities have been identified for the clinical and industrial translation of these products?

These questions were formulated to integrate extraction technologies, formulation strategies, reported biological activities, methodological reporting, sustainability considerations, and translational limitations within a single systematic synthesis.

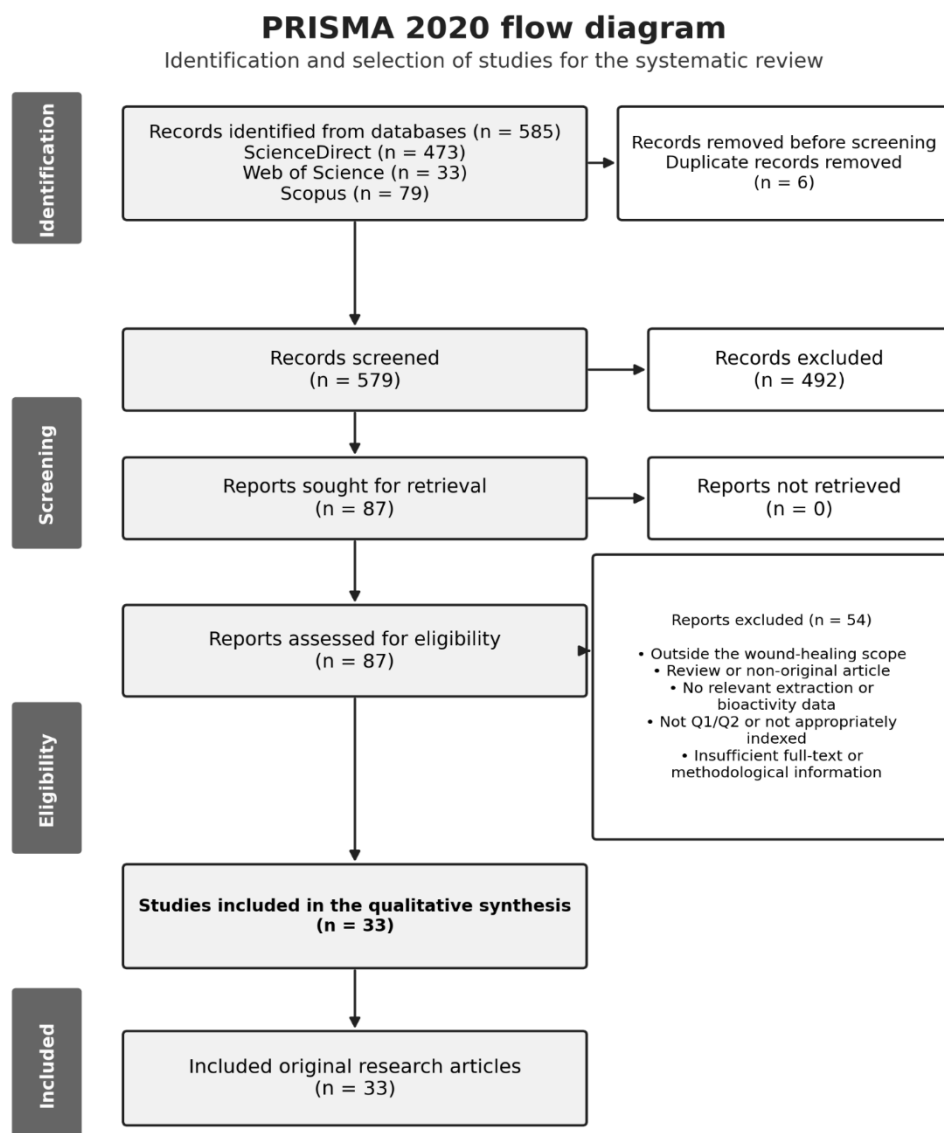
2.3. Search strategy

The literature search followed the PRISMA identification, screening, eligibility, and inclusion pathway summarised in Figure 2.2. Scopus, Web of Science Core Collection, and ScienceDirect were searched for publications dated from January 2019 to October 2024. The initial search retrieved multiple publication types, but only peer-reviewed original research articles were eligible for inclusion following screening and full-text assessment. Conference

papers, reviews, book chapters, and other non-original publications were subsequently excluded according to the eligibility criteria.

The search strategy addressed three principal concepts: (i) wound healing and related wound-care applications; (ii) conventional, green, or intensified extraction technologies and formulation strategies; and (iii) bioactive compounds obtained from natural

waste- or plant-derived sources. Boolean operators, phrase searching, and truncation were applied where supported by the respective database. Database-specific field restrictions and syntax were used because the three platforms differed in their search interfaces and indexing structures. The complete search strings applied to ScienceDirect, Web of Science, and Scopus are presented in Table 2.1.



Adapted from the PRISMA 2020 flow-diagram structure.

Figure 2.2. PRISMA 2020 flow diagram for the identification, screening, eligibility assessment, and inclusion of studies.

Table 2.1. Database-specific search fields and search strings used for literature identification

Database	Search field	Search string
ScienceDirect	Title, abstract, or keywords	(“wound healing” OR “wound care”) AND (“extraction technologies” OR “extraction methods” OR “formulation strategies”) AND (“bioactive compounds” OR “plant extracts”)
Web of Science Core Collection	Topic (TS)	TS = (“wound healing” AND (“extraction technologies” OR “extraction methods”) AND “bioactive compounds” AND “plant extracts”)
Scopus	Title, abstract, and keywords	TITLE-ABS-KEY (“wound healing” AND “plant extract” AND “extraction method” AND “bioactive compound” AND “natural”)

Note: Search syntax and field tags were adapted to the requirements of each database. Consequently, the exact combinations of terms differed across platforms while retaining the principal concepts of wound healing, extraction or formulation, and natural or plant-derived bioactive compounds.

2.4. Screening

The database searches identified 585 records: 473 from ScienceDirect, 33 from Web of Science, and 79 from Scopus. After six duplicate records were removed, 579 unique records remained for title and abstract screening. Of these, 492 records were excluded because they were unrelated to the wound-healing scope, represented reviews or other non-original publication types, or did not satisfy the language criterion. The remaining 87 reports were sought for full-text retrieval, and all were successfully obtained (reports not retrieved, $n = 0$). The identification and screening process is presented in Figure 2.2.

2.5. Eligibility

The full texts of 87 reports were assessed against the predefined eligibility criteria presented in Table 2.2. Studies were eligible if they were peer-reviewed original research articles published in English between January 2019 and October 2024 in Q1 or Q2 journals indexed in Scopus or Web of Science. The studies were also required to investigate natural waste- or related plant-derived bioactive materials, report an identifiable extraction or preparation approach, and examine at least one wound-healing-related biological or formulation outcome. Relevant outcomes included antioxidant, antimicrobial, anti-inflammatory, cytocompatibility, cell-migration, collagen-

deposition, tissue-regeneration, or in vivo wound-closure measures. Journal quartile was applied solely as an eligibility criterion and was not interpreted as an indicator of the methodological quality of an individual study. Following full-text assessment, 54 reports were excluded, and 33 original research articles were included in the qualitative synthesis (Figure 2.2).

2.6. Preliminary Methodological Reporting Assessment

Owing to the heterogeneity of the included evidence, which comprised extraction investigations, formulation-characterisation studies, in vitro bioactivity assays, and in vivo wound-healing experiments, a preliminary descriptive assessment of methodological reporting was conducted. The assessment examined whether each of the 33 included articles provided sufficient information to identify: (i) the bioactive source; (ii) the extraction or preparation method; (iii) the investigated biological activity or wound-healing-related outcome; and (iv) the experimental study design. The availability of information within these domains was recorded during data extraction to support consistent study classification and evidence synthesis. No aggregate methodological-quality score was calculated. Journal quartile was used only as an eligibility criterion and was not considered a

substitute for methodological quality or risk-of-bias assessment. Owing to the diversity of study designs and the limited reporting of design-

specific information, a formal risk-of-bias score was not assigned.

Table 2.2. Eligibility criteria applied during full-text assessment.

Criterion	Inclusion	Exclusion
Publication type	Peer-reviewed original research article	Review, systematic review, book chapter, conference paper, editorial, commentary, or other non-original publication
Publication period	Published from January 2019 to October 2024	Published outside the specified period
Language	Full text available in English	Full text not available in English
Journal eligibility	Published in a Q1 or Q2 journal indexed in Scopus or Web of Science	Journal did not meet the predefined indexing or quartile requirement
Bioactive source	Natural waste- or related plant-derived material relevant to the review scope	Synthetic-only material or source unrelated to the review scope
Extraction or preparation	An identifiable extraction, recovery, or preparation approach was reported	No relevant extraction or preparation information could be identified
Biological or formulation relevance	At least one wound-healing-related biological, formulation, or regenerative outcome was investigated	No outcome relevant to wound healing, bioactivity, or wound-care formulation was reported
Full-text information	Sufficient full-text information was available to establish eligibility and extract the principal study characteristics	Insufficient information to determine relevance or extract the minimum required study characteristics

2.7. Descriptive Analysis

The 33 eligible articles were examined and synthesised according to the predefined research questions. Extracted information included the bioactive source, extraction or preparation method, reported biological activity, experimental study design, formulation characteristics, operating conditions, and principal findings. The study-design categories, extraction methods, and biological activities were summarised using frequencies and percentages where appropriate. Extraction-method and biological-activity categories were not mutually exclusive because individual studies could employ more than one extraction technique or investigate multiple biological activities. Owing to substantial heterogeneity in study design, experimental conditions, formulations, and outcome measures, the findings were synthesised qualitatively and no

meta-analysis or pooled effect estimate was calculated.

3. Results and Discussions

3.1. Study Classification and Design Overview

The final dataset comprised 33 original research articles published between 2019 and 2024. Of these, 19 studies (57.6%) were classified as *in vitro* and seven studies (21.2%) as *in vivo*. The experimental design of the remaining seven studies (21.2%) could not be determined confidently from the reported information and was therefore classified as unclear. Collectively, the evidence base was dominated by preclinical investigations, with no full clinical trials identified, indicating that this field remains at an early stage of clinical translation.

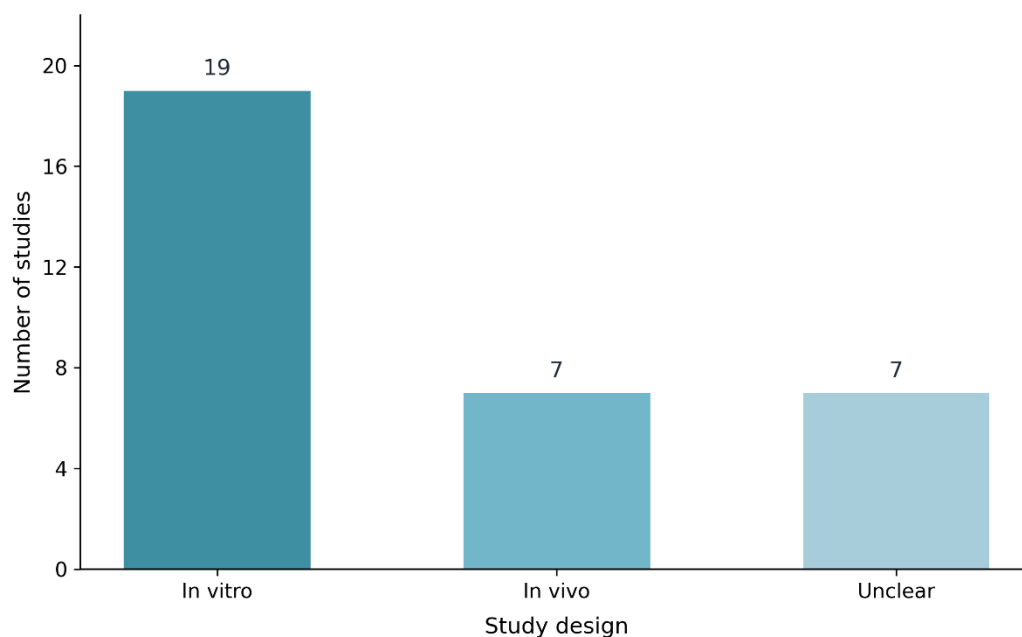


Figure 3.1. Distribution of experimental study designs among the 33 included studies

3.2. Methodological Reporting of the Included Studies

The preliminary methodological reporting assessment identified considerable variation in the completeness of reporting across the 33 included studies. Extraction or preparation methods were identifiable in 32 studies, whereas one study did not provide sufficient methodological detail for clear categorisation. Bioactive sources and investigated biological activities were generally reported, but information concerning independent replication, sample-size justification, randomisation, blinding, and other procedures used to minimise experimental bias was not consistently available. Furthermore, the experimental design

of seven studies could not be classified confidently from the reported information. Consequently, direct comparisons among studies and definitive judgements regarding methodological quality remain limited.

The principal characteristics of the 33 included studies are summarised in Table 3.1. The table presents the bioactive source, extraction or preparation method, reported biological activities, experimental study design, and reference for each study. Biological activities were standardised into broad wound-healing-related categories to support consistent descriptive synthesis across the heterogeneous evidence base.

Table 3.1. Characteristics of the 33 studies included in the systematic review

No	Bioactive Source	Extraction Method	Reported Bioactivity	Study Type	References
1	<i>Ipomoea pes-capre</i> leaf	Maceration	Anti-inflammatory, wound healing, and antiophidic properties.	Unclear	(Xavier-Santos et al., 2022)
2	<i>Woodfordia fruticosa</i> flowers	Distillation	Antioxidant; Antimicrobial; Anti-inflammatory	In vitro	(Maheshwari, 2024)
3	Propolis and honey	Heated ethanol extraction	Antioxidant; Antimicrobial; Anti-inflammatory	In vitro	(Abdelsattar et al., 2022)
4	Barley	High-power ultrasound (HPU)	Anti-inflammatory; Wound healing/regeneration	Unclear	(Di Michele et al., 2023)

5	<i>Plumbago zeylanica</i> , <i>Datura stramonium</i> and <i>Argemone mexicana</i>	Methanolic extraction	Antimicrobial; Anti- inflammatory; Wound healing/regeneration	Unclear	(Dev et al., 2019)
6	<i>Curcuma longa</i> (turmeric)	Soxhlet	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vitro	(Abbas et al., 2019)
7	<i>Lansium domesticum</i> (duku)	Microwave- assisted extraction (MAE)	Antioxidant; Anti-inflammatory; Wound healing/regeneration	In vivo	(Febriani et al., 2024)
8	<i>Verbascum thapsus</i>	Maceration + liquid-liquid extraction	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vitro	(Razani et al., 2024)
9	<i>Moringa oleifera</i> flowers	Aqueous extraction	Antimicrobial; Wound healing/regeneration	Unclear	(Butt et al., 2024)
10	<i>Lawsonia inermis</i> and <i>Scrophularia striata</i>	Maceration	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vivo	(Akbarpour et al., 2024)
11	<i>Stachys byzantina</i>	Soaking	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vitro	(Abbasinia et al., 2024)
12	<i>Centella asiatica</i>	MAE + UAE	Anti-inflammatory; Wound healing/regeneration	In vivo	(Rachpirom et al., 2023)
13	<i>Lavandula stoechas</i>	Not clearly reported	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vitro	(Mutlu et al., 2023)
14	<i>Punica granatum</i> (pomegranate)	Hydroalcoholic percolation	Antimicrobial; Wound healing/regeneration	Unclear	(Costa et al., 2020)
15	<i>Punica granatum</i> (pomegranate)	Hydroalcoholic percolation	Antimicrobial; Wound healing/regeneration	Unclear	(Santoso et al., 2024)
16	<i>Centella asiatica</i> herb	Ultrasound- assisted extraction (UAE)	Wound healing/regeneration	In vivo	(Witkowska et al., 2023)
17	<i>Mauritia flexuosa</i>	Aqueous/ethanol extraction	Antioxidant; Antimicrobial; Anti- inflammatory	In vitro	(Ferreira et al., 2022)
18	<i>Tamarindus indica</i> seeds	Soxhlet	Antimicrobial	In vitro	(Yadav et al., 2024)
19	<i>Punica granatum</i> (pomegranate) and <i>Moringa oleifera</i>	Aqueous decoction	Antioxidant; Antimicrobial; Anti- inflammatory	In vitro	(Meghwar et al., 2024)
20	<i>Vernonia amygdalina</i> leaf	Percolation	Anti-inflammatory	Unclear	(Canh Pham et al., 2024)
21	<i>Acca sellowiana</i> (feijoa)	Hot-water, acid and alkaline extraction	Antioxidant; Wound healing/regeneration	In vitro	(Zhu et al., 2023)
22	<i>Tecoma undulata</i> (Sm.) seem, commonly known as "Rohira" or "Rohitaka"	Soxhlet + maceration	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vitro	(Sable et al., 2023)
23	<i>Strychnos</i> <i>camptoneura</i>	Maceration	Wound healing/regeneration	In vivo	(Moulari et al., 2023)
24	<i>Sophora japonica</i> flowers	Modified maceration	Antioxidant; Antimicrobial; Anti- inflammatory; Wound healing/regeneration	In vitro	(Đorđević et al., 2022)
25	<i>Olea europaea</i> and <i>Acacia dealbata</i>	Solid-liquid extraction, UAE, Soxhlet + MAE	Antioxidant; Antimicrobial	In vitro	(Borges et al., 2020)

26	<i>Ginkgo biloba</i>	Maceration, reflux, shaking + Soxhlet	Antioxidant; Antimicrobial; Wound healing/regeneration	In vitro	(Sati et al., 2019)
27	<i>Lepidium sativum</i>	Maceration, UAE, ASE + SFE	Antioxidant; Antimicrobial; Wound healing/regeneration	In vivo	(Rafińska et al., 2019)
28	<i>Mangifera indica</i> peel	Ultrasound-assisted extraction (UAE)	Antioxidant; Antimicrobial; Anti-inflammatory; Wound healing/regeneration	In vitro	(Guandalini et al., 2019)
29	<i>Pogostemon cablin</i>	Caprylic/capric triglyceride extraction	Anti-inflammatory; Wound healing/regeneration	In vivo	(Choo et al., 2024)
30	<i>Moringa oleifera</i>	Pressurised liquid extraction (PLE)	Antioxidant; Anti-inflammatory; Wound healing/regeneration	In vitro	(Chatzimita kos et al., 2024)
31	<i>Commiphora gileadensis</i>	Microwave-assisted extraction (MAE)	Other reported bioactivity	Unclear	(Bin Mokaizh et al., 2024)
32	<i>Citrus reticulata</i> (kinnow)	Ultrasound-assisted extraction (UAE)	Antioxidant	In vitro	(Kaur et al., 2023)
33	<i>Citrus amblycarpa</i> , <i>C. aurantiifolia</i> and <i>C. hystrix</i>	UAE with 70% ethanol	Antioxidant; Anti-inflammatory; Wound healing/regeneration	In vitro	(Desmiaty et al., 2024)

Note: Reported biological activities were standardised into broad wound-healing-related categories. Extraction method and biological-activity categories were not mutually exclusive because individual studies could report more than one method or activity. UAE, ultrasound-assisted extraction; MAE, microwave-assisted extraction; ASE, accelerated solvent extraction; PLE, pressurised liquid extraction; SFE, supercritical fluid extraction.

3.3. Extraction Technologies

As summarised in Table 3.1, both conventional and intensified extraction methods were represented across the included studies. Maceration was the most frequently reported method, appearing in nine studies, followed by ultrasound-assisted extraction in eight studies. Soxhlet extraction was reported in five studies and microwave-assisted extraction in four studies, whereas pressurised or accelerated solvent extraction and supercritical fluid extraction were reported in two studies and one study, respectively. As several studies evaluated or combined multiple techniques, these frequencies represent method occurrences and should not be interpreted as mutually exclusive study categories.

Overall, conventional extraction methods remained widely represented, while green and intensified techniques were also evident within the current evidence base. However, meaningful comparisons of extraction performance remain challenging because of substantial differences in feedstock characteristics, solvent systems, operating conditions, extraction-yield

definitions, and analytical methods across the included studies. The reported frequencies therefore indicate the prevalence of method use rather than the relative effectiveness of each extraction technology.

The reported biological activities were also quantitatively synthesised using the standardised categories presented in Table 3.1. Wound-healing or regenerative outcomes were reported in 23 studies (69.7%), followed by anti-inflammatory activity in 21 studies (63.6%). Antimicrobial and antioxidant activities were each reported in 20 studies (60.6%). These categories were not mutually exclusive because most studies evaluated more than one biological activity. The distribution indicates that the evidence primarily focuses on wound-regenerative, anti-inflammatory, antimicrobial, and antioxidant outcomes rather than clinical measures of treatment effectiveness.

Figure 3.2 provides a conceptual synthesis of the research pathway identified across the included studies, beginning with the selection of natural waste- or plant-derived bioactive sources and progressing through extraction, chemical composition, formulation, and reported wound-

healing-related activities. Conventional and intensified extraction techniques were used to recover phenolic compounds, flavonoids, terpenoids, and other bioactive constituents. These extracts or bioactive materials were subsequently incorporated into hydrogels, gels, films, nanofibrous dressings, and related delivery systems associated with antioxidant, antimicrobial, anti-inflammatory, and wound-regenerative activities. The framework

highlights that the potential performance of a wound-care formulation depends on interconnected factors, including source characteristics, extraction conditions, recovered chemical composition, and formulation design. However, not every included study investigated all stages of this pathway, and the framework should therefore be interpreted as an integrated conceptual synthesis rather than a causal mechanism validated within a single study.



Figure 3.2. Conceptual framework illustrating the pathway from natural waste- or plant-derived bioactive sources through extraction and formulation to reported wound-healing-related biological activities. Created with BioRender.com.

3.4. The Formulation Strategies, Their Advantages and Challenges

Table 3.2 summarises the representative formulation strategies identified among the included studies. The formulations comprised topical gels, chitosan-based hydrogels, polysaccharide-based hydrogels, polyherbal gels, and composite delivery systems containing natural products or plant-derived extracts. Chitosan and plant-derived polysaccharides

were frequently employed as formulation matrices because of their biocompatibility, gel-forming properties, and suitability for incorporating bioactive constituents. However, the formulations differed substantially in polymer composition, extract concentration, preparation method, physicochemical characterisation, and biological assessment, limiting direct comparisons of their performance.

Table 3.2. Representative formulation strategies reported among the included studies.

Bioactive source or material	Formulation strategy	Reported purpose or biological activity	Reference
<i>Ipomoea pes-caprae</i> phenolic-rich leaf extract	Topical gel	Anti-inflammatory, wound-healing and antiophidic activities	(Xavier-Santos et al., 2022)
Asiaticoside-rich <i>Centella asiatica</i> extract	Chitosan-based hydrogel	Controlled delivery and wound-healing activity	(Witkowska et al., 2023)
<i>Mauritia flexuosa</i> oil	Chicha gum–chitosan gel	Potential wound-healing activity	(Ferreira et al., 2022)
Carboxymethyl tamarind kernel gum and ciprofloxacin	Hydrogel containing synthetic polymers	Antimicrobial drug delivery	(Yadav et al., 2024)
<i>Plumbago zeylanica</i> , <i>Datura stramonium</i> and <i>Argemone mexicana</i> extracts	Polyherbal gel	Antimicrobial, anti-inflammatory and wound-healing activities	(Dev et al., 2019)
Honey, propolis and bee venom	Chitosan-based hydrogel	Antimicrobial, anti-inflammatory and wound-healing activities	(Abdelsattar et al., 2022)

Note: The reported purposes and biological activities are based on the outcomes described in the individual studies and should not be interpreted as evidence of comparative clinical effectiveness. The table presents representative formulation-focused studies from the final dataset.

Among the six formulation-focused studies summarised in Table 3.2, five employed gel- or hydrogel-based delivery systems, while one investigated a hydrogel-based antimicrobial drug-delivery system. This distribution indicates that gel-forming polymeric matrices predominated among the selected formulation studies. Nevertheless, the limited number of studies and substantial differences in polymer composition, bioactive concentration, preparation method, and outcome assessment prevent conclusions regarding the superiority of any formulation type.

The therapeutic activities reported for the reviewed formulations were associated with

several structurally diverse plant-derived constituents. Phenolic compounds and flavonoids were frequently reported, while more specifically identified constituents included asiaticoside from *Centella asiatica*, ellagic acid and punicalagin from pomegranate, and curcumin from turmeric. Figure 3.3 presents the representative chemical structures of these selected constituents, which belong to different chemical classes and therefore possess distinct physicochemical characteristics that may influence their extraction, formulation, stability, and biological performance.

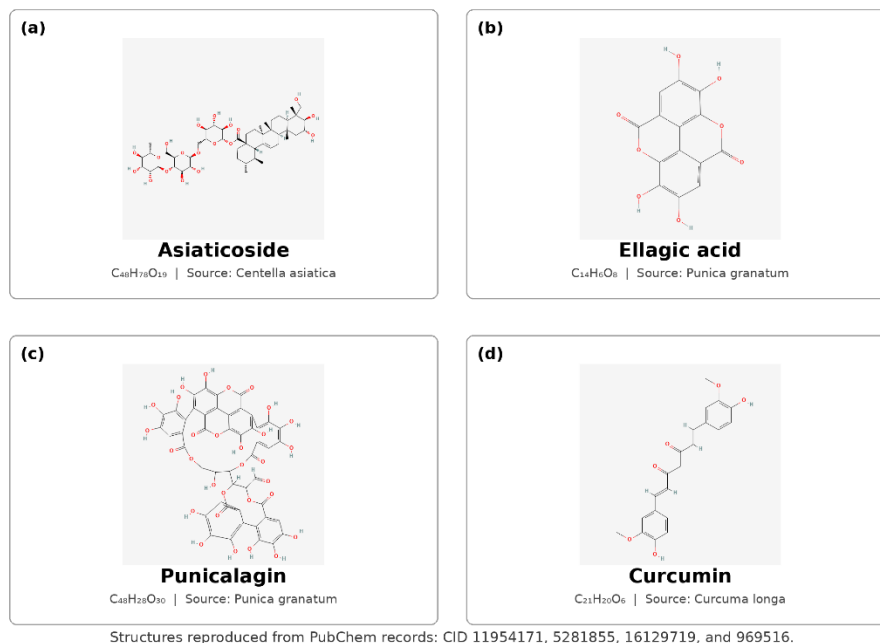


Figure 3.3. Representative chemical structures of selected bioactive constituents reported among the included studies: (a) asiaticoside, (b) ellagic acid, (c) punicalagin, and (d) curcumin. Chemical structures were reproduced from PubChem records (CID 11954171, 5281855, 16129719, and 969516, respectively).

As illustrated in Figure 3.3, the selected constituents differ considerably in molecular size and structural complexity. Asiaticoside is a relatively large triterpenoid glycoside containing multiple sugar moieties, whereas ellagic acid is a smaller polyphenolic compound. Punicalagin is a structurally complex hydrolysable tannin with numerous hydroxyl groups, while curcumin possesses a comparatively flexible diarylheptanoid structure. These structural differences may affect compound polarity, solvent compatibility, extraction behaviour, formulation requirements, and susceptibility to degradation. Nevertheless, the reviewed studies did not evaluate these compounds under standardised extraction, concentration, formulation, and biological-assay conditions. Consequently, Figure 3.3 should be interpreted as an illustration of the chemical diversity within the evidence base rather than as a direct comparison of biological potency or wound-healing effectiveness.

3.5. Reported Efficacy, Stability, and Safety of Natural Waste-Derived Wound-Healing Products

Across the included studies, natural waste- and plant-derived extracts demonstrated multiple wound-healing-related biological activities. Pomegranate-derived extracts were associated with antimicrobial and wound-regenerative activities (Costa et al., 2020), while turmeric-derived curcuminoids demonstrated antioxidant, antimicrobial, anti-inflammatory, and wound-healing-related effects (Abbas et al., 2019). Duku leaf extract was also associated with antioxidant, anti-inflammatory, and wound-regenerative activities (Febriani et al., 2024). These findings suggest that natural bioactive extracts may act through several complementary mechanisms, including the reduction of oxidative stress, modulation of inflammation, inhibition of microbial growth, and support of tissue regeneration.

Nevertheless, the available evidence does not establish that natural waste-derived formulations are equivalent or superior to established synthetic wound-care products. Most of the reported outcomes were obtained from in vitro or preclinical in vivo investigations, and direct comparisons were limited by differences in formulation composition, extract concentration, experimental model, treatment duration, and outcome assessment. Therefore, the findings

should be interpreted as evidence of therapeutic potential rather than confirmation of comparative clinical effectiveness.

The principal mechanisms through which the reported bioactive constituents may contribute to wound healing are conceptually summarised in Figure 3.4. The framework links the antioxidant, antimicrobial, and anti-inflammatory activities identified across the included studies with their potential cellular and tissue-level effects.

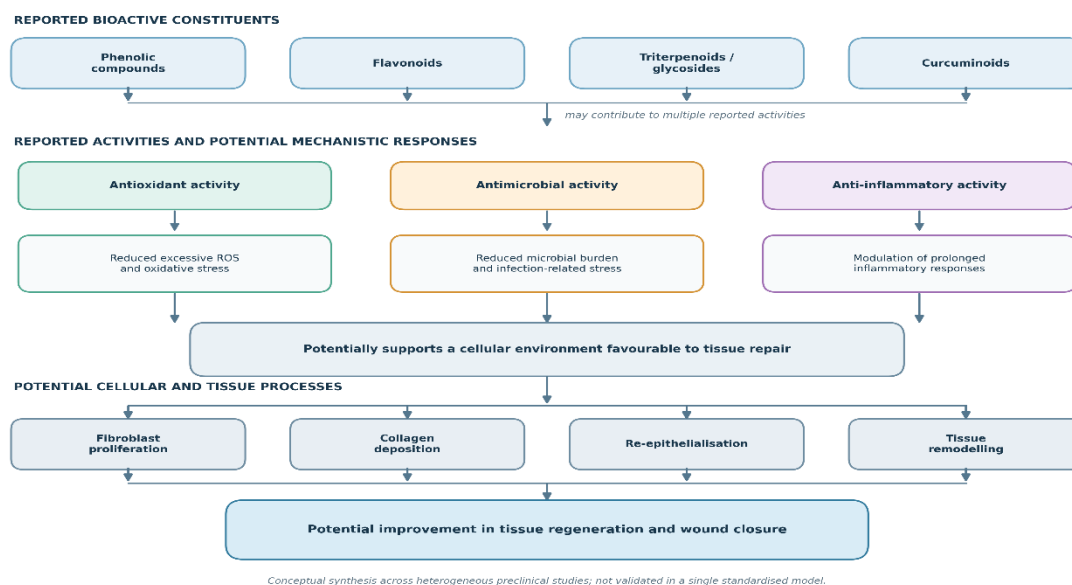


Figure 3.4. Conceptual mechanistic pathway linking the reported bioactive constituents and biological activities to potential cellular and tissue processes involved in wound healing. The pathway represents a synthesis across heterogeneous preclinical studies and was not validated using a single standardised experimental model.

As illustrated in Figure 3.4, the wound-healing potential of the reported bioactive constituents may involve several complementary pathways. Antioxidant activity may reduce excessive reactive oxygen species and oxidative stress, while antimicrobial activity may decrease microbial burden and infection-related stress. Anti-inflammatory activity may help modulate prolonged inflammatory responses. Collectively, these effects may create conditions that support fibroblast proliferation, collagen deposition, re-epithelialisation, and tissue remodelling, potentially improving tissue regeneration and wound closure. However, the pathway represents a conceptual synthesis of

findings obtained from different studies rather than a mechanism validated within a single experimental model. It should therefore be interpreted cautiously because the included studies differed in bioactive sources, extraction methods, formulations, concentrations, biological models, and outcome measures.

Stability represents an important limitation in the development of natural waste- and plant-derived wound-healing products. Bioactive constituents may undergo oxidation, hydrolysis, thermal degradation, or loss of biological activity during processing and storage, while differences in extract composition and formulation conditions may further affect

product reproducibility. Formulations containing aqueous extracts or natural polymers may also be susceptible to microbial contamination if appropriate preservation and packaging systems are not employed. However, storage stability, shelf life, microbial quality, and the retention of bioactivity over time were not consistently evaluated across the included studies (Gościński et al., 2025; Sharifi & Bahrami, 2024). Future investigations should therefore incorporate standardised stability testing under defined temperature, humidity, light, and storage-duration conditions. Strategies such as encapsulation, freeze-drying, antioxidant protection, appropriate preservation systems, and moisture- or oxygen-barrier packaging may be investigated, but their effectiveness and compatibility with the final formulation must be experimentally validated.

Safety evidence was similarly limited across the included studies. Natural origin should not be interpreted as evidence that an extract or formulation is inherently safe, because biological responses may vary according to chemical composition, bioactive concentration, impurities, residual solvents, formulation components, and duration of exposure agents (Rezvani Ghomi et al., 2019; Yousefian et al., 2023). Although some included studies reported cytocompatibility or preclinical biological activity, information concerning irritation, sensitisation, systemic toxicity, repeated exposure, and long-term safety was not consistently available (Ferreira et al., 2022; Witkowska et al., 2023). Standardised dose-response, cytotoxicity, irritation, sensitisation, and biocompatibility assessments are therefore required before these formulations can progress towards clinical evaluation.

The incorporation of natural bioactive constituents into polymeric delivery systems may offer a means of combining biological activity with improved handling, protection, and controlled release at the wound site (Razani et

al., 2024). Nevertheless, hybrid natural-synthetic formulations should be evaluated as complete products because interactions between the extract, polymer matrix, preservatives, and other formulation components may influence stability, safety, release behaviour, and biological performance (Sharifi & Bahrami, 2024). Direct comparisons with established wound-care products under standardised experimental conditions are required before claims of equivalent or superior efficacy, stability, or safety can be supported.

3.6. Technological Advances and Sustainability of Extraction Processes

Recent advances in the recovery of bioactive compounds from natural waste- and plant-derived materials have increasingly focused on intensified extraction technologies. Among the included studies, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurised or accelerated solvent extraction (PLE/ASE), and supercritical fluid extraction (SFE) were investigated as alternatives or complements to conventional extraction methods. These techniques offer potential advantages, including shorter extraction times, improved mass transfer, reduced solvent consumption, and greater control over operating conditions. However, their performance varied according to the feedstock, solvent system, temperature, pressure, extraction time, liquid-to-solid ratio, and analytical response employed. Consequently, the available evidence does not establish that any single intensified extraction technique is universally superior. Representative operating conditions and study-specific outcomes reported for selected intensified extraction techniques are summarised in Table 3.3. The table illustrates the variation in process parameters and analytical responses among the included studies rather than providing a direct performance comparison between extraction methods.

Table 3.3. Representative operating conditions and reported outcomes of intensified extraction methods among the included studies.

Extraction method	Bioactive source	Reported operating conditions	Principal reported outcome	Reference
Ultrasound-assisted extraction (UAE)	<i>Citrus reticulata</i> (kinnow) peel	Amplitude: 31%; liquid-to-solid ratio: 30:1 mL/g; temperature: 41 °C; extraction time: 15 min	TPC: 36.17 mg GAE/g extract; DPPH radical-scavenging activity: 64.70%	(Kaur et al., 2023)
Microwave-assisted extraction (MAE)	<i>Commiphora gileadensis</i> leaf	Microwave power: 300 W; solvent-to-sample ratio: 10 mL/g	Recovery and characterisation of phenolic constituents; the reported outcome was method-specific and was not directly comparable with the UAE or PLE studies	(Bin Mokaizh et al., 2024)
Pressurised liquid extraction (PLE)	<i>Moringa oleifera</i> leaves	Liquid-to-solid ratio: 70 mL/g; pressure: 1700 psi; temperature: 150 °C; extraction time: 15 min	TPC: 24.28 mg GAE/g dry weight; TFC: 17.20 mg RtE/g dry weight	(Chatzimitakos et al., 2024)

Note: The values are reproduced as reported in the individual studies. They should not be compared directly because the studies differed in plant material, solvent system, extraction scale, analytical method, and response definition. TPC, total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalents; RtE, rutin equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

Table 3.3 presents representative operating conditions and outcomes for selected intensified extraction techniques among the included studies. UAE achieved phenolic recovery from kinnow peel under moderate temperature and a relatively short extraction period, while PLE employed elevated temperature and pressure to recover phenolic and flavonoid compounds from *Moringa oleifera* leaves. MAE was also applied to the recovery and characterisation of phenolic constituents from *Commiphora gileadensis* leaves. Although these findings demonstrate the potential of intensified extraction technologies, the reported outcomes cannot be used to rank the methods directly. Differences in feedstock composition, solvent system, operating scale, extraction conditions, yield definition, and analytical response prevent a reliable determination of whether UAE, MAE, or PLE provides superior extraction performance.

Beyond the recovery of bioactive compounds, the valorisation of fruit peels and other plant-derived residues may contribute to waste reduction and circular-resource

utilisation. Converting these materials into potentially useful extracts and wound-care formulations could reduce the disposal of bioactive-rich residues while generating products with possible biomedical value. This approach is conceptually aligned with Sustainable Development Goal 12, particularly the reduction of waste through prevention, recycling, and reuse, and may also support Sustainable Development Goal 3 through the development of wound-care materials with potential health applications (Sorooshian, 2024). Nevertheless, the environmental advantages of these extraction processes should not be assumed solely from their classification as green or intensified technologies. Their sustainability depends on solvent toxicity, energy consumption, extraction yield, equipment requirements, waste generation, process scalability, and the overall life cycle of the resulting formulation. Future studies should therefore incorporate quantitative environmental and techno-economic assessments before definitive sustainability claims are made.

3.7. Limitations and Future Directions

Despite the promising biological activities reported across the included studies, several limitations currently restrict the translation of natural waste- and plant-derived wound-healing products into clinical practice. The evidence base remains predominantly preclinical, and the studies differed substantially in bioactive source, extraction method, formulation composition, treatment concentration, experimental model, and outcome assessment. Variability in plant origin, harvesting conditions, post-harvest processing, and extraction parameters may also affect the chemical composition and biological activity of the resulting extracts. In addition, the limited reporting of sample-size justification, independent replication, randomisation, blinding, and other procedures used to minimise experimental bias reduces confidence in direct comparisons among studies. Standardised extraction, compound-characterisation, formulation, and biological-testing protocols are therefore required to improve reproducibility and facilitate the validation of promising wound-care products.

Stability represents another important limitation in the development of natural waste- and plant-derived wound-healing products. Bioactive constituents may undergo oxidation, hydrolysis, thermal degradation, or loss of biological activity during processing and storage. Formulations containing aqueous extracts or natural polymers may also be susceptible to microbial contamination if appropriate preservation and packaging systems are not employed. However, storage stability, shelf life, microbial quality, and the retention of bioactivity over time were not consistently evaluated across the included studies. Future investigations should therefore incorporate standardised stability testing under defined temperature, humidity, light, and storage-duration conditions. Strategies such as encapsulation, freeze-drying, antioxidant

protection, appropriate preservatives, and moisture- or oxygen-barrier packaging may be investigated, but their effectiveness and compatibility with the final wound-care formulation must be experimentally validated.

The principal limitations identified across the included studies and the corresponding priorities for future research are summarised in Table 3.4. These limitations encompass extraction standardisation, variability in bioactive composition, formulation characterisation, stability and safety assessment, methodological reporting, clinical translation, scalability, and environmental sustainability. The table links these recurring evidence gaps with appropriate assessment strategies rather than attributing every limitation to each study. As summarised in Table 3.4, the future development of natural waste- and plant-derived wound-healing products requires improvement across the entire research pathway, from raw-material control and extraction standardisation to formulation characterisation, stability testing, safety assessment, and clinical validation. These limitations are interconnected. Variability in feedstock and extraction conditions may alter bioactive composition, which subsequently affects formulation behaviour, biological activity, reproducibility, and shelf life. Similarly, promising *in vitro* activity alone does not establish product safety or clinical effectiveness. Future studies should therefore adopt integrated development strategies that combine chemical standardisation, robust experimental design, formulation-performance testing, and staged translational evaluation.

Future research should adopt a staged translational pathway rather than focusing solely on improvements in extraction yield or short-term biological activity. Initial studies should establish raw-material specifications, validated chemical markers, reproducible extraction procedures, and complete formulation characterisation.

Table 3.4. Current limitations and recommended future directions for natural waste- and plant-derived wound-healing products.

Current limitation	Evidence pattern identified in the included studies	Recommended future direction	Suggested assessment or approach
Heterogeneous extraction conditions	Studies differed in feedstock preparation, solvent system, liquid-to-solid ratio, temperature, pressure, power, and extraction time	Develop standardised and reproducible extraction protocols for specific bioactive sources	Design of experiments, response-surface methodology, extraction mass balance, and reporting of complete operating parameters
Variability in extract composition	Bioactive composition may vary according to plant origin, processing conditions, extraction method, and analytical procedure	Establish chemical specifications and marker compounds for each extract	Validated HPLC, LC–MS, GC–MS, or spectroscopic fingerprinting and batch-to-batch comparison
Incomplete formulation characterisation	Formulation composition, extract concentration, mechanical properties, and release behaviour were not consistently reported	Standardise formulation preparation and physicochemical characterisation	Rheology, swelling, porosity, mechanical strength, moisture transmission, degradation, and controlled-release testing
Limited stability and shelf-life evidence	Storage stability, microbial quality, and retention of bioactivity over time were infrequently or inconsistently evaluated	Conduct stability testing under defined storage and packaging conditions	Accelerated and real-time stability studies, microbial-limit testing, bioactivity-retention analysis, and packaging evaluation
Limited methodological reporting and risk-of-bias control	Replication, sample-size justification, randomisation, blinding, and other bias-minimisation procedures were not consistently reported	Adopt study-design-specific reporting and quality-assessment procedures	Appropriate in vitro and animal-study reporting guidelines, preregistered protocols, randomisation, blinding, and justified sample sizes
Predominantly preclinical evidence	Most studies employed in vitro or animal models, with no full clinical trials identified	Progress from standardised preclinical testing to carefully designed clinical investigations	Cytotoxicity, irritation, sensitisation, dose–response, validated animal models, and phased clinical evaluation
Uncertain scalability and sustainability	Laboratory-scale extraction and formulation processes were rarely supported by scale-up, energy, cost, or life-cycle data	Evaluate technical, economic, and environmental feasibility before commercial translation	Pilot-scale processing, solvent-recovery assessment, energy analysis, techno-economic assessment, and life-cycle assessment

Note: The limitations represent patterns identified across the 33 included studies rather than deficiencies attributed to every individual study.

Promising formulations should subsequently undergo standardised stability, cytotoxicity, irritation, sensitisation, and dose–response testing before evaluation in validated in vivo models. Only formulations supported by reproducible efficacy and safety evidence should progress to clinical investigation. In parallel, pilot-scale production, solvent recovery, energy demand, manufacturing cost, and life-cycle impacts should be assessed to determine whether the proposed products are technically, economically, and environmentally feasible.

4. Conclusion

This systematic review synthesised evidence from 33 original research studies on extraction technologies and formulation strategies for natural waste- and plant-derived wound-healing products. The evidence base was dominated by preclinical studies, comprising 19 in vitro studies, seven in vivo studies, and seven studies whose experimental designs could not be classified confidently from the reported information. Conventional extraction methods remained widely represented, while intensified techniques, particularly ultrasound-assisted extraction, microwave-assisted extraction, pressurised or accelerated solvent extraction, and supercritical fluid extraction, demonstrated potential for recovering bioactive compounds under method-specific operating conditions. The recovered extracts were incorporated into gels, hydrogels, films, nanofibrous dressings, and other delivery systems associated with antioxidant, antimicrobial, anti-inflammatory, and wound-regenerative activities.

Nevertheless, the available evidence does not establish that these extraction techniques or natural-product-based formulations are superior to conventional extraction processes or established wound-care products. Direct comparisons were limited by substantial heterogeneity in feedstock characteristics, extraction conditions, analytical procedures, formulation composition, bioactive concentration, experimental models, and outcome measures. Methodological reporting

was also incomplete, particularly regarding independent replication, sample-size justification, randomisation, blinding, stability, safety, and procedures used to minimise experimental bias. Consequently, the reported findings should be interpreted as evidence of preclinical potential rather than confirmation of clinical effectiveness.

Future research should prioritise standardised raw-material specifications, validated extraction and analytical procedures, complete formulation characterisation, and reproducible biological-testing protocols. Promising formulations should undergo systematic stability, safety, dose–response, and validated in vivo evaluation before progressing to clinical investigation. Pilot-scale processing, techno-economic assessment, solvent recovery, energy consumption, and life-cycle impacts should also be evaluated to determine whether these technologies are practically and environmentally sustainable. Addressing these limitations is essential for translating natural waste- and plant-derived bioactive materials into reliable, safe, and clinically relevant wound-care products.

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Research article

COMPARATIVE ANALYSIS OF INFRARED AND CONVENTIONAL OVEN DRYING ON THE KINETICS, EFFECTIVE MOISTURE DIFFUSIVITY AND SHRINKAGE OF CALAMANSI (*CITRUS MICROCARPA*)

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ABSTRACT

Calamansi (*Citrus microcarpa*) is a Southeast Asian citrus fruit valued for its distinctive sour taste and bioactive compounds. However, its high moisture content contributes to rapid deterioration, making drying an important preservation method. Drying is a common preservation technique that lowers water activity and enhances the stability of food. This study investigated the effect of infrared (IR) and oven drying at 50, 60, 70, and 80 °C on the drying kinetics, effective moisture diffusivity, and shrinkage of calamansi slice. The results showed that higher temperatures and the use of the IR dryer led to a faster drying rate. Five thin-layer drying models -Newton, Page, Modified Page, Henderson and Pabis, and Midilli were fitted to the experimental moisture ratio data to describe the drying behaviour. The effective moisture diffusivity values were found to range from 0.35×10^{-8} to 5.05×10^{-8} m²/s and generally increased with drying temperature. Shrinkage increased at higher drying temperatures and was more pronounced during IR drying. The Midilli model nevertheless demonstrated high flexibility across IR and high temperature oven drying, achieving $R^2 \geq 0.999$ at IR drying at 60 °C and oven drying at 80 °C. Overall, IR drying provided moisture removal faster than conventional oven drying of calamansi slices under the investigated conditions.

1. Introduction

Calamansi (*Citrus microcarpa*), a small citrus fruit native to Southeast Asia, is widely cultivated and consumed in countries such as the Philippines and Malaysia. It is a natural hybrid of mandarin and kumquat (Cheong et al., 2012) and is prized for its aromatic, tangy juice, which is commonly used in beverages, marinades, dipping sauces, and traditional culinary

preparations. Despite its growing popularity, Calamansi remains highly perishable due to its high moisture content (85–90 %) and delicate peel structure. Seasonal overproduction often leads to significant post-harvest losses, as the fruit deteriorates quickly without proper preservation. This short shelf life limits its availability and increases food waste,

particularly in regions with insufficient cold-chain infrastructure.

Nutritionally, Calamansi is a rich source of vitamin C and various bioactive compounds, including phenolics and flavonoids, which contribute to its antioxidant capacity and associated health benefits (Carr & Maggini, 2017). Preservation of Calamansi not only extends its marketability beyond harvest season but also retains its functional properties, supporting the development of value-added products in the nutraceutical, beverage, and cosmetic sectors (Ma et al., 2017).

Drying is one of the most widely employed preservation methods for fruits, offering enhanced shelf stability by lowering water activity and inhibiting microbial and enzymatic degradation (Kondareddy et al., 2019). Conventional hot-air drying, while effective, is often associated with long drying times, uneven heat distribution, shrinkage, and considerable energy consumption due to indirect convective heat transfer. To overcome these drawbacks, alternative drying technologies such as fluidized bed, microwave, osmotic, and infrared (IR) drying have been developed. Among these, infrared drying has garnered increasing interest in the food industry due to its rapid heating mechanism, energy efficiency, and potential to better preserve color, texture, and nutrient content.

Infrared radiation penetrates food surfaces, inducing molecular vibration and volumetric heating, which accelerates moisture diffusion and evaporation. This method has shown promising results in the dehydration of various food materials, including fruits, vegetables, herbs, and grains (Sakare et al., 2020). Studies comparing hot-air and infrared drying techniques have demonstrated that IR drying can significantly reduce drying times while improving rehydration capacity and maintaining better physical and nutritional qualities (Abidin et al., 2025; Li et al., 2021; Nwakuba et al., 2021; Oshima et al., 2021; Rojas et al., 2019; Alwi et al., 2017). However, excessive IR exposure can cause surface hardening or color degradation, requiring precise process control (Alwi et al., 2022).

Despite these developments, information on the IR drying behaviour of calamansi remains limited. Most previous studies examined other fruits or citrus species, which differ from calamansi in terms of fruit size, peel structure, composition and internal moisture distribution. In particular, limited comparative information is available on the drying kinetics, moisture transport and shrinkage of calamansi subjected to IR and conventional oven drying over different temperatures. Mathematical modelling of its drying behaviour also remains insufficiently reported despite its importance predicting moisture removal. Therefore, this study aimed to compare the drying kinetics of calamansi using IR and oven drying at 50, 60, 70 and 80 °C; evaluate the suitability of selected thin-layer drying model; determine the effective moisture diffusivity and assess the resulting shrinkage. This work provides calamansi specific drying data and identifies the differences between IR and oven drying under the investigated conditions.

2. Materials and methods

2.1. Materials

2.1.1. Plant Material and Sample Preparation

Fresh Calamansi (*Citrus microcarpa*) fruits were purchased from a local market in Shah Alam, Malaysia. Fruits of similar size, shape, colour and apparent maturity without visible damage were selected and rinsed with distilled water. Each fruit was cut with a stainless-steel knife into circular slices of 10 mm thickness and approximately 25 mm diameter and the seeds were removed. The weight for each slice was approximately 3.0 ± 0.2 g, with the initial moisture content determined gravimetrically. The prepared slices were immediately sealed in airtight polyethylene containers and stored at 4 °C prior to the drying experiment.

2.2. Drying Procedures

2.2.1. Infrared Drying

Infrared drying was conducted in a custom-fabricated (IR) dryer with ceramic emitters. Three infrared heaters were installed inside the vessel made of ceramic tubes and installed with a fiber wire with a current voltage of 240 V and

a frequency of 50 Hz. Two mineral-insulated thermocouple Type K sensors were used to monitor and set the temperature during drying. For each drying run, five samples were arranged in a single layer on the stainless tray without overlapping. Drying was conducted at 50 °C, 60 °C, 70 °C, and 80 °C. The samples were weighed at intervals of 10 minutes using a balance with an accuracy of 0.001 g and drying was terminated when the mass change between two consecutive measurements was constant (approximately less than 0.01g).

2.2.2. Oven Drying

For comparison, a forced-convection oven (Memmert GmbH, Germany) was used under identical temperature settings (50–80 °C). Slices were similarly arranged on trays and dried until constant weight. Similarly, each drying temperature was tested with five replicate samples. The oven was preheated at the desired temperature prior to the drying experiments. Sample mass was recorded at 10-min intervals and the same endpoint criterion used for IR drying (mass change <0.01g between two consecutive measurements) was applied.

2.3. Drying Characteristics

2.3.1 Moisture Content

At each drying time, moisture content on a dry basis MC_{db} (g water/g dry matter) was calculated using the equation:

$$\text{Moisture Content}(MC_{db}) = \frac{M_w - M_{db}}{M_{db}} \times 100 \quad (1)$$

where m_w is the wet sample mass (g) and m_{db} is the dry weight (g) of the sample determined at the drying endpoint.

2.3.2. Drying Rate and Moisture Ratio

The dimensionless moisture ratio (MR) and drying rate (DR) were calculated at 10-minute intervals as follows:

$$\text{Moisture Ratio (MR)} = \frac{M_t - M_e}{M_0 - M_e} \approx \frac{M_t}{M_0} \quad (2)$$

$$\text{Drying Rate (DR)} = \frac{M_{t+\Delta t} - M_t}{\Delta t} \quad (3)$$

where M_0 , M_t , $M_{t+\Delta t}$, and M_e are the initial, current, subsequent and equilibrium moisture contents on a dry basis respectively and Δt is the measurement interval. Because M_e was small relative to M_0 , MR was approximated as M_t/M_0 .

2.3.3. Drying Kinetics Modeling

Nonlinear regression analysis was performed using Microsoft Excel Solver® to fit experimental MR data to five common thin-layer drying models as shown in Table 1.

Table 1. Thin-layer drying models used in this study

Model Name	Model Equation
Lewis	$MR = \exp(-kt)$
Page	$MR = \exp(-kt^n)$
Modified Page	$MR = \exp[-(kt)^n]$
Henderson & Pabis	$MR = a \exp(-kt)$
Midilli	$MR = a \exp(-kt^n) + bt$

Model performance was evaluated based on the coefficient of determination (R^2), reduced

chi-square (χ^2), and root mean square error (RMSE) as shown in Table 2.

Table 2. Statistical metrics used for model validation

Metric	Formula
Coefficient of Determination (R^2)	$R^2 = 1 - \frac{\sum(MR_{\text{exp}} - MR_{\text{pred}})^2}{\sum(MR_{\text{exp}} - \bar{MR}_{\text{pred}})^2}$
Chi-Square (χ^2)	$\chi^2 = \sum \frac{(MR_{\text{exp}} - MR_{\text{pred}})^2}{N - n}$
Root Mean Square Error (RMSE)	$RMSE = \sqrt{\frac{1}{N} \sum (MR_{\text{exp}} - MR_{\text{pred}})^2}$

The model exhibiting the highest R^2 and the lowest χ^2 and RMSE values was considered the most suitable for describing calamansi drying behaviour (Kim et al., 2021).

2.3.4 Effective Moisture Diffusivity (D_{eff})

Effective moisture diffusivity was estimated using Fick's second law of diffusion for slab geometry. The moisture ratio was modeled using the simplified form:

$$MR = \frac{8}{\pi^2} \exp\left(-\frac{\pi^2 D_{\text{eff}} t}{4L^2}\right) \quad (4)$$

Taking the natural logarithm:

$$\ln(MR) = \ln\left(\frac{8}{\pi^2}\right) - \frac{\pi^2 D_{\text{eff}} t}{4L^2} \quad (5)$$

D_{eff} was calculated from the slope of $\ln(MR)$ versus time. The characteristic length (L) was taken as half of the initial slice thickness ($L = 0.005$ m). The slab approximation was adopted because moisture transport was assumed to occur predominantly through the

two broad faces of the 10-mm thick, approximately 25-mm diameter slices.

2.3.5. Shrinkage Measurement

Shrinkage was evaluated as a percentage reduction in mass before and after drying:

$$\text{Shrinkage}(\%) = \frac{m_i - m_f}{m_i} \times 100 \quad (6)$$

where m_i and m_f are the initial and final sample masses, respectively. Samples were allowed to cool to ambient temperature prior to final weighing.

2.4. Data analysis

Drying curves were fitted by nonlinear regression using Microsoft Excel Solver. Model performance was assessed using R^2 , RMSE and χ^2 .

3. Results and discussion

3.1. Calamansi Drying Time

Drying time decreased as temperature increased for both methods. At 80 °C, IR drying reached the endpoint after approximately 130 min, whereas oven drying required approximately 310 min. IR drying at 50 °C produced a drying duration broadly comparable with oven drying at 80 °C as shown in Figure 1. These observations demonstrate faster moisture removal by IR compared to conventional oven. Compared with oven drying, the shorter processing time achieved by IR drying at 50 °C

may reduce cumulative thermal exposure and potentially improve the retention of heat-sensitive constituents. Previous studies have similarly shown that IR drying can shorten processing time relative to oven drying and that appropriately selected IR temperatures may help preserve antioxidant activity in plant materials (Alwi et al., 2025).

The shorter drying time achieved by IR drying can be attributed to the penetration and absorption of radiant energy within the surface and near-surface regions of the sample. The absorbed energy excites molecular vibrational modes, particularly those associated with water, producing rapid heating and steeper temperature and moisture gradients. These gradients enhance internal moisture diffusion towards the surface and accelerate evaporation (Sakare et al., 2020).

3.2. Drying Characteristics

3.2.1. Moisture Content Profiles

Figure 1 illustrate the moisture ratio profiles of calamansi slices dried by infrared (IR) and conventional oven methods across a temperature range of 50–80 °C. In all conditions, moisture content declined progressively over time, with

more pronounced reductions at elevated temperatures. This behavior is further reflected in the moisture ratio (MR), a dimensionless indicator of relative moisture loss, which also decreased with time in all cases. IR drying exhibited a steeper decline in both moisture content and MR during the initial stages, particularly at 80 °C, highlighting its higher drying intensity and efficiency.

As drying progressed, the rate of moisture removal slowed considerably, indicating a shift into the falling-rate period—characterized by internal moisture diffusion as the controlling mechanism. The falling-rate period was longer for oven-dried samples, particularly at lower temperatures. IR drying at 80 °C achieved significant moisture reduction and a sharp drop in MR within the first two hours, whereas oven drying required up to five hours to reach a comparable MR level. These trends are consistent with the drying behavior of biological materials, where water becomes increasingly bound within the cellular matrix as drying continues (Bolaji et al., 2021; Ampah et al., 2022).

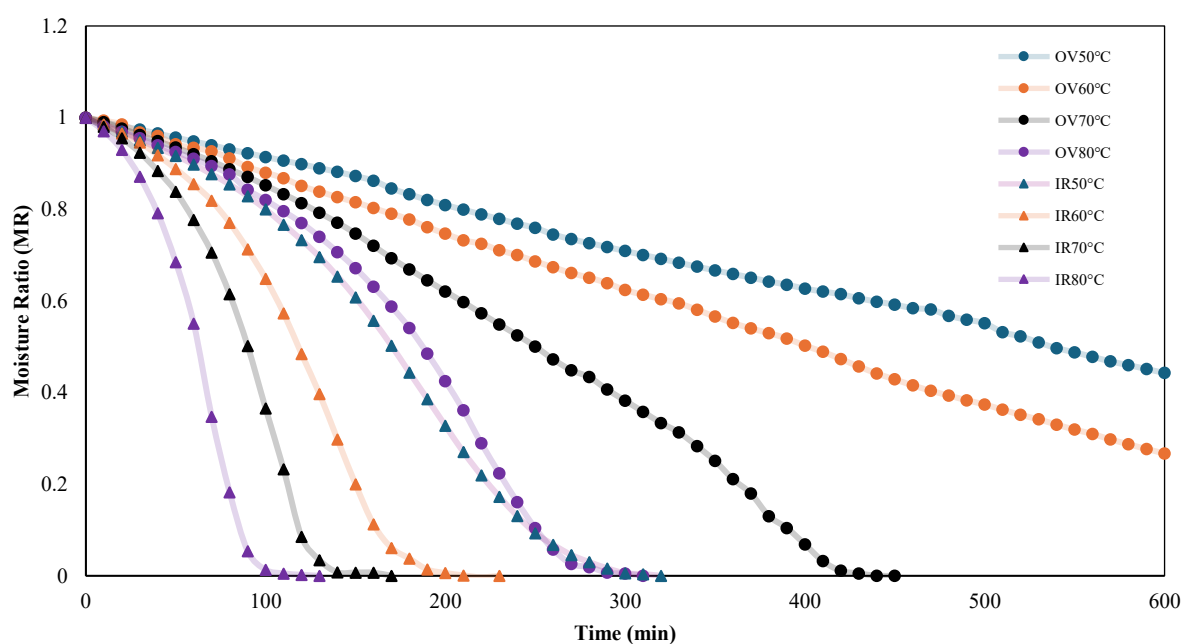


Figure 1. Moisture ratio of dried calamansi under influence of IR and conventional oven drying at various temperatures; 50 °C, 60 °C, 70 °C, 80 °C.

3.2.2. Drying Rate Analysis

Figures 2 and 3 show drying rate as a function of moisture content for IR and oven drying respectively at 50, 60, 70 and 80 °C. For IR drying (Figure 2), the highest observed drying rate occurred at 80 °C (approximately 0.13 g water/g dry matter.min) followed by 70,

60 and 50 °C. This ordering reflects the greater heat- and mass-transfer driving forces at higher temperatures and the rapid absorption of IR radiation by the exposed sample layers, which enhances heating, internal moisture diffusion, and surface evaporation (Sakare et al., 2020; Abdullah et al., 2022).

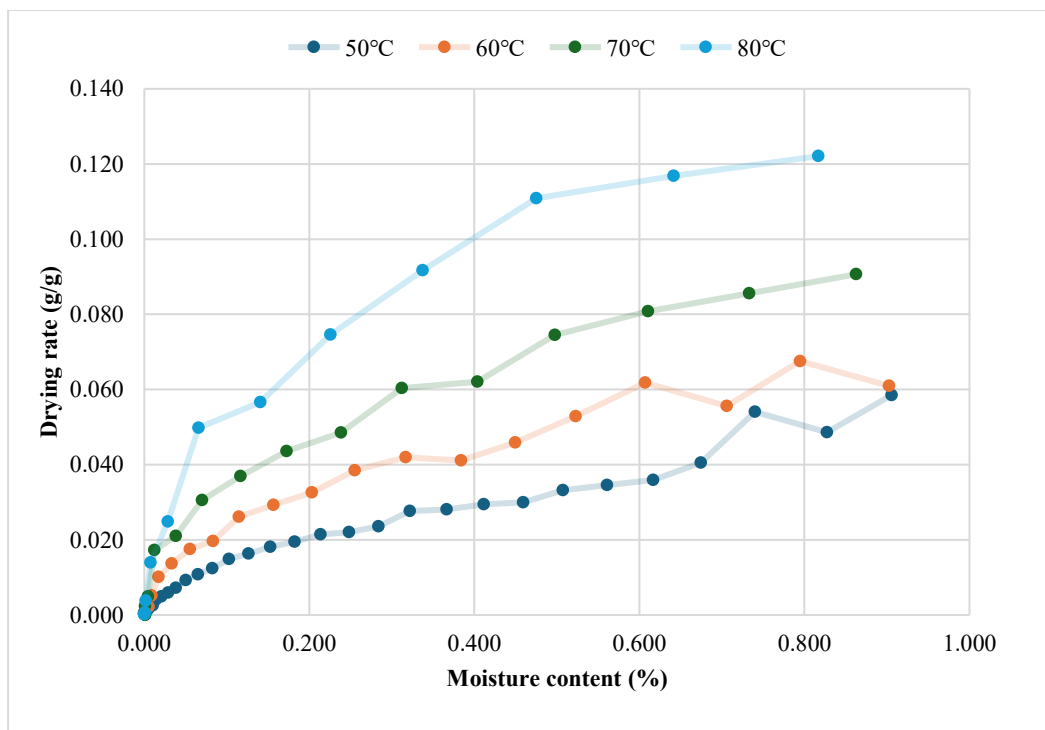


Figure 2. The relationship between drying rate (g/g) and moisture content (%) of Calamansi slices dried using an infrared (IR) dryer

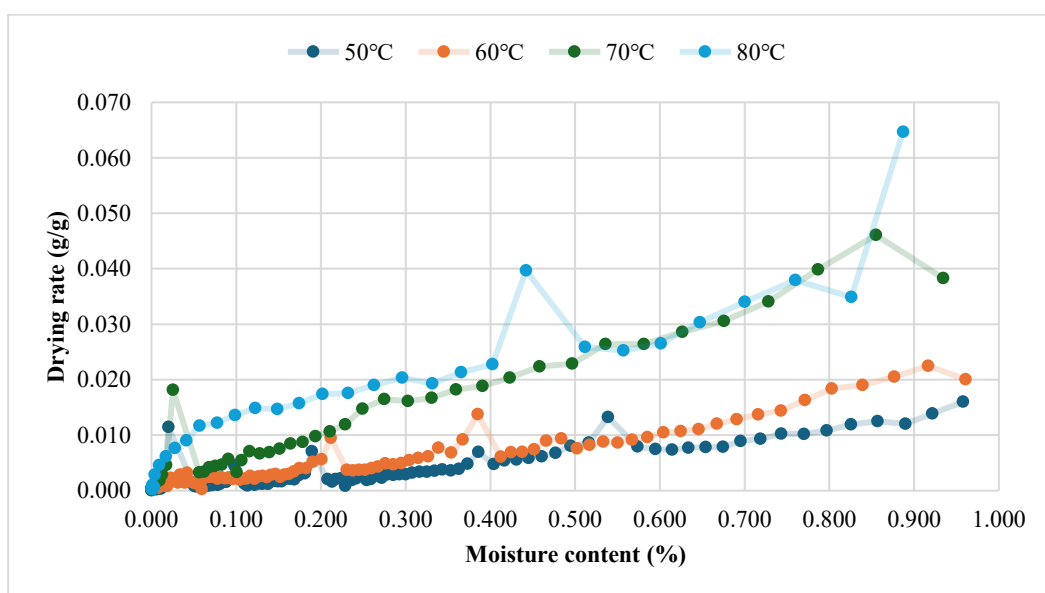


Figure 3. The relationship between drying rate (g/g) and moisture content (%) of Calamansi slices dried using conventional oven.

The drying rate generally decreased as moisture content declined, confirming predominantly falling-rate behaviour. Free and readily available moisture was removed first, whereas the later stages depended increasingly on internal diffusion of more strongly bound water. The observed kinetic trends agree with reports of accelerated moisture removal during IR drying of sliced plant materials (Çağlar & Selvi, 2020; Rojas et al., 2019; Younis et al., 2018).

In contrast, the oven drying data in Figure 3 displayed lower drying rates across all temperatures, with a maximum drying rate of approximately 0.07 g/g at 80 °C. Moreover, the curves were more scattered and irregular, indicating non-uniform drying behavior. This variability can be attributed to the slower and indirect heat transfer mechanism of conventional ovens, which primarily rely on convective heat transfer from the surrounding air. The thermal inertia and longer heat-up time in oven drying limit its capacity to initiate rapid moisture removal during early drying phases, particularly at lower temperatures (Kim et al., 2021; Akpınar, 2010).

3.2.3 Mathematical Modeling of Drying Behaviour

The experimental drying behavior of calamansi slices under different temperatures and drying methods was evaluated using five established thin-layer drying models: Newton, Page, Modified Page, Henderson and Pabis, and Midilli. Model fitting was assessed through the coefficient of determination (R^2), root mean square error (RMSE), and reduced chi-square (χ^2) as tabulated in Table 3. A model was considered suitable when it demonstrated high R^2 values (close to 1) and minimal RMSE and χ^2 values.

Among the evaluated models, the Midilli model exhibited the highest predictive performance, particularly under infrared (IR) drying conditions at 60 °C and 70 °C, and under oven drying at 80 °C, with R^2 values exceeding 0.9990. This model's inclusion of both exponential and linear terms allows it to effectively capture both the initial rapid drying

phase and the subsequent falling-rate period, where moisture diffusion becomes dominant. These findings align with prior studies highlighting the Midilli model's robustness across varying food matrices and drying conditions (Ça & Selvi, 2020; Younis et al., 2018).

The Page model also demonstrated strong agreement with experimental data, especially for IR drying at 60 °C and oven drying at 60 °C, achieving $R^2 > 0.9995$ and near-zero residual errors. Its flexible structure proved advantageous in modeling non-linear moisture removal dynamics, particularly in mid-range temperatures. This is consistent with its widespread application in modelling fruit and vegetable dehydration processes (Ampah et al., 2022).

The Newton and Modified Page models showed satisfactory performance under specific conditions, particularly IR drying at 50 °C, with R^2 values up to 0.9986. However, their predictive capacity declined at elevated temperatures, likely due to their inability to capture the complexities of internal moisture migration and structural shrinkage occurring in advanced drying stages. The Henderson and Pabis model, although less accurate overall, maintained moderate fit with R^2 values between 0.97 and 0.98, particularly under IR drying. Its comparatively simple formulation may have limited its capacity to reflect the full range of drying kinetics, especially in the transition from constant to falling-rate periods.

The Midilli model emerged as the most appropriate model for describing the drying kinetics of calamansi slices across both drying methods and temperature ranges. Its superior adaptability and consistency highlight its suitability for process modelling and optimization in drying operations. The Page model served as a reliable secondary model, particularly under moderate drying conditions. Conversely, the Newton, Modified Page, and Henderson and Pabis models were limited in scope, with their performance confined to specific drying conditions.

Table 3. Mathematical drying models and statistical values for dried calamansi.

Model Name	Drying Method	Drying Temperature	a	b	k	n	RMSE	X2	R2
Newton	IR Dryer	50°C	–	–	0.0023	–	0.0001	0.0002	0.9889
		60°C	–	–	0.0044	–	0.0017	0.0036	0.9875
		70°C	–	–	0.0085	–	0.0101	0.0214	0.9908
		80°C	–	–	0.0150	–	0.0146	0.0315	0.9885
	Oven	50°C	–	–	0.0009	–	0.0000	0.0000	0.9986
		60°C	–	–	0.0008	–	0.0000	0.0000	0.9979
		70°C	–	–	0.0016	–	0.0000	0.0000	0.9942
		80°C	–	–	0.0020	–	0.0001	0.0001	0.9901
Page	IR Dryer	50°C	–	–	0.0002	1.4899	0.0000	0.0000	0.9958
		60°C	–	–	0.0000	2.3208	0.0001	0.0002	0.9995
		70°C	–	–	0.0000	2.7144	0.0007	0.0016	0.9996
		80°C	–	–	0.0000	2.7013	0.0005	0.0013	0.9996
	Oven	50°C	–	–	0.0001	1.4899	0.0000	0.0000	0.9995
		60°C	–	–	0.0001	1.4899	0.0000	0.0000	0.9998
		70°C	–	–	0.0004	1.2846	0.0000	0.0000	0.9974
		80°C	–	–	0.0000	2.3208	0.0001	0.0003	0.9996
Modified Page	IR Dryer	50°C	–	–	0.0038	1.4906	0.0000	0.0000	0.9958
		60°C	–	–	0.0072	2.2896	0.0001	0.0002	0.9991
		70°C	–	–	0.0103	3.1205	0.0006	0.0013	0.9997
		80°C	–	–	0.0147	3.0565	0.0004	0.0009	0.9997
	Oven	50°C	–	–	0.0009	1.0039	0.0000	0.0000	0.9986
		60°C	–	–	0.0008	1.0093	0.0000	0.0000	0.9980
		70°C	–	–	0.0024	1.2846	0.0000	0.0000	0.9974
		80°C	–	–	0.0032	1.4111	0.0000	0.0000	0.9950
Henderson and Pabis	IR Dryer	50°C	1.0250	–	0.0026	–	0.0001	0.0001	0.9902
		60°C	1.0868	–	0.0054	–	0.0012	0.0027	0.9899
		70°C	1.1709	–	0.0105	–	0.0079	0.0178	0.9930
		80°C	1.0000	–	0.0150	–	0.0146	0.0341	0.9885
	Oven	50°C	1.0000	–	0.0009	–	0.0000	0.0000	0.9986
		60°C	1.0006	–	0.0008	–	0.0000	0.0000	0.9979
		70°C	1.0127	–	0.0017	–	0.0000	0.0000	0.9948
		80°C	1.0175	–	0.0022	–	0.0000	0.0001	0.9909
Midilli	IR Dryer	50°C	1.0111	0.0000	0.0000	2.7248	0.0005	0.0011	0.9992
		60°C	1.0111	0.0000	0.0000	2.7248	0.0004	0.0010	0.9997
		70°C	0.9889	0.0000	0.0000	2.7099	0.0007	0.0019	0.9996
		80°C	0.9970	0.0000	0.0000	2.7005	0.0005	0.0015	0.9996
	Oven	50°C	1.0111	0.0000	0.0000	2.7248	0.0009	0.0020	0.9929
		60°C	1.0111	0.0000	0.0000	2.7248	0.0012	0.0024	0.9924
		70°C	1.0111	0.0000	0.0000	2.7248	0.0009	0.0020	0.9942
		80°C	1.0111	0.0000	0.0000	2.7248	0.0004	0.0010	0.9990

Similarly in oven drying, five widely used thin-layer drying models were fitted to the experimental moisture ratio (MR) data, and model performance was assessed using R^2 , RMSE, and χ^2 as tabulated in Table 3. The Newton, Page, Modified Page, and Henderson & Pabis models showed high accuracy in oven-drying applications, particularly at 50–60 °C. The Newton and Modified Page models achieved R^2 values of 0.9986 with zero RMSE and χ^2 under those conditions.

The Midilli model, however, demonstrated superior flexibility across both IR and high-temperature oven settings. It showed optimal fit at 60 °C in IR drying and 80 °C in oven drying, with R^2 values exceeding 0.999 and minimal RMSE (0.0004) and χ^2 (0.0010). This is consistent with other studies showing that the Midilli model effectively captures drying kinetics in non-linear, high-energy

environments, especially under IR drying of herbs and citrus materials (Çağatay Selvi & Gatay, 2020; Nasr et al., 2021; Górnicki et al., 2020). These results confirm that while simpler models may suffice under lower-temperature convection drying, the Midilli model is most appropriate for predicting the complex behavior of Calamansi drying under variable thermal conditions.

3.3. Effective Moisture Diffusivity (D_{eff})

Table 4 presents the effective moisture diffusivity (D_{eff}) values of Calamansi slices dried at 50 °C to 80 °C. The values of D_{eff} are critical for understanding the mass transfer characteristics during drying, reflecting how efficiently moisture migrates from the interior to the surface of the product.

Table 4. Effective moisture diffusivity of calamansi slice under IR & oven drying at different temperatures.

Type of Dryer	Drying Temperature	Effective Moisture Diffusivity ($\text{m}^2\cdot\text{s}^{-1}$)
IR Dryer	50 °C	1.45×10^{-8}
	60 °C	2.52×10^{-8}
	70 °C	3.31×10^{-8}
	80 °C	5.05×10^{-8}
Oven	50 °C	0.35×10^{-8}
	60 °C	0.35×10^{-8}
	70 °C	1.00×10^{-8}
	80 °C	1.38×10^{-8}

The IR dryer demonstrated significantly higher D_{eff} values than the oven at all temperatures. At 80 °C, IR drying achieved the highest of $5.05 \times 10^{-8} \text{ m}^2/\text{s}$, compared to only $1.38 \times 10^{-8} \text{ m}^2/\text{s}$ for oven drying. This difference underscores the greater drying efficiency of IR drying, attributed to its direct radiation heating mechanism, which provides rapid surface heating and volumetric moisture transport. The difference is consistent with the steeper MR gradients and shorter drying times observed during IR drying. In contrast, the oven relies solely on convective heat transfer, which results in slower internal water diffusion and lower

D_{eff} values (Abdullah et al., 2022; Wang et al., 2021).

D_{eff} increased with temperature for both methods. For IR drying, it rose from $1.45 \times 10^{-8} \text{ m}^2/\text{s}$ at 50 °C to $5.05 \times 10^{-8} \text{ m}^2/\text{s}$ at 80 °C. For oven drying, it increased from 0.35×10^{-8} to $1.38 \times 10^{-8} \text{ m}^2/\text{s}$. Higher temperature increases the vapour-pressure driving force and molecular mobility, thereby promoting internal moisture migration (Kim et al., 2021; Younis et al., 2018). These findings show faster apparent moisture transport under IR drying.

3.4. Shrinkage Effects

Figure 4 presents the shrinkage ratios of calamansi slices subjected to IR and oven drying at temperatures ranging from 50 to 80 °C. The IR-dried samples exhibited higher shrinkage ratios (approximately 0.85–0.865) than the oven-dried samples (approximately 0.77–0.84) across all temperatures. This greater shrinkage may be associated with the faster heating and moisture removal achieved during IR drying, which intensifies moisture gradients and promote the collapse of the cellular structure (Talebzadeh et al., 2022).

For both drying methods, the shrinkage ratio generally increased as the drying temperature increased from 50 to 70 °C. However, a slight reduction was observed for oven drying at 80 °C, possibly due to surface hardening and the formation of a rigid outer layer that restricted further structural contraction (Thuwapanichayanan et al., 2011). In contrast, the shrinkage ratio of IR-dried samples continued to increase slightly at 80 °C, indicating greater structural deformation under the investigated conditions.

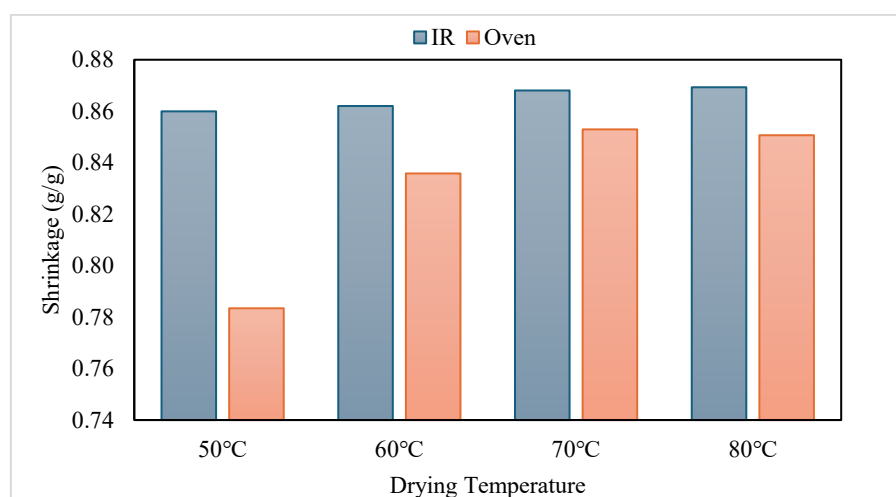


Figure 4. The shrinkage ratio (g/g) of calamansi slices subjected to infrared (IR) and conventional oven drying

4. Conclusion

This study comprehensively evaluated the drying kinetics and physical properties of Calamansi slices subjected to infrared (IR) and oven drying at temperatures ranging from 50 °C to 80 °C. The findings highlight the significant impact of drying method and temperature on drying rate, moisture removal efficiency, and structural changes. IR drying reached the defined endpoint more rapidly and produced higher D_{eff} values than oven drying under the investigated conditions. The Midilli model nevertheless demonstrated high flexibility across IR and high temperature oven drying, achieving $R^2 \geq 0.999$ at IR drying at 60 °C and oven drying at 80 °C. The increase in effective moisture diffusivity with rising temperature and under IR drying conditions was attributed to enhanced vapour-pressure gradients and the

radiative heat transfer mechanism of IR drying. Future investigations should focus on the effects of these drying methods on the nutritional, chemical, and sensory attributes of Calamansi. Such studies would complement the current findings and support the development of optimized, value-added products using sustainable drying technologies.

5. References

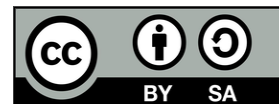
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Research article

EFFECT OF EXTRACTION SOLVENT ON PHENOLIC AND FLAVONOID CONTENTS AND ANTIOXIDANT ACTIVITY OF *CAESALPINIA SAPPAN* L.

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ABSTRACT

Caesalpinia sappan L. heartwood contains phenolic and flavonoid compounds with reported antioxidant properties. Solvent selection and extraction conditions can substantially affect the recovery of these compounds. This study evaluated the effects of 70% (v/v) ethanol–water and 70% (v/v) methanol–water on total phenolic content (TPC), total flavonoid content (TFC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity of *Caesalpinia sappan* L. heartwood extracts. Response surface methodology (RSM) was used to investigate extraction temperature (30–70 °C) and time (15–45 minutes) and to identify conditions that simultaneously maximize the measured responses. The highest experimentally observed TPC and TFC values were 7.8 and 8.1 mg/g, respectively, while the highest reported DPPH inhibition was 93% for the ethanol extract at 65 °C for 40 minutes. RSM-based optimization predicted a desirability solution for ethanol at 65 °C and 38 minutes, with predicted responses of 4.60 mg/g TPC, 6.80 mg/g TFC, and 85% inhibition; the corresponding methanol solution was 52 °C and 35 minutes, with predicted responses of 1.52 mg/g TPC, 2.35 mg/g TFC, and 74% inhibition.

1.Introduction

Caesalpinia sappan L. or Sappan Wood and Brazilwood Tree is a small tree (6m -15m) from Leguminosae family (NParks, 2021). Sappan wood has a spherical, brownish-green stem and rufous-pubescent branches. Its diameter ranges from 15 to 25 cm. The main active ingredient is Brazilin, although there are also additional compounds like triterpenoids, flavonoids, lipids, steroids, and amino acids. Heartwood of the

Caesalpinia sappan L. is a component in traditional medicines (Vij et al., 2023).

Karneng (2024) stated that the community has utilized the *Caesalpinia sappan* L. as a natural remedy to treat a variety of illnesses, including tetanus, blood clots, and pain. It is believed to have potential as a plant with plenty of antioxidant components. In Southeast Asia, sappan wood has also been used to colour foods like rice and noodles, produce fine furniture and decorative goods, and dye textiles made of silk

and cotton. Anthraquinones, phenolic acids, and flavonoids are the main components of *Caesalpinia sappan* L. (Vij et al., 2023). Xanthones, coumarins, chalcones, flavones, and brazilin are among the phytochemical components found in *Caesalpinia sappan* L. or Secang wood (Koeswantika Putri & Indah Sabila, 2021). In recent years, the food, pharmaceutical, cosmetic, and functional food industries have mostly used natural sources to produce bioactive chemicals. Phytochemical substances found in medicinal plants are abundant and have positive health effects. The possible antibacterial, anti-inflammatory, and antioxidant properties of *Caesalpinia sappan* L. extracts have been the main topic of numerous research (Insuan et al., 2024). Sappan wood thus is packed with many other biochemicals such as xanthones, flavones, chalcones, and brazilin. Brazilin-a principal active element of *Caesalpinia sappan* L. is a white phenolic compound grouped based on its molecular structure into bioflavonoids which is characterized by two aromatic rings (Vij, 2023).

The phenolic compounds in sappan wood are very beneficial for human health. Phenolic compounds can provide good health by reducing the metabolic disorders. Rahman et al. (2022) states that "Many studies have indicated that phenolic compounds have diverse effects of anti-inflammatory, anti-aging, and antioxidant properties". Phenolic compounds are a diverse group of secondary metabolites formed during the metabolism of plants. Many experts focus on phenol compounds for the reason of health benefits, particularly those found in food. Vitamins C and E are the most often utilized antioxidant supplements. Compared to vitamin C and vitamin E, flavonoids have a stronger antioxidant capacity. Bone health is improved by flavonoids because of their well-known and enhanced antioxidant and anti-inflammatory effects. There are many opportunities for bone tissue engineering when flavonoids are included in biomaterials. Flavonoids, which may be used as sunscreens, are found in the secang tree. Conjugated aromatic benzene groups in flavonoid compounds could absorb ultraviolet

(UV) light and are thought to protect skin exposed to direct sunlight (Rohmah et al., 2022).

Brazilin is the main compounds in Sappan wood that has numerous industrial uses in the medicinal, cosmetic, and textile sectors (Vij et al., 2023). According to Vij (2023), Potential pharmacological activities of brazilin include immunostimulant, anti-inflammatory, anti-tumor, and anti-diabetic effects. Brazilin can bind iron to both ferrous and ferric ions (Taveepanich et al., 2024). Since brazilin is the primary active component of the flavonoid group, the red dye found in sappan wood is referred to as a brazilin group compound (Koeswantika Putri & Indah Sabila, 2021). The antioxidants could slow, stop, and reverse the lipid oxidation process, which can harm the body's cells. The antioxidants work in the body is by binding free radicals too. The body's regular metabolism produces free radicals, which can lead to oxidative damage, including damage to DNA, membranes, and cells (Koeswantika Putri & Indah Sabila, 2021). Synthetic antioxidants and natural antioxidants are the two categories of antioxidants. When an antioxidant substance is produced via a chemical reaction, it is referred to as a synthetic antioxidant. Antioxidant molecules that are derived from natural components are known as natural antioxidants. Vegetables, seeds, stems, and fruits are typically plants that contain natural antioxidants. Flavonoids and phenolic *Caesalpinia sappan* L. have various benefits in human health, but none are apparently nullified by evidence toward their extraction techniques from *Caesalpinia sappan* L. Moreover, even fewer studies have been carried out on appropriate solvents used to recover these bioactive compounds generated from *Caesalpinia sappan* L. Accordingly, extraction solvents are an important part in the success rate of bioactive compound extraction from plant products, especially from *Caesalpinia sappan* L.

A successful extraction process depends on factors such as the method applied, solvent, and the nature of the target compounds. Conventional techniques like maceration and Soxhlet extraction have been in use, also modern extraction techniques such as Ultrasound-

Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE) might be more efficient. The extraction efficiency depends on the type of the solvent used so that the bioactive compound is not lost or destroyed during the extraction process. The solvent system used is mostly determined by the bioactive molecule being targeted. Various solvent systems are available to extract bioactive compounds from natural sources. Hydrophilic chemicals are extracted using polar solvents such as methanol, ethanol, or ethyl acetate (Sasidharan et al., 2011). Ethanol has been widely used for the extraction of phenolic compounds because it is suitable, nontoxic for researchers and environment. Methanol also expresses highest selectivity towards phenolic compounds (Pintac et al., 2018).

Response surface methodology (RSM) is used as a substitute procedure to lessen the difficulties associated with conventional testing and to assess the impact of several factors. RSM is an effective mathematical and statistical method for examining empirical models that explain how independent factors and their interactions affect response variables (Mansour et al., 2023). As stated by Maccarronello et al. (2024), RSM will enhance the recovery of bioactive chemicals by determining the optimal operating conditions. Compared to the conventional one-factor at a time method, RSM saves time and money by reducing the number of experiments needed for the study. RSM has previously been used to optimize the extraction of polyphenols from a variety of plant-based foods and their byproducts. The objectives of this study are to determine the total flavonoid content, total phenolic content and antioxidant activities in Brazilin extracted from *Caesalpinia sappan* L. using different types of solvent extraction and to compare the effectiveness of different solvents on the total flavonoid content, total phenolic content and antioxidant activities in Brazilin extracted from *Caesalpinia sappan* L.

2. Materials and Methods

2.1. Materials and Sample Preparation

The materials and reagents included *Caesalpinia sappan* L. heartwood, ethanol, methanol, distilled water, Folin–Ciocalteu reagent, sodium carbonate (Na_2CO_3), dimethyl sulfoxide, aluminium chloride (AlCl_3), quercetin, potassium acetate, diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), gallic acid (Sigma-Aldrich, USA), and laboratory-grade consumables. The instruments included a rotary evaporator, UV–Vis spectrophotometer (GENESYS), water bath, hot plate with magnetic stirring, grinder, and filtration apparatus.

Caesalpinia sappan L. heartwood was obtained from a local supplier and authenticated before use. The material was washed to remove surface impurities and dried in a hot-air oven at 60 °C. The dried heartwood was ground and sieved to a particle size of 300 µm. The powder was stored in airtight containers at room temperature and protected from light until extraction.

2.2. Experimental Design and Response Surface Methodology (RSM)

Response surface methodology (RSM) was employed to investigate and optimize the effects of extraction temperature, extraction time, and solvent type on the extraction of bioactive compounds from *Caesalpinia sappan* L. heartwood. A central composite design (CCD) was used for the two quantitative process variables, namely extraction temperature (X_1) and extraction time (X_2), while solvent type (X_3) was incorporated as a categorical factor. The temperature was investigated over the range of 30–70 °C, with a centre point of 50 °C, whereas the extraction time was varied from 15 to 45 min, with a centre point of 30 min. Two extraction solvents, ethanol and methanol, were evaluated as discrete categorical levels.

For the quantitative variables, the actual experimental levels were transformed into coded values of –1, 0, and +1 according to the standard linear transformation used in RSM. Thus, coded levels of –1, 0, and +1 corresponded to 30, 50, and 70 °C for temperature and 15, 30, and 45

min for extraction time, respectively. Solvent type was coded as a categorical variable, with ethanol and methanol represented by the two categorical levels. The numerical coding assigned to the solvents was used only for experimental-design purposes and did not imply an ordered quantitative relationship between the two solvents.

2.3. Solvent preparation of quercetin and gallic acid standard calibration curve

The quercetin calibration curve was prepared following Masturi et al. (2021) with modifications. Briefly, 50 mg of quercetin was dissolved in 50 mL ethanol. Aliquots of 0.50, 1.00, 1.50, 2.00, and 2.50 mL were diluted with distilled water to 10 mL, giving nominal concentrations of 50, 100, 150, 200, and 250 µg/mL. For each standard, 3 mL of quercetin solution was mixed with 3 mL of 2% (w/v) AlCl_3 and 3 mL of 120 mM potassium acetate. The mixtures were incubated in the dark for 30 minutes, and absorbance was measured at 425 nm against a reagent blank.

The gallic acid calibration curve was prepared according to Masturi et al. (2021) with modifications. Gallic acid (50 mg) was dissolved in ethanol to 50 mL. Aliquots of 1.0–4.0 mL were diluted to 10 mL to obtain nominal concentrations of 100, 150, 200, 250, 300, 350, and 400 µg/mL. One millilitre of each standard was mixed with 3 mL Folin–Ciocalteu reagent in a 50 mL volumetric flask and allowed to react for 5 minutes. Subsequently, 8 mL of 7.5% (w/v) Na_2CO_3 was added and the volume was made up with distilled water. The mixture was incubated in the dark at room temperature. Absorbance was measured at 765 nm.

2.4. Solvent Extraction Method

The extraction procedure was adapted from Settharaksa et al. (2019), with modifications to solvent composition, temperature, and extraction time. Five grams of dried *Caesalpinia sappan* L. heartwood powder was mixed with 100 mL of either 70% (v/v) ethanol–water or 70% (v/v) methanol–water, giving a solvent-to-solid ratio of 20 mL/g. The suspension was heated with continuous magnetic stirring at the

temperature and for the duration specified by the RSM design. After extraction, the mixture was cooled to room temperature and filtered through Whatman No. 1 filter paper. The filtrate was stored at 4 °C until analysis.

2.5. Determination of total phenolic content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method described by Masturi et al. (2021), with modifications in the reagent and sample volumes. Briefly, 1 mL of sample extract or gallic acid standard solution was transferred into a volumetric flask, followed by the addition of 3 mL of Folin–Ciocalteu reagent and 30 mL of distilled water. The mixture was thoroughly mixed and allowed to stand for 10 minutes at room temperature. Subsequently, 8 mL of 7.5% (w/v) sodium carbonate solution was added, and distilled water was added to make up the final volume to 50 mL. The resulting solution was mixed thoroughly and incubated in the dark at room temperature for 30 minutes to allow colour development. After incubation for 45 minutes, the absorbance of the reaction mixture was measured at 765 nm using a UV–Vis spectrophotometer. A calibration curve was constructed using gallic acid standard solutions (0 – 100 mg/L), and the total phenolic content of the samples was calculated from the calibration equation.

2.6. Determination of total flavonoid content

The determination of total flavonoid content (TFC) was carried out according to the method of Masturi et al. (2021), with slight modifications in sample and reagent volumes. Briefly, 2 mL of sample extract and 3 mL of quercetin standard solution were separately added to 4 mL of 0.1 mol/L aluminium chloride solution. The mixtures were then combined and diluted with 70% (v/v) ethanol to a final volume of 25 mL. The reaction mixture was thoroughly mixed and allowed to stand for 30 minutes at room temperature to ensure complete complex formation. Subsequently, the absorbance of the solution was measured using a UV–visible spectrophotometer at 425 nm against an appropriate blank.

2.7. Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was performed according to Masturi et al. (2021), with slight modifications in sample preparation and assay conditions. A DPPH working solution was prepared in methanol. Subsequently, 3 mL of DPPH solution was mixed with 4 mL of methanol and incubated in the dark at room temperature for 45 minutes. The absorbance of the resulting solution was measured using a UV-visible spectrophotometer at 517 nm.

For sample preparation, 2 mg of crude extract and quercetin (standard) were separately dissolved in 10 mL of methanol and then transferred into a 100 mL volumetric flask. The solutions were made up to volume with methanol to obtain a stock concentration of 1000 µg/mL. Serial dilutions were prepared to obtain final concentrations of 10, 20, 30, 40, and 50 µg/mL. 3 mL of each sample concentration was mixed with 1 mL of DPPH solution in methanol. The reaction mixtures were incubated in the dark at room temperature for 30 minutes. Methanol containing DPPH without sample was used as the control, while methanol alone served as the blank.

All measurements were performed in triplicate, and the percentage of DPPH radical scavenging activity was calculated using Equation (1).

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100 \quad (1)$$

where A_{control} is the absorbance of the DPPH control and A_{sample} is the absorbance of the extract-containing reaction mixture after the same incubation period.

3. Results and discussion

The extraction experiments compared 70% (v/v) ethanol–water and 70% (v/v) methanol–water over extraction temperature of 30–70 °C and extraction time of 15–45 minutes.

3.1. Total phenolic content

The response surface plot demonstrates that extraction temperature and time exert a significant combined effect on total phenolic content (TPC) when ethanol is used as the

solvent, as evidenced by the dome-shaped curve (Figure 1). An increase in temperature and extraction time initially enhances TPC, which can be attributed to improved solubility of phenolic compounds in ethanol, reduced solvent viscosity, and increased cell wall permeability that collectively promote mass transfer from the plant matrix into the solvent. However, beyond the optimum region, further increases in temperature and prolonged extraction time result in a decline in TPC, most likely due to thermal degradation, oxidation, or polymerization of heat-sensitive phenolic compounds. The elliptical contour pattern confirms a strong interaction by temperature and time combination, indicating that optimal phenolic recovery is achieved only within a moderate range of both parameters rather than at extreme conditions. These findings are consistent with previous studies reporting that ethanol-based extraction of phenolics is governed by a balance between enhanced diffusion kinetics and compound stability, where excessive thermal exposure negatively affects phenolic integrity (Cacace & Mazza, 2003; Lapornik et al., 2005; Dai & Mumper, 2010).

The highest concentration for TPC obtain is 7.8 mg/ml as shown in Figure 1 by using ethanol as solvent for extraction. For methanol solvent, the highest value of the concentration is 2.8 mg /ml after compared with standard curve based on Figure 2. The range for TPC concentration value is (0.7- 7.8) mg/ml for ethanol and for methanol is (0.08- 2.8) mg/ml. Based on Figure 1 and Figure 2, the colour blue represents the lowest value of concentration obtained for the total phenolic content while the red colour represents the highest concentration value. The experimental value obtained conclude that ethanol extracts more phenolic content compared to methanol consistent with Kurniasari et al. (2024), who reported that ethanol gave the highest phenolic content. This supports the study that ethanol has superior efficacy for extracting phenolic compounds like brazilin due to its polarity and thermal profile.

The extraction time that will give higher concentration of TPC is 40 minutes; however, 45 minutes give lower value of TPC. This means

longer extraction time can result in degradation of phenolic compound resulting in lower TPC value. As stated by Che Sulaiman et al. (2017), at fixed temperature, an increase in extraction time slightly decreased the yield. Also, at 65°C extraction temperature results in the highest

concentration of total phenolic content because theoretically, under high temperatures, plant tissues are softened, and the weak interactions affect the cell membranes. As a result, phenolic compounds can be easily extracted into the solvent (Che Sulaiman et al., 2017).

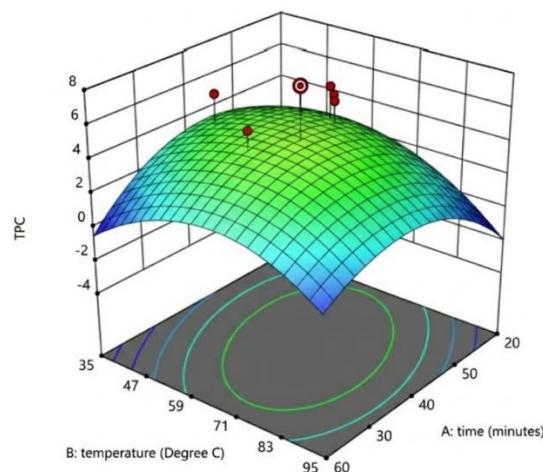


Figure 1. Effect of temperature and time on TPC using ethanol as solvent.

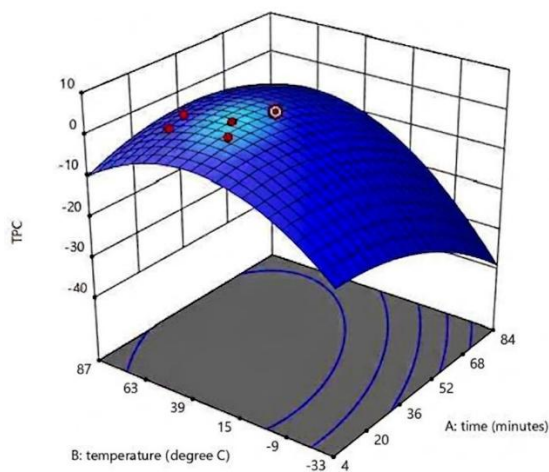


Figure 2. Effect of temperature and time on TPC using methanol as solvent.

To evaluate the adequacy and predictive accuracy of the established response surface methodology (RSM) model for total phenolic content (TPC) extraction using ethanol and methanol systems, the predicted values derived from the model equations were plotted against the experimentally observed actual values (Figure 3). The predicted versus actual plot for total phenolic content (TPC) demonstrates an

overall good agreement between experimental and model-predicted values for both ethanol and methanol extracts, as indicated by the clustering of most data points near the 45° reference line. The diagnostic plot reveals a positive linear progression, indicating that the model successfully captures the overarching upward trend of TPC yields across the experimental design space. However, a detailed analysis of the

residual distribution around the 45-degree line indicates a moderate correlation coefficient ($R^2 = 0.42$). The colour coding represents different sample groups corresponding to extraction. Dark blue points represent the lowest observed TPC extraction yields, meanwhile light blue points indicate intermediate TPC extraction yields. The green points correspond to high TPC extraction yields. The data points associated with ethanol extraction are generally distributed at higher actual and predicted TPC values, reflecting the greater extraction efficiency of ethanol, while methanol data points are concentrated in the lower TPC range. Minor

deviations from the parity line, particularly at lower concentrations, suggest some variability in model prediction, which may be attributed to experimental noise or the complex influence of extraction parameters on phenolic release. Nonetheless, the absence of systematic over- or under-prediction and the random dispersion of residuals indicate that the developed model is robust and adequately captures the relationship between process variables and TPC for both solvents, supporting its suitability for process optimization and prediction within the studied experimental domain.

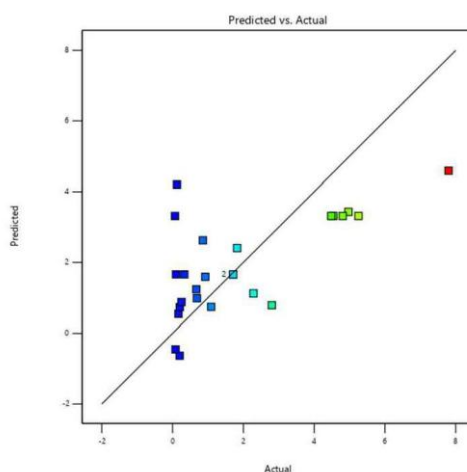


Figure 3. Graph predicted vs actual for the concentration of TPC of both solvent ethanol and methanol.

3.2. Total flavonoid content

Figure 4 illustrates that extraction temperature and time significantly influence total flavonoid content (TFC) when ethanol is used as the solvent, with a clear interaction between both variables. An initial increase in temperature and extraction time leads to a marked rise in TFC, which can be attributed to enhanced solubility of flavonoids in ethanol, improved mass transfer, and greater disruption of plant cell walls that facilitate the release of intracellular compounds. However, beyond the optimal region, further increases in temperature and prolonged extraction time result in a decline in TFC, indicating the susceptibility of flavonoids to thermal degradation, oxidation, and possible structural transformation under excessive heat exposure. The curved response surface and elliptical contour lines confirm that

moderate extraction conditions are more favorable than extreme settings, as excessive thermal energy combined with long extraction durations may accelerate degradation kinetics and reduce flavonoid stability. Overall, the observed trend highlights the balance between improved extraction efficiency and compound stability, emphasizing that optimal temperature–time combinations are critical for maximizing flavonoid recovery while minimizing degradation during ethanol-based extraction.

Compared with the ethanol-based extraction, extraction using methanol as solvent indicates a similar general trend in which total flavonoid content (TFC) initially increases with increasing temperature and extraction time but reaches its optimum at comparatively lower conditions, followed by a more pronounced decline at higher temperatures and prolonged

durations (Figure 5). While ethanol shows a broader optimum region and better flavonoid retention at moderate–high temperature–time combinations, the methanol system appears more sensitive to thermal and time-related effects, as reflected by the steeper curvature of the surface and tighter contour lines. This behavior can be attributed to the higher polarity and volatility of methanol, which enhances rapid solubilization of flavonoids at shorter times but also accelerates degradation, oxidation, or solvent loss under excessive thermal exposure. In contrast, ethanol provides a more stable extraction environment, reducing flavonoid degradation and allowing sustained extraction efficiency over a wider operational range. Overall, the comparison suggests that methanol favors faster extraction under milder conditions, whereas ethanol is more suitable for achieving higher and more stable flavonoid yields when moderate increases in temperature and time are applied.

Total flavonoid content value is the highest at 65°C for 40 minutes of extraction using

ethanol solvent which gives the concentration of 8.1 mg/ml as shown in Figure 4. Based on Figure 5, for methanol solvent, highest concentration is at 30 minutes and 70°C which is 5 mg/ml. From the result, the concentration of TFC for ethanol solvents ranges from 0.14 mg/ml to 8.1 mg/ml while for methanol the concentration ranges from 0.11 mg/ml to 5 mg/ml.

The highest value for the concentration of total phenolic content was obtained when extracted by using ethanol-water mixture. From this, flavonoid content is easier to extract by using ethanol than methanol. Among the solvent extracts, the flavonoid content of the water and ethanol extracts was higher than that of the methanol extract (Seo et al., 2014). Figure 6 shows the predicted versus actual plot for total flavonoid content (TFC) shows a strong agreement between experimental and model-predicted values, as most data points are closely distributed around the 45° reference line, indicating good predictive accuracy of the developed model.

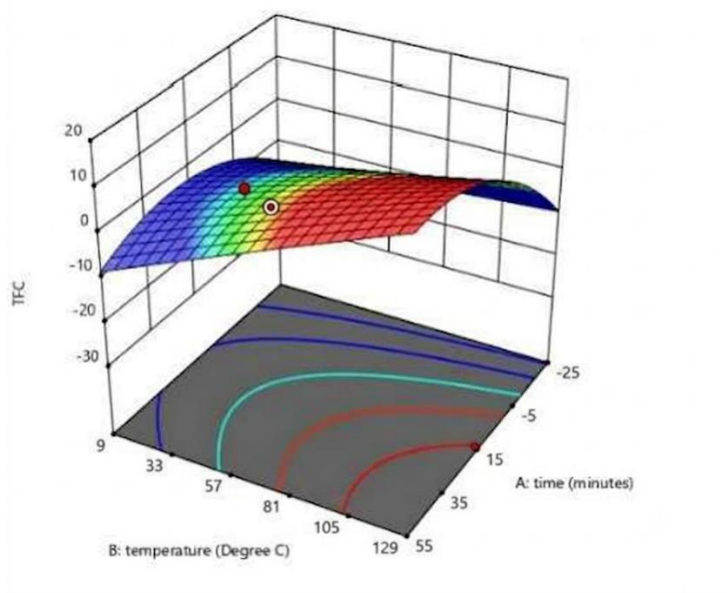


Figure 4. Effect of temperature and time on TFC using ethanol as solvent.

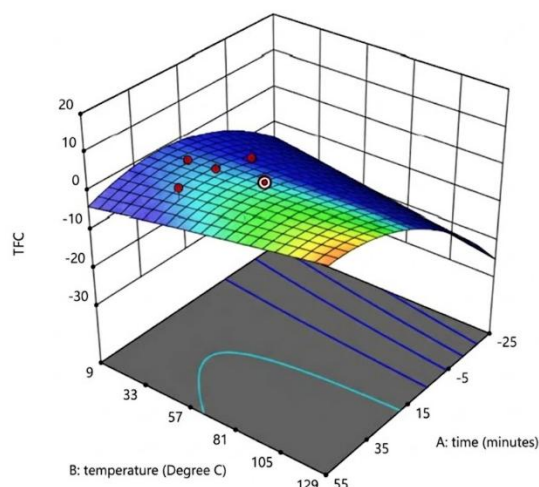


Figure 5. Effect of temperature and time on TFC using methanol as solvent.

This translates to an approximate coefficient of determination, $R^2 = 0.72$, confirming that the model successfully accounts for a substantial majority of the experimental variance driven by the extraction variables. Low concentration regime of TFC represents dark blue data points, meanwhile intermediate regime of TFC represents by green data points. Yellow and red data points show high yield regime of TFC. Data points at lower TFC concentrations are more tightly clustered, suggesting higher model reliability in the low to moderate flavonoid range, while slightly larger deviations are

observed at higher concentrations, which may be attributed to increased experimental variability or nonlinear extraction behavior at elevated temperature–time conditions. Importantly, no systematic over- or under-prediction is evident, as points are symmetrically scattered around the parity line. Overall, the plot confirms that the model adequately captures the relationship between extraction parameters and TFC concentration, supporting its suitability for prediction and optimization of flavonoid extraction within the studied experimental domain.

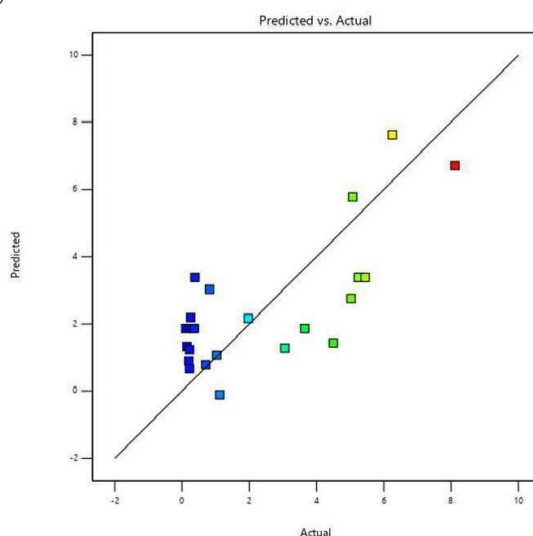


Figure 6. Graph predicted vs actual for the concentration of TFC of both solvent ethanol and methanol.

3.3. Antioxidant activity

Antioxidant activity is being measured by the percentage (%) inhibition calculated from equation (1). From both Figure 7 and Figure 8 the highest percentage inhibition obtained from

solvent ethanol at 65°C for 40 minutes of extraction which is 93% followed by extraction using methanol for 30 minutes at 45°C which is 91%.

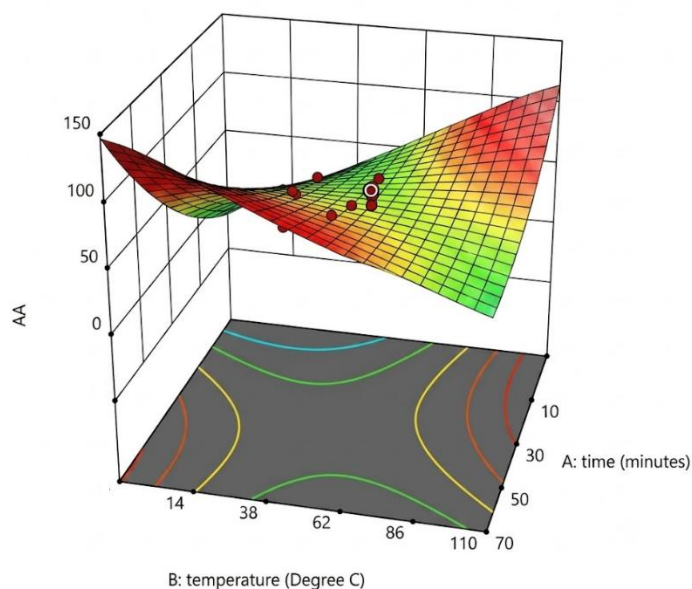


Figure 7. Effect of temperature and time on Antioxidant activity using ethanol as solvent.

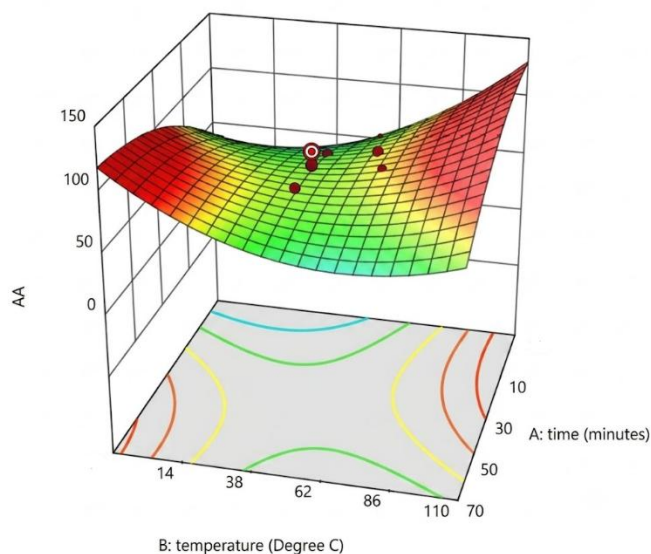


Figure 8. Effect of temperature and time on Antioxidant activity using methanol as solvent.

Antioxidant activity (AA) extracted using ethanol is significantly influenced by both temperature and extraction time, with a strong interaction between the two variables. Antioxidant activity increases markedly as

temperature rises from low to moderate levels, indicating enhanced solubility and extraction efficiency of antioxidant compounds in ethanol. Similarly, increasing extraction time from short to moderate durations leads to a substantial

increase in AA, reflecting improved diffusion and solvent–solute contact. The highest antioxidant activity is observed at moderate to high temperatures (approximately 80–100 °C) combined with moderate extraction times (around 30–40 minutes). However, at very high temperatures and prolonged extraction times, the increase in AA becomes less pronounced or slightly declines, suggesting possible degradation of ethanol-extractable antioxidants. Overall, the figure demonstrates the existence of an optimal temperature–time region for maximizing antioxidant activity when ethanol is used as the solvent. Based on the response surface plot, antioxidant activity extracted using methanol is influenced by both temperature and extraction time, with a clear interaction between these factors. Antioxidant activity increases as temperature and time rise from low levels, indicating enhanced extraction efficiency due to improved solubility and mass transfer of antioxidant compounds in methanol. However, at excessively high temperatures or prolonged extraction times, the antioxidant activity begins to decline, likely due to thermal degradation or oxidation of heat-sensitive compounds. The highest antioxidant activity is achieved at moderate to high temperatures combined with moderate extraction times, suggesting the presence of an optimum extraction condition where maximum antioxidant yield can be obtained without compromising compound stability.

The results show that both methanol and ethanol extracts exhibit a significant dependence of antioxidant activity on temperature and extraction time, with clear interaction effects. In both solvents, antioxidant activity increases from low values at short extraction times and low temperatures, reaching maximum levels at moderate extraction times (≈ 30 – 40 minutes) and elevated temperatures (≈ 80 – 100 °C), indicating that heat and sufficient contact time enhance mass transfer and solubility of antioxidant compounds. However, the methanol system shows a more pronounced decline in antioxidant activity at higher temperatures and longer

extraction times, suggesting greater susceptibility to thermal degradation or solvent–compound interactions. In contrast, ethanol extraction demonstrates a broader high-activity region and a more gradual response at elevated conditions, implying better stability of antioxidants or a milder extraction environment. Overall, while both solvents are effective, ethanol appears to provide a slightly more stable antioxidant response over a wider range of temperature and time compared to methanol.

Figure 9 shows the predicted vs actual graph for the inhibition of antioxidant activity of both solvents. Based on the graph, the distribution of points shows a moderate alignment along the diagonal line, indicating that the model has a fair predictive potential. However, certain points are far above or below the line, showing underprediction or overprediction in some samples. Also, ethanol solvents give the highest value for antioxidant activity. As stated by Ghazzawi et al. (2021), the antioxidant activity increases with increasing of ethanol concentration up to 70% and depended on total phenolic content. In line with this study, which highest TPC obtained is by using ethanol as solvent.

The predicted versus actual plot for antioxidant activity of both ethanol and methanol extracts shows that most data points are closely distributed around the 45° reference line, indicating good agreement between the experimental and model-predicted values. This suggests that the developed models are reliable in predicting antioxidant activity for both solvents within the studied range. Minor scatter of points away from the diagonal line is observed, particularly at lower and higher antioxidant activity values, reflecting small prediction errors that may arise from experimental variability or solvent-specific extraction behavior. Overall, the close alignment between predicted and actual values confirms the adequacy and validity of the models for describing antioxidant activity in both ethanol and methanol extractions.

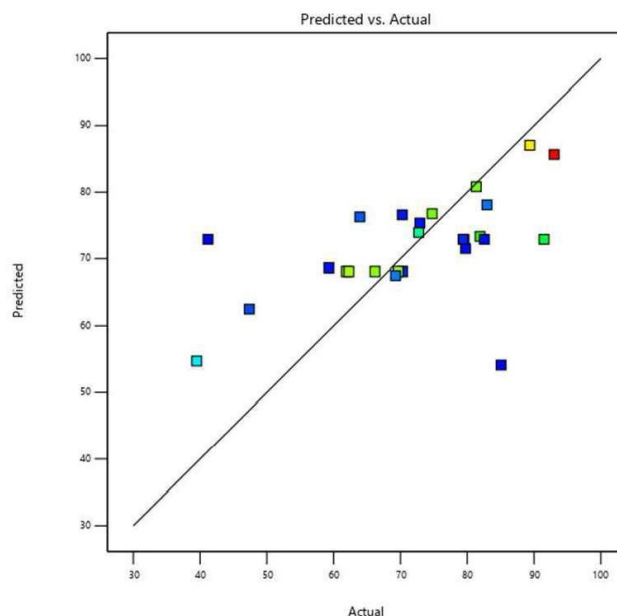


Figure 9. Graph predicted vs actual for the Antioxidant activity of both solvent ethanol and methanol.

Table 1 shows the summary of the final quadratic regression equations and statistical performance metrics for Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity (AA) in terms of actual factors (X_1 = time and X_2 = temperature) across ethanol and methanol solvents. The regression model for TFC demonstrated the highest predictive fit, reaching near-statistical significance ($p = 0.0563$) and explaining 53.68% of the variance ($R^2 = 0.5368$), whereas the models for TPC ($p = 0.2159$, $R^2 = 0.4201$) and AA ($p = 0.5723$, $R^2 = 0.2862$) were statistically

non-significant ($p > 0.05$). Across all three responses, low Lack of Fit values (1.12–1.87) indicate that the second-order model structure is appropriate without severe misspecification. Furthermore, the positive interaction terms (X_1X_2) for TPC and TFC reflect a slight synergistic effect when increasing extraction time and temperature simultaneously, while the negative interaction term for AA points to potential thermal degradation of bioactive compounds during prolonged, high-temperature extractions.

Table 1. Regression Equations and Model Fitting Statistics for TPC, TFC, and AA

Response	Solvent	Regression Equation (X_1 = Time, X_2 = Temperature)	p - value	Lack of Fit	R^2
Total Phenolic Content (TPC)	Ethanol	$Y = -9.055 + 0.108X_1 + 0.352X_2 + 0.003X_1X_2 - 0.004X_1^2 - 0.003X_2^2$	0.2159	1.87	0.4201
	Methanol	$Y = -5.353 + 0.109X_1 + 0.233X_2 + 0.003X_1X_2 - 0.004X_1^2 - 0.003X_2^2$			
Total Flavonoid Content (TFC)	Ethanol	$Y = -6.236 + 0.218X_1 + 0.110X_2 + 0.002X_1X_2 - 0.005X_1^2$	0.0563	1.37	0.5368
	Methanol	$Y = -3.232 + 0.269X_1 + 0.025X_2 + 0.002X_1X_2 - 0.005X_1^2$			
Antioxidant Activity (AA)	Ethanol	$Y = 13.523 + 2.022X_1 + 0.075X_2 - 0.014X_1X_2 - 0.011X_1^2 + 0.010X_2^2$	0.5723	1.12	0.2862
	Methanol	$Y = 54.255 + 1.486X_1 - 0.366X_2 - 0.014X_1X_2 - 0.011X_1^2 + 0.010X_2^2$			

3.4.Optimization

Table 2 shows the validation of the response surface methodology model, comparing predicted and experimental values for Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity under optimized conditions for 70% Ethanol (65°C, 38 minutes) and 70% Methanol (52°C, 35 minutes). Across all responses, the percentage errors remained consistently low between 5.81% and

8.09%, well within the acceptable 10% threshold, confirming the accuracy and reliability of the predictive models. Ethanol consistently outperformed, achieving higher experimental yields for TPC (4.31 ± 0.12), TFC (6.29 ± 0.18), and Antioxidant Activity (79.8 ± 1.5), which demonstrates that a higher temperature and longer duration in an ethanol system optimize the recovery of these bioactive compounds.

Table 2. Predicted and Experimental Values at Optimization Condition

Target Response	Solvent	Conditions	Predicted Value	Experimental Value	Percentage Error (%)
TPC	70%	65 °C, 38 min	4.6	4.31 ± 0.12	6.30%
	Ethanol				
	70%	52 °C, 35 min	1.52	1.40 ± 0.05	7.89%
	Methanol				
TFC	70%	65 °C, 38 min	6.8	6.29 ± 0.18	7.50%
	Ethanol				
	70%	52 °C, 35 min	2.35	2.16 ± 0.09	8.09%
	Methanol				
Antioxidant activity	70%	65 °C, 38 min	85	79.8 ± 1.5	6.12%
	Ethanol				
	70%	52 °C, 35 min	74	69.7 ± 1.2	5.81%
	Methanol				

* Experimental values are presented as Mean $\pm$ SD (n = 3)

The table indicates that ethanol is a more effective solvent than methanol for extracting bioactive compounds under the studied conditions. At 65 °C for 38 minutes, ethanol produced nearly three times higher total phenolic content compared to methanol extracted at 52 °C for 35 minutes, consistent with findings that solvent polarity and hydrogen-bonding interactions influence phenolic and flavonoid solubility and extraction efficiency. Previous research has shown that aqueous ethanol often yields higher flavonoid extraction and greater antioxidant activity compared to aqueous methanol in plant materials, reflecting solvent-dependent extraction patterns (Do et al., 2014).

Consequently, the ethanol extract exhibited significantly greater antioxidant activity (85%) than the methanol extract (74%), reinforcing the positive correlation between the concentration of extracted phenolics and flavonoids and antioxidant potential observed in other plant systems. Overall, despite requiring slightly higher temperature and longer extraction time, ethanol proved superior in maximizing extraction of key antioxidant compounds and enhancing antioxidant potential, a trend similarly reported in studies where ethanol-based extracts showed stronger radical scavenging effects than methanol-based extracts in selected plant matrices (Przybylski et al., 2025).

Based on the RSM optimization using ethanol as the extraction solvent, the optimal condition for simultaneously maximizing total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (AA) was achieved at an extraction time of approximately 30 minutes and a temperature of around 65 °C (Figure 10). Under these conditions, the model predicted high responses with TPC of about 4.60 mg/g, TFC of approximately 6.80 mg/g, and antioxidant activity reaching 85%, indicating a strong positive influence of temperature and moderate extraction time on the release of bioactive compounds. The desirability value (~0.75) further confirms that this combination represents a suitable compromise to optimize all responses simultaneously, suggesting that higher temperatures enhance solvent penetration and mass transfer efficiency, while excessive extraction time is not required when ethanol is used as an effective and green solvent. When compared with methanol as the extraction solvent (Figure 11), ethanol demonstrated

superior performance in optimizing TPC, TFC, and antioxidant activity under the RSM-identified conditions. Although methanol also showed an increase in these responses with increasing temperature and time, the maximum predicted values obtained using methanol were consistently lower than those achieved with ethanol. Specifically, methanolic extraction generally required slightly lower optimal temperatures and shorter extraction times, yet resulted in reduced TPC and TFC yields as well as weaker antioxidant activity (74%) compared to ethanol (85%). This difference can be attributed to ethanol's better affinity for a wider range of phenolic and flavonoid compounds, as well as its enhanced ability to disrupt plant cell matrices at elevated temperatures. Overall, the comparison indicates that ethanol not only provides higher extraction efficiency for bioactive compounds from *Caesalpinia sappan* L. but also offers a more effective and safer solvent option than methanol for maximizing antioxidant-related responses.

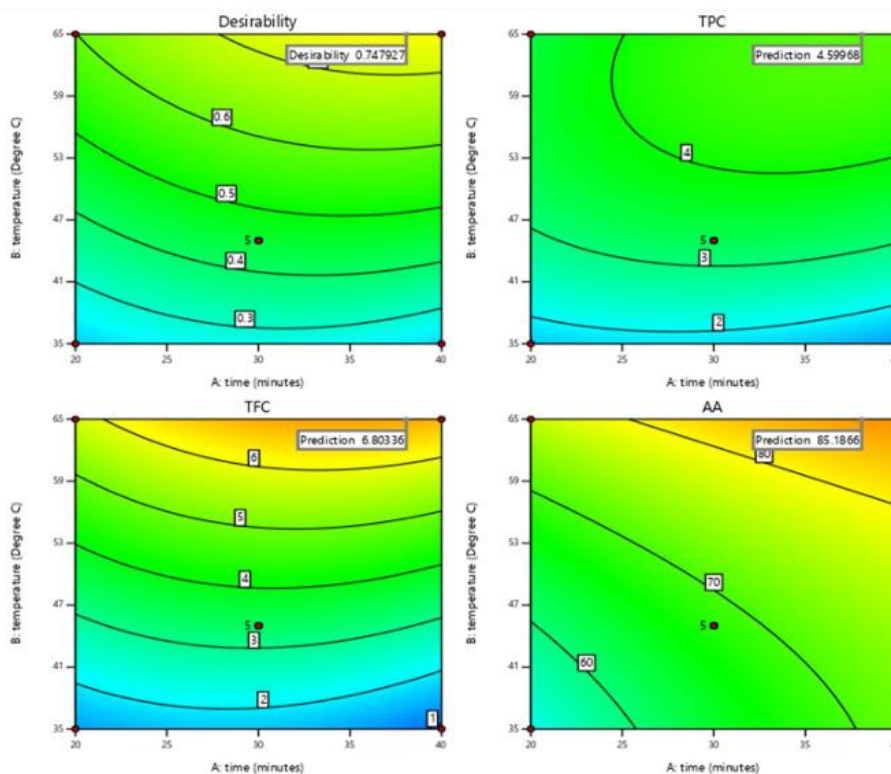


Figure 10. Graph optimization condition using ethanol as solvent

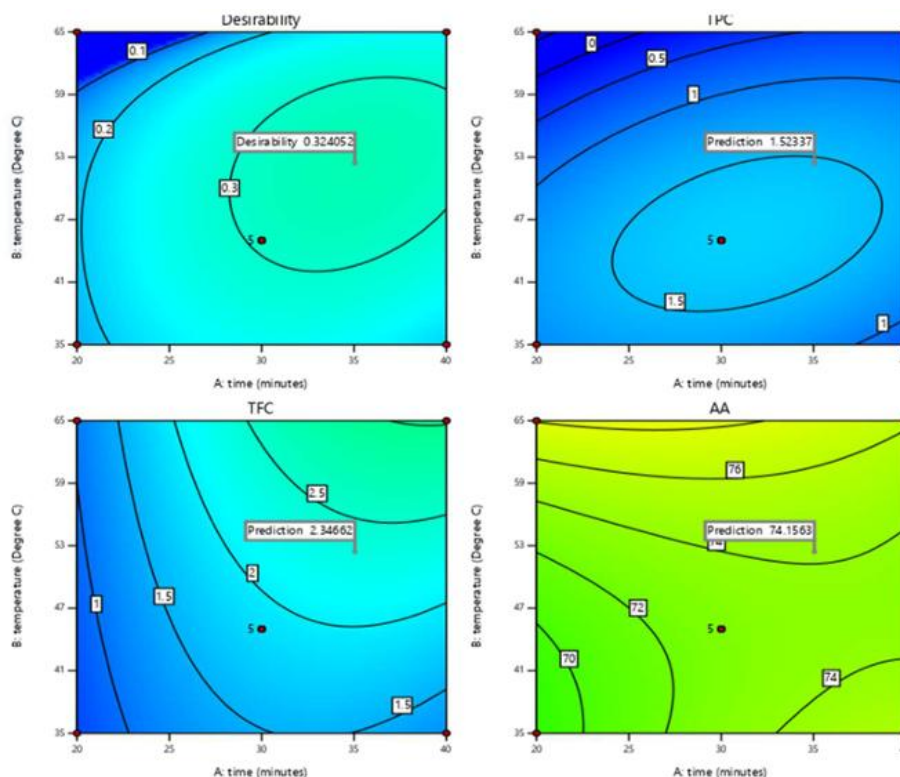


Figure 11. Graph optimization condition using methanol as solvent

Both total phenolic content, total flavonoid content, and antioxidant activity were maximized when ethanol was employed as the extraction solvent, indicating its superior extraction efficiency compared to methanol. This can be attributed to the favourable polarity of ethanol, which enables effective solubilization of a wide range of polyphenolic compounds while maintaining compound stability during extraction (Do et al., 2014). In addition to its extraction performance, ethanol is widely recognized as a food-grade solvent and is extensively used in food additives, personal care products, and alcoholic beverages, whereas methanol is primarily limited to industrial applications due to its higher toxicity (Bulk Chemicals, 2021). Considering the intended application of *Caesalpinia sappan* L. as a natural food colourant, the use of ethanol is particularly appropriate, as it ensures both high bioactive compound recovery and compliance with food safety requirements. Overall, these findings confirm that ethanol is the most suitable solvent for extracting bioactive compounds from *Caesalpinia sappan* L., supporting its potential application in food and nutraceutical industries.

4. Conclusions

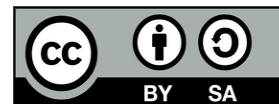
This study compared 70% aqueous ethanol and 70% aqueous methanol for extraction of phenolic and flavonoid constituents and antioxidant activity from *Caesalpinia sappan* L. heartwood within 30–70 °C and 15–45 minutes. The results clearly demonstrate that ethanol is the more effective solvent for recovering phenolic and flavonoid compounds, reported experimental TPC and TFC values were 7.8 and 8.1 mg/mL, respectively, and the highest reported DPPH inhibition for ethanol was 93% at 65 °C and 40 minutes. RSM predicted solvent-specific compromise conditions of 65 °C for 38 minutes for ethanol and 52 °C for 35 minutes for methanol. This study confirms the suitability of ethanol as an efficient and safer food-grade solvent for the extraction of bioactive compounds from *Caesalpinia sappan* L., highlighting its potential application in food, pharmaceutical, and cosmetic industries.

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*Research article***ANTIOXIDANT POTENTIAL AND PHENOLIC COMPOSITION OF COLUMN-CHROMATOGRAPHIC FRACTIONS FROM *GYMNEMA INODORUM* LEAF EXTRACT**

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ABSTRACT

Gymnema inodorum (Lour.) Decne, a plant traditionally used in Northern Thailand for its antidiabetic properties has shown potential as a source of bioactive compounds with antioxidant activity. Despite its traditional use, the phenolic profile and specific antioxidant contributions of *Gymnema inodorum* fractions remain inadequately characterized. Given the growing interest in identifying plant-based molecules to combat oxidative stress, further exploration is necessary. This study aims to explore the antioxidant properties and phenolic profile of column chromatographic fractionated *G. inodorum* leaf extract. The ethanolic reflux extracts of *G. inodorum* leaves were subjected to column chromatography for the isolation of bioactive molecules. Total phenolic and flavonoid contents were assessed using Folin–Ciocalteu and aluminium chloride colorimetric methods, respectively. Total antioxidant activity was evaluated by employing different antioxidant assays, including DPPH, ABTS scavenging assay and FRAP assay. High-Performance Liquid Chromatography (HPLC) with a C18 column was employed to determine the level of gymnemic acid, while Liquid Chromatography Quadrupole Time-of-Flight/Mass Spectrometry (LC-QTOF/MS) was used to identify potential phenolic compounds. The three separated fractions obtained after column chromatographic separation were designated as Fraction A (fraction 1-4, eluted with low polarity mobile phase), Fraction B (fractions 5-10, intermediate polarity), and Fraction C (fractions 11-12, higher polarity) based on UV absorbance profiles. These fractions exhibited remarkable antioxidant activity, with total phenolic content of 0.294 ± 0.056 , 0.085 ± 0.039 , and 0.227 ± 0.041 mg QE/mL, and total flavonoid content of 0.274 ± 0.050 , 0.309 ± 0.110 , and 0.384 ± 0.007 mg QE/mL, respectively. Fraction A exhibited the highest DPPH and ABTS scavenging activity, while fraction C demonstrated significantly higher ferric-reducing power. HPLC analysis revealed a highest concentration of gymnemic acid in fraction C (0.393 ± 0.003 mg/g) compared to fraction B (0.159 ± 0.003 mg/g) and fraction A (0.142 ± 0.000 mg/g). The LC-QTOF/MS phenolic screening

showed seven potential phenolic compounds in fraction A, seven compounds in fraction B, and four compounds in Fraction C. Pearson correlation analysis using the individual replicate measurements underlying Table 1 and Table 2 ($n = 9$) showed a significant positive correlation between total phenolic content and DPPH scavenging ($r = 0.68$, $p = 0.044$), and significant correlations between total flavonoid content and DPPH ($r = -0.79$, $p = 0.012$), ABTS ($r = -0.95$, $p < 0.001$), and FRAP ($r = 0.81$, $p = 0.009$), suggesting that phenolic and flavonoid compounds are important contributors to the antioxidant activity of *G. inodorum*.

1. Introduction

Apocynaceae is a family of plants that includes the herb *Gymnema* spp. It is extensively dispersed in tropical and subtropical areas of Asia and Africa, and currently, more than 50 species are listed (*Gymnema* - The Plant List, 2023). *Gymnema inodorum*, a medicinal plant locally known as Chiang-da in Northern Thailand (Dunkhunthod et al., 2021), has a wide range of therapeutic uses. In northern Thailand, Chiang-da is mainly used as a vegetable for cooking and as a key botanical ingredient in various dishes, undergoing essential preparation methods like boiling and stir-frying. It is also air-dried for tea production, especially in the countryside. Traditionally, it has been used for treating diabetes and obesity. Because of the plant's numerous therapeutic benefits, including its possible antioxidant action, Ayurvedic medicine has long been used (Kasote et al., 2015).

Gymnema sylvestre, a common *Gymnema* species found in Asia has been extensively researched for its antioxidant and medicinal properties (Dai & Mumper, 2010). Many studies have reported the medical usage of this species. This species is also studied for potential phytoconstituents, including triterpene saponins known as gymnemic acids, *Gymnemasaponins*, and polyphenols (Ounjaijean et al., 2021).

G. inodorum belongs to the same genus as *G. sylvestre* and occupies a same ecological niche. However, despite being cultivated in regions same to *G. sylvestre*, *G. inodorum* has received comparatively limited scientific attention. Previous research has demonstrated that *G. inodorum* extract possesses glucose absorption inhibiting activity, primarily due to gymnemic acids (Suwan et al., 2022). Further, *G. inodorum* leaf extracts contain gymnemic acid and ginsenosides responsible for anti-

fungal and antioxidant activity (Jeytawan et al., 2022). Additionally, *G. inodorum* has been investigated for phytoconstituents and gymnemic acid and is believed as one of the potential phytochemicals that act as a blood sugar regulator (Chiabchalard et al., 2010) along with antimalarial properties (Boonyapranai et al., 2022). Nuchuchua et al. (2023) studied the relationship between phytochemical and antioxidant activities in *G. inodorum* leaf extracts, reporting significant antioxidant capacity in the crude ethanol extract, with polyphenolic compounds identified as key contributors. Similarly, (Haideri et al., 2024) compared different extraction techniques for *G. inodorum*, noting that while the crude extracts showed promising phenolic profiles, specific bioactive compounds responsible for antioxidant activity remained insufficiently characterized (Cianciosi et al., 2018). These studies underscore a critical gap: the reliance on crude extracts limits the identification and attribution of antioxidant activity to specific phenolic fractions.

Plants' phenolic chemicals have been reported to be the source of their antioxidant action (Baba & Malik, 2015). Phenolic compounds have been extensively researched due to their non-toxic nature, efficacy at low concentrations across various biological activities, eco-friendly extraction methods at low cost, showcase diverse pharmacological benefits (Gil et al., 2002). They exhibit potent antioxidants, anti-inflammatory properties, and robust inhibition against cardiovascular diseases, cancer, and diabetes (Phanjaroen et al., 2024). Phytochemicals have been widely utilized in healthcare and studied for their antioxidant potential, reactive ability of compounds to stop the oxidation of free radicals

(Tiwari et al., 2014). Exploration into extracting antioxidants from plant tissues has garnered significant interest due to their natural availability and cost efficiency. Enhancing foods, beverages, or medicinal items with additional antioxidants presents a promising approach to mitigating the adverse impacts of oxidative stress (Abu et al., 2017). The limited prior research on the active polyphenols of *G. inodorum* responsible for antioxidant activity underscores the importance of our current study.

As far as we know, our study is the first to explore fractions of *G. inodorum* extract because most literature received is of crude extract. The crude extract provides limited information about phenols activity and fractionation is done to enhance the activity of extract as well as to identify the essential phenols responsible for higher activity. Hence, the screening and investigation of the *G. inodorum* extract, which bears a resemblance to the well-known *G. sylvestre*, along with limited information on its phenolic profile, becomes imperative. The detection and measurement of phenolic compounds in the leaf extract of *G. inodorum* can shed light on the compound's potential health benefits, as phenolic compounds have considerable antioxidant qualities (Miliauskas et al., 2004).

Various extraction methods have been studied for purification of phytoconstituents, but *Gymnema* leaf contains various other compounds, creating difficulty in screening specific phytochemicals. An additional column separation technique was applied to overcome this shortcoming and find targeted polyphenols, which assisted in screening polyphenols in *Gymnema* leaf extract.

The primary objective of this study was to investigate the phenolic profile and antioxidant activity of *G. inodorum* leaf extract. The study focused on specific column separation technique for fractionation of crude extract to find out potential phenolic compounds, for the analysis of antioxidant capacity, as well as quantification of the total phenolic compounds, total flavonoid contents, and gymnemic acid levels. The results support the plant's potential polyphenols as specific phytochemical constituents in the

extract and antioxidant potential, highlighting the prospective use in pharmaceutical and nutraceutical applications.

2. Materials and Methods

2.1 Chemicals and reagents

The solvent methanol, chloroform, acetonitrile, chemicals and gymnemic acid standard of analytical grade (AR) were obtained from Sigma Chemical Company (Sigma-Aldrich, St. Louis MO, USA).

2.2. Plant material and preparation of extract

The shoot and first three pairs of *G. inodorum* leaves were harvested from Sanmahaphon Organic Herbs Community Enterprise, Chiang-da Garden, Chiang Mai, Thailand. Subsequently, the leaves were washed thoroughly under running tap water with to remove dust and soil particles and rinsed with distilled water, followed by air-drying at room temperature under shade conditions. The dried leaves were ground and sieved through a sieve shaker using 60-mesh sieve size to yield the particle size 150 - 250 μm . Dried leaves were powdered, and 10 g was packed into a cellulose thimble. Next, a 95 percent ethanol solution (100 mL) was added to the thimble at a ratio of 1:10 (w/v). The extraction process was performed thrice at 80 °C using an automatic Soxhlet extractor, each running for 2 h. After this extraction, the resulting solution was subjected to evaporation using a rotary evaporator to yield the crude extract. Subsequently, the extract was desiccated in a chemical hood, reweighed, and stored at 4°C for further analysis (Gini & Jeya Jothi, 2018). Based on the same extraction protocol applied to the same source material and reported separately (Dewanto et al., 2002), the corresponding crude ethanol reflux extract yield was 20.12% (w/w) relative to dry leaf powder.

2.3. Column chromatographic fractionation of extract

The crude extract was subject to column chromatography for fraction separation using slurry method for column packing. 20 g of 'silica gel 60' was packed into transparent (80 cm long,

5 cm diameter) glass column, as a stationary adsorbent without introducing air bubbles. Petroleum ether and methanol (2:1) was used as mobile phase in the column. The column was passed with mobile phase before adding silica slurry, and then the mobile phase was gradually increased to develop a packed solid column. Subsequently, 2 g of crude extract was added from top without disturbing the solid phase. Sea sand with a particle size 0.3 – 0.9 mm was applied to settle the layers properly, separating the crude extract and mobile phase. After proper layer formation between the sample and mobile phase, stopcock was opened to run the mobile phase down through column. The mobile solvent was gradually added to column to develop gradient elution. Fractions of the extract were received 4 mL, each with specific time interval. 12 fractions were collected in glass tubes with a flow rate of 0.99 mL/min (Islam et al., 2023). Each fraction was scanned and grouped by similarity of its whole UV-Vis absorbance profile (λ_{max} ranging ~250–670 nm across fractions), then later re-checked at 450 nm and 660 nm and pooled into the final 3 fractions (A=1–4, B=5–10, C=11–12) were passed through rotary evaporator to remove the organic solvent, followed by lyophilization to dry for further analysis.

2.4. Phytochemical Analysis

The fresh fraction extract was analyzed for detecting phytochemicals, total phenolic content (TPC) and total flavonoid content (TFC) through spectroscopy.

2.4.1. Total Phenolic Content

Quantitative analysis of phenolic content was analyzed using the Folin-Ciocalteu colorimetric method (Jeenoo et al., 2022), using Quercetin as the standard reference. To perform the analysis, a mixture of 20 μL of *G. inodorum* fraction extract and 20 μL of the diluted (10 times in distilled water) Folin-Ciocalteu solution was prepared. The mixture was combined with 80 μL of 7% Na_2CO_3 and 200 μL of DW. The plate was observed for any turbidity, and immediately at 760 nm, the absorbance was recorded by spectrophotometer to calculate total phenolic content. The findings were expressed

in mg of quercetin equivalents (mg QE/g sample) or milligrams per gram of sample.

2.4.2. Total Flavonoid Content

Total flavonoid content was determined by using quercetin as standard through colorimetric assays (Arnao et al., 2001). To perform this, 25 μL of the sample, blank or various concentrations of standard solution were added in a 96-well microtiter plate with 12.5 μL of DW and 7.5 μL of 7% NaNO_3 solution. The mixture was allowed to settle at ambient temperature for 5 min. Afterwards, 10% AlCl_3 15 μL solution was added and thoroughly mixed. The plate was stored for another 5 min at room temperature. Finally, 27.5 μL of distilled water and 50 μL of 1 M NaOH solution were added and incubated at room temperature for an additional 5 min. At 510 nm, the absorbance was measured using water as a reference, and the results were reported in terms of the amount of quercetin equivalents per g of sample (mg QE/g sample). The calibration curve (quercetin, 0.00625–0.4 mg/mL) followed $y = 8.346x + 0.217$ ($R^2 = 0.952$); estimated LOD and LOQ were 0.003 and 0.009 mg/mL. Quercetin was also used as the TPC calibration standard (Section 4.3.1) to keep a single reference compound across the phenolic and flavonoid assays; TPC is therefore expressed as quercetin equivalents rather than the more conventional gallic acid equivalents.

2.5. Antioxidant Analysis

The fractions of *G. inodorum* leaf extract were evaluated for their potential antioxidant capacity and free radical scavenging activities.

2.5.1. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to assess the free radical scavenging capabilities of *G. inodorum* extracts. The procedure involved creating a DPPH stock solution in methanol and adding 195 μL of the DPPH solution with 10 μL of *G. inodorum* extract in a 96-well micro-titer plate. After a 30-minute dark incubation period, the absorbance at 517 nm was obtained using a SPECTROstar nano microplate reader (BMG LABTECH) (Chaves et al., 2020).

2.5.2. ABTS Decolorization Assay

Arnao's described method (Arnao et al., 2001) was used to evaluate the antioxidant potential of *G. inodorum* fraction extract using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay. The method involved preparing a 7mM ABTS reagent using 2,2'-azino-bis-6-sulfonic acid, diammonium salt in H₂O and 2.45 mM of K₂S₂O₈ (potassium persulfate), which was kept in dark incubation for 12 h at room temperature. 80% ethanol was added to dilute the solution in a 96-well microtiter plate along with 10 µL of *G. inodorum* fraction extract. After a 30-minute dark incubation at 734 nm wavelength, the absorbance was measured and reported as the 50% inhibition concentration value, which reflects the correlation between the % inhibition of the test sample concentration and ABTS. The inhibition was measured at 734 nm absorbance, determined as a percentage and calculated by comparison with Trolox as a standard reference.

2.5.3. FRAP Assay

The method described by Aljadai (Devi & Jain, 2014) was used to determine the ferric-reducing antioxidant power (FRAP) value of *G. inodorum* extract. The process involved preparing the FRAP reagent by mixing 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) with 2.5 mL of 20 mM ferric chloride (FeCl₃), 40 mM HCl, and 25 mL of 300 mM acetate buffer at pH 3.6. The *G. inodorum* fraction extract was then diluted two times with DW (distilled water), and ten microliters of the *G. inodorum* fraction extract was added with 190 microliters of the FRAP standard reagent in a 96-well microtiter plate for a 30-minute dark incubation period. Finally, at 593 nm, the sample's absorbance was observed using a SPECTROstar nano microplate reader and calculated by comparison with Trolox as a standard reference.

2.6. Detection and Quantification of Gymnemic Acid

The quantification of gymnemic acid was conducted according to the validated HPLC method described by Devi and Jain (Devi & Jain, 2014) and modifications by Kamble et al.

For the detection of gymnemic acid, 10 mg of the crude gymenema extract was dissolved in 50 mL of a 50% (V/V) methanol solution for chromatographic analysis. To this solution, after adding 10 mL of 11% KOH, heated under reflux for one hour in a boiling water bath before being cooled. After that, 9 mL of 12 N hydrochloric acid (HCl) was mixed and refluxed for another hour in the water bath. After lowering the temperature, the solution was mixed with 50% (V/V) methanol marked 100 mL final volume and further subjected to analyzation with HPLC (Shimadzu LC, Kyoto, Japan). The percentage of gymnemic acid was calculated using the sample's area under the curve, and it was compared to the standard value of deacyl gymnemic acid. Because gymnemic acid lacks a readily available analytical reference standard, deacyl gymnemic acid was used for calibration, consistent with the method of Devi and Jain (Bajpai et al., 2016); gymnemic acid content reported in this study is therefore expressed as deacyl gymnemic acid equivalents. Chromatography was conducted using LC systems, HPLC with analytical C18 column (250 x 4.60 mm i.d. x 5.0 µm), mobile phase consisting of (A) Acetonitrile solution and (B) Type 1 water (50:50) at 30°C column temperature, a flow rate of 2.0 mL/min, and detected at the wavelength of 218 nm. Deacyl gymnemic acid was used as a standardization concentration (3.91-500 µg/mL), and a duplicate sample was injected along with 20 µL of the sample solution to perform the HPLC estimation (Manthey, 2000). Because gymnemic acid was quantified against a deacyl gymnemic acid standard, in accordance with Devi and Jain (Pietta, 2000), all gymnemic acid concentrations reported in this study are expressed as deacyl gymnemic acid equivalents. The standard injection series (40–80 µL) gave a linear peak-area response ($R^2 = 0.993$) across the validated range.

2.7 Characterization of Active Phytochemicals

The characterization of active phytoconstituents from *G. inodorum* leaf extract fractions was performed by a quadrupole time-

of-flight mass spectrometer, following the method described by Chiwat (Zhang et al., 2015). Initially, the collected fractions were dissolved in methanol, followed by cleaning with dispersive C18 SPE. Before analysis, the solution was filtered through HPLC micro-filters (0.22 μm). Further analysis was conducted by utilizing the series of Agilent 1290 Infinity II in conjugation with a 6546 LC-QTOF/MS apparatus with resolution 1.3 Da and 3 ppm accuracy of m/z determination. The LC conditions involve the use of a ZORBAX Eclipse Plus C18 column Shimadzu (HPLC-UV) with an injection quantity of 10 μL , a flow rate of 0.2 mL/min, and a mobile gradient system starting with a mixture of 95% water (1% formic acid) and 5% acetonitrile, gradually increasing to a final composition of 95% acetonitrile. Chromatographic separation was achieved through mass spectrometry conditions that include a positive mode electrospray ionization (ESI) probe, a nebulizer pressure of 20 psi, a flow rate of 7 L/min of N_2 , a capillary temperature of 300°C, a flow rate of 8 $\mu\text{L}/\text{min}$, a mass-to-charge ratio range of 50-1000 m/z , a capillary voltage of 4500 V, and a temperature of 280°C dry heater. The phenolic compounds were identified using the database EMBL – EBI (Bajpai et al., 2016).

3. Results and Discussion

3.1. Results

The preliminary screening of ethanol reflux extract based on Thin Layer Chromatography (TLC) indicated active compounds in the *G. inodorum* leaf extract. The active compounds in the extract were separated on the TLC plate and compared with the R_f values of employed standards (Quercetin, Gallic Acid, Caffeine, and Trolox). The column chromatographic fractionation of crude extract yielded 12 fractions in the best-suited solvent system of TLC as a mobile phase and silica gel as a stationary phase. Solvent-system screening identified ethyl acetate:methanol:water (10:1:1) as the system giving the clearest separation on

silica gel, with R_f values of 0.93–0.975 (two spots) for the extract, 0.93–0.97 for quercetin, and 0.812 for gallic acid; caffeine remained at the origin ($R_f = 0$) under this system, visualized with vanillin sulfuric acid spray. The 12 fractions were assessed by using a 218 nm UV spectrophotometer and produced 3 pool fractions based on UV absorbance values. Newly named as Fraction A (fraction 1 - 4 of column chromatography), Fraction B (fraction 5 – 10), and Fraction C (fraction 11 – 12). TLC screening prior to column chromatography tested seven mobile-phase systems on the ethanol reflux extract against Quercetin, Gallic acid, and Caffeine standards, visualized with vanillin–concentrated H_2SO_4 spray; ethyl acetate:methanol:water (10:1:1 v/v/v) gave the cleanest multi-spot resolution (sample $R_f \approx 0.93$ –0.98; Quercetin $R_f \approx 0.93$ –0.97; Gallic acid $R_f \approx 0.81$) and was used to select the mobile-phase polarity range for the column gradient.

The phenolic and flavonoid content of the three fractions derived from the leaf extract of *G. inodorum* were examined. Total phenolic content revealed that fraction A has the highest at 0.294 ± 0.056 mg QE/mL, followed by fraction C (0.227 ± 0.041 mg QE/mL) and fraction B (0.085 ± 0.039 mg QE/mL), as shown in (Table 1).

The analysis of total flavonoid content in the fractions of *G. inodorum* found that fraction C has the highest flavonoid content at 0.384 ± 0.007 mg QE/mL, followed by fraction B and A (0.309 ± 0.110 mg QE/mL, 0.274 ± 0.050 mg QE/mL), respectively (Table 1). The statistical analysis showed significant differences ($p < 0.05$) among fractions for phenolic, flavonoid, and antioxidant activities. Summing the individual fraction yields in Table 1 gives a total recovery of 63.94% (w/w) of the crude extract mass loaded onto the column, with the remainder attributable to material retained on the column and losses during evaporation and lyophilization.

Table 1. The total phenolic and flavonoid content and percent yield of the *G. inodorum* leaf extract fraction.

Samples	Total Phenolic Content (mg QE/mL)	Total Flavonoid Content (mg QE/mL)	% Yield (w/w)
Fraction A	0.294 ± 0.056 ^a	0.274 ± 0.050 ^b	12.60
Fraction B	0.085 ± 0.039 ^c	0.309 ± 0.110 ^{ab}	39.93
Fraction C	0.227 ± 0.041 ^b	0.384 ± 0.007 ^a	11.41

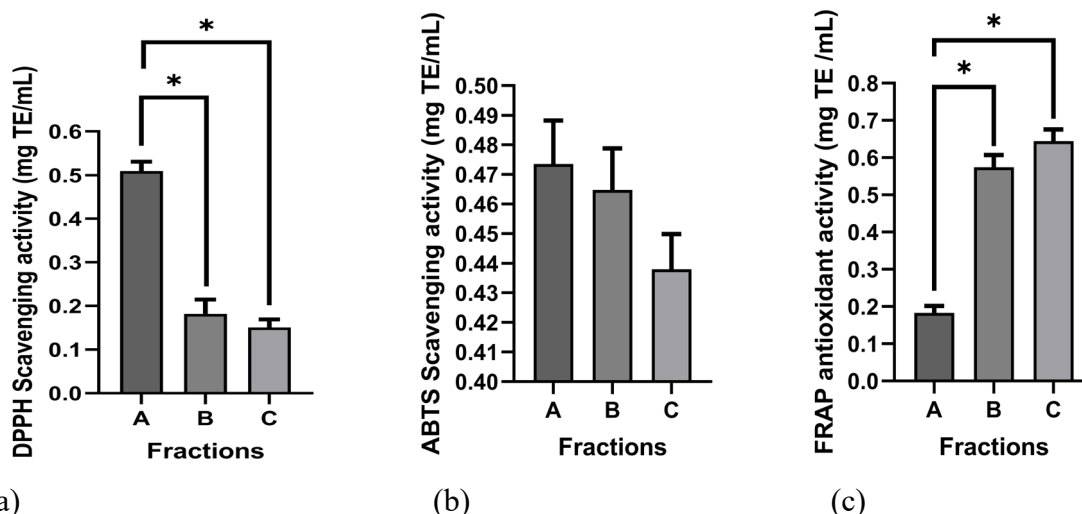


Figure 1. The bar graph shows the antioxidant activities of 3 different pool fractions (A, B, and C): (a) DPPH radical scavenging activity, (b) ABTS radical scavenging activity, and (c) Ferric reducing antioxidant activity. The statistical significance were determined using one-way ANOVA, followed by Duncan's multiple range test at $p < 0.05$ and represented by star marks. The Star marks represent the significant mean difference between the fractions (A; fraction 1 - 4 of column chromatography, B; fraction 5 - 10, C; fraction 11 - 12). Data are expressed as mean $\pm$ SD ($n = 3$).

Total phenolic content was quantified using the Folin-Ciocalteu method and expressed as mg quercetin equivalents (QE) per milliliter of extract (mg QE/mL). Total flavonoid content was measured using a colorimetric method and also expressed as mg quercetin equivalents (QE) per milliliter of extract (mg QE/mL). Statistical significance ($p < 0.05$) was assessed using one-way ANOVA followed by Duncan's multiple range test. Results are presented as mean $\pm$ SD ($n = 3$) for total phenolic content, total flavonoid content, and % yield (w/w) with distinct superscript letters (a,b,c) to highlight significant differences.

The antioxidant activity of the three fractions received from the leaf extract of *G. inodorum* was assessed by employing DPPH, ABTS, and FRAP assays. In the DPPH assay, it was observed that fraction A exhibited the most pronounced antioxidant activity (71.25 ± 3.15

mg TE/mL), followed by fraction B (31.57 ± 6.42 mg TE/mL), with the lowest activity observed in fraction C (27.92 ± 3.64 mg TE/mL). Summing the percent yields of Fractions A, B, and C (12.60%, 39.93%, and 11.41% w/w, respectively) gives a total recovery of 63.94% (w/w) of the crude extract loaded onto the column.

Likewise, the ABTS assay yielded results indicating that fraction A displayed the highest radical scavenging activity (95.45 ± 0.89 mg TE/mL), closely followed by fraction B (93.46 ± 1.43 mg TE/mL), whereas fraction C demonstrated activity of a comparable magnitude (87.29 ± 0.45 mg TE/mL).

Conversely, the assessment of reducing properties using the FRAP assay unveiled that fraction C exhibited the most robust antioxidant activity (2.97 ± 0.25 mg TE/mL). In comparison, fractions B and A also demonstrated

considerably lower ferric ion reducing power (2.65 ± 0.26 mg TE/mL and 0.85 ± 0.14 mg TE/mL, respectively) (Figure 1). The three fractions were analyzed through HPLC with C18 column to quantify the Gymnemic acid level. After analyzing the sample, fraction C showed the highest gymnemic acid

concentration at 0.3933 ± 0.003 mg/g, followed by fractions B and A at 0.1591 ± 0.003 mg/g and 0.1417 ± 0.000 mg/g, respectively (Table 2). The HPLC-C18 chromatogram in Figure 2 shows the extract's standard and gymnemic acid peaks.

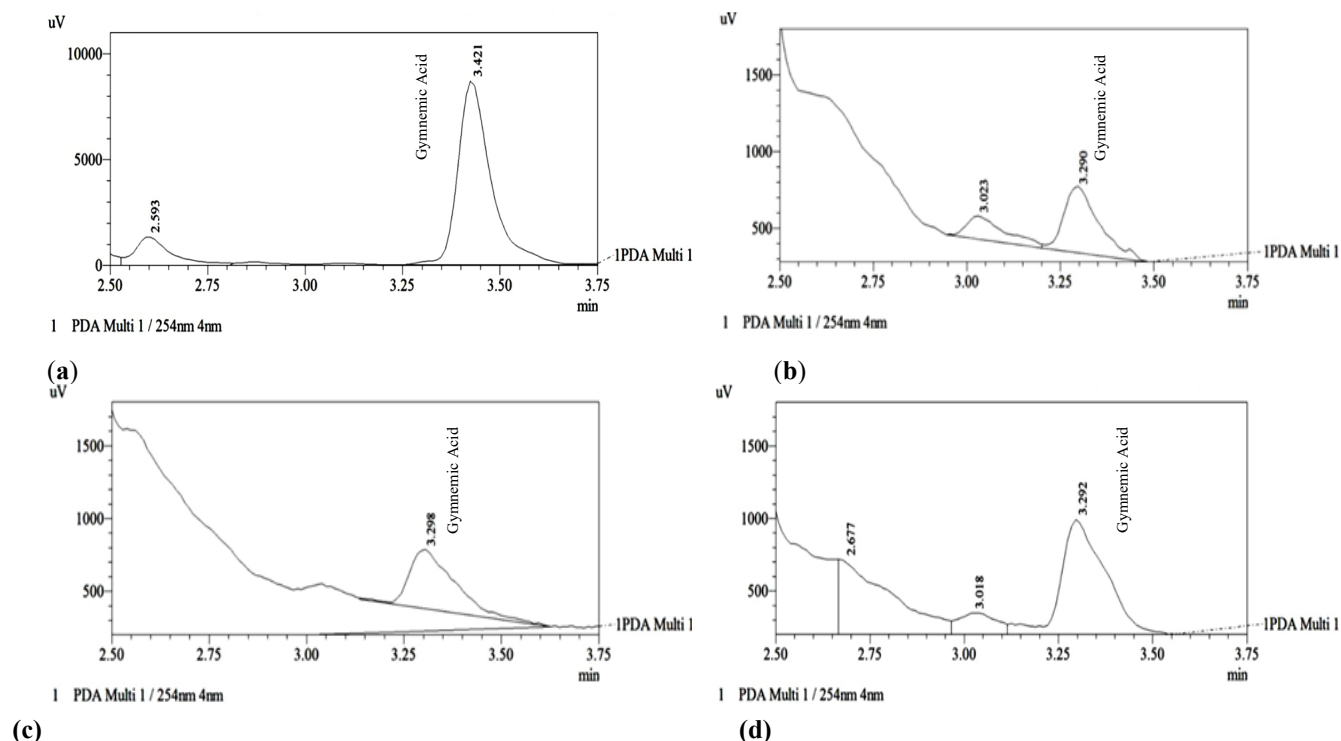


Figure 2. HPLC-UV chromatograms of gymnemic acid. Figure (a) shows the chromatogram of a gymnemic acid standard at 100 µg/mL. Figure (b), (c), and (d) display gymnemic acid content in fractions A, B, and C, respectively. All analyses were performed at a detection wavelength of 218 nm, matching the method used for gymnemic acid detection in Section 3.6. The presence of distinct peaks in each fraction shows isolation and quantification of gymnemic acid, indicating variability in concentration across the fractions.

Table 2. The gymnemic acid concentration in fraction of *G. inodorum* leaf extract.

Fractions	Gymnemic Acid (mg/g)
Fraction A	0.1417 ± 0.000^c
Fraction B	0.1591 ± 0.003^b
Fraction C	0.3933 ± 0.003^a

The concentration of gymnemic acid was determined using HPLC technique, with quantified using authentic standard and expressed as milligrams per gram of extract. The statistical significance was at $p < 0.05$ expressed as mean $\pm$ SD ($n = 3$) denoted by different superscript letters (a, b, c) within the column.

Phenolic compounds were screened in fractions of *G. inodorum* leaf reflux extract by LC-QTOF/MS. The LC-QTOF/MS analyses revealed phytochemical compounds in fractions. From the LC-QTOF/MS result for each fraction, the compounds were analyzed for active phenolic compound screening and the database EMBL – EBI and ChemSpider by Royal Society of Chemistry was used to authenticate the compounds. Fraction A contained the phenolic compounds Bufotalinin, Erysothiopine, Schisanhenol B, Zhebeiresinol, Kanzonol P, 8-[(2Z)-3,6-Dimethyl-2-hepten-1-yl]-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one, and Toyocamycin based on tentative database matching; other potential antioxidants listed include fatty acids and drugs.

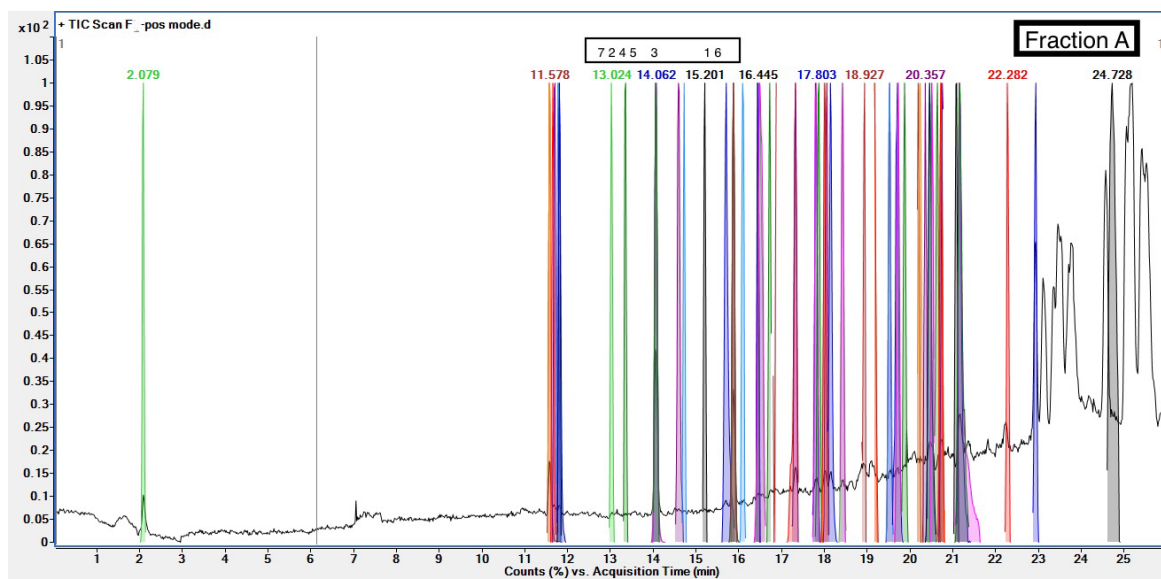
The fraction B of *G. inodorum* leaf reflux extract contained seven phenolic compounds, including Erysothiopine, Schisanhenol B, Kanzonol P, Fluocinolone, Bufotalinin, Chamuvaritin and Tomentolide A among the

active compounds. The 6 H Reflux extract of *G. inodorum* leaf, fraction C, screened for four potential phenolic compounds, namely 2,2-Dimethyl-3,4-bis(4-methoxyphenyl)-2H-1-benzopyran-7-ol acetate, Clausarinol, 8-Deoxy-11,13-dihydroxygrosheimin, and Eudesmin. The catalog of these phenolic compounds is mentioned in Table 3. The Phenolic compounds in a fraction of *G. inodorum* leaf identified by LC-QTOF/MS, and Figure 3 shows chromatograms for LC-QTOF/MS of phenolic compounds. Compounds with a database mass difference greater than 10 ppm or a matching score below 85% are flagged here as lower-confidence annotations pending confirmation with authentic reference standards or MS/MS fragmentation, consistent with accepted metabolomics reporting standards; several Table 3 entries (mass differences of 18–23 ppm) fall into this range.

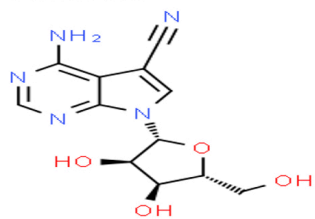
Table 3. The Phenolic compounds in a fraction of *G. inodorum* leaf identified by LC-QTOF/MS

Sample	Peak No	Retention Time	Mass	[M+H] ⁺	Mass Diff (db/ppm)	Matching Score (%)	Formula	Tentative Phenolic Compound
Fraction A	1	15.48	414.20	415.2123	1.01	97.57%	C ₂₄ H ₃₀ O ₆	Bufotalinin
	2	13.65	407.10	425.1368	18.03	96.67%	C ₁₉ H ₂₁ NO ₇ S	Erysothiopine
	3	14.11	386.17	387.1805	1.01	95.54%	C ₂₂ H ₂₆ O ₆	Schisanhenol B
	4	13.65	280.09	281.1021	1.01	85.66%	C ₁₄ H ₁₆ O ₆	Zhebeiresinol
	5	13.66	368.16	369.1698	1.00	83.60%	C ₂₂ H ₂₄ O ₅	Kanzonol P
	6	15.48	396.19	397.2010	1.01	82.43%	C ₂₄ H ₂₈ O ₅	8-[(2Z)-3,6-Dimethyl-2-hepten-1-yl]-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one
	7	13.47	291.09	309.1315	18.04	82.22%	C ₁₂ H ₁₃ N ₅ O ₄	Toyocamycin
Fraction B	1	13.65	407.10	425.1368	18.03	96.67%	C ₁₉ H ₂₁ NO ₇ S	Erysothiopine
	2	14.03	386.17	387.1812	1.01	94.48%	C ₂₂ H ₂₆ O ₆	Schisanhenol B
	3	13.66	368.16	369.1698	1.00	83.60%	C ₂₂ H ₂₄ O ₅	Kanzonol P
	4	14.03	412.17	413.1777	1.00	82.57%	C ₂₁ H ₂₆ F ₂ O ₆	Fluocinolone
	5	15.48	414.20	415.2123	1.01	81.92%	C ₂₄ H ₃₀ O ₆	Bufotalinin
	6	15.85	452.16	453.1682	1.00	80.18%	C ₂₉ H ₂₄ O ₅	Chamuvaritin

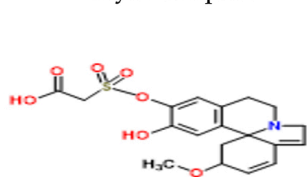
	7	14.49	402.14	425.1369	22.99	79.90%	C ₂₅ H ₂₂ O ₅	Tomentolide A
Fraction C	1	15.87	414.20	437.1931	22.99	98.00%	C ₂₄ H ₃₀ O ₆	Clausarinol
	2	14.06	386.17	409.1627	22.99	91.51%	C ₂₂ H ₂₆ O ₆	Eudesmin
	3	15.87	430.17	453.1669	22.99	87.85%	C ₂₇ H ₂₆ O ₅	2,2-Dimethyl-3,4-bis(4-methoxyphenyl)-2H-1-benzopyran-7-ol acetate
	4	15.48	280.13	281.1388	1.00	85.47%	C ₁₅ H ₂₀ O ₅	8-Deoxy-11,13-dihydroxygrosheimin



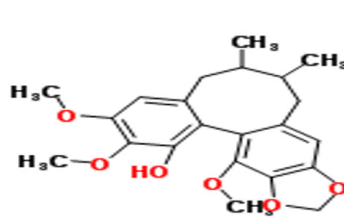
Bufotalinin



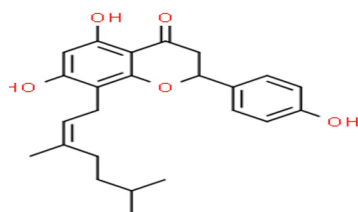
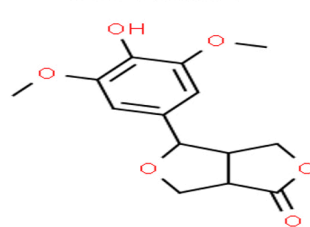
Erysothiopine



Schisanhenol B

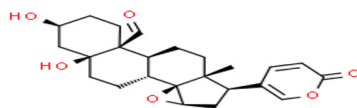


Zhebeiresinol

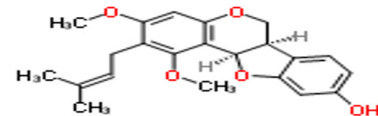


Kanzonol P

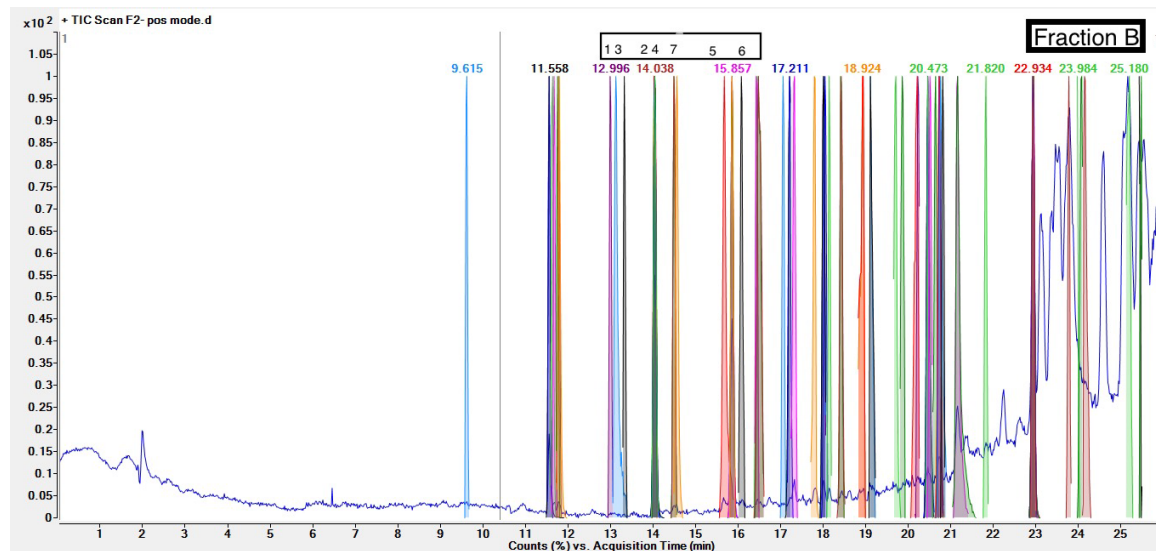
(a)



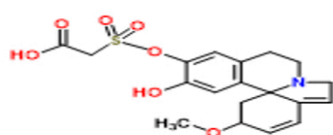
(2Z)-3,6-Dimethyl-2-hepten-1-yl



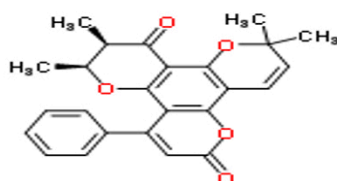
Toyocamycin



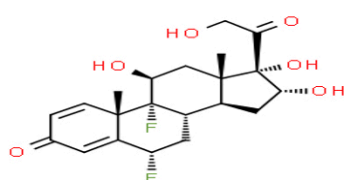
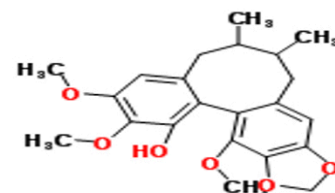
Erysothiopine



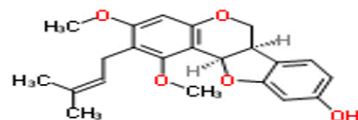
Schisanhenol B



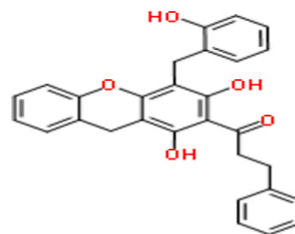
Kanzonol P



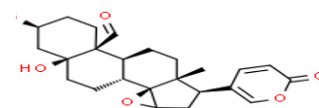
Fluocinolone



Bufotalinin



Chamuvaritin



Tomentolide A

(b)

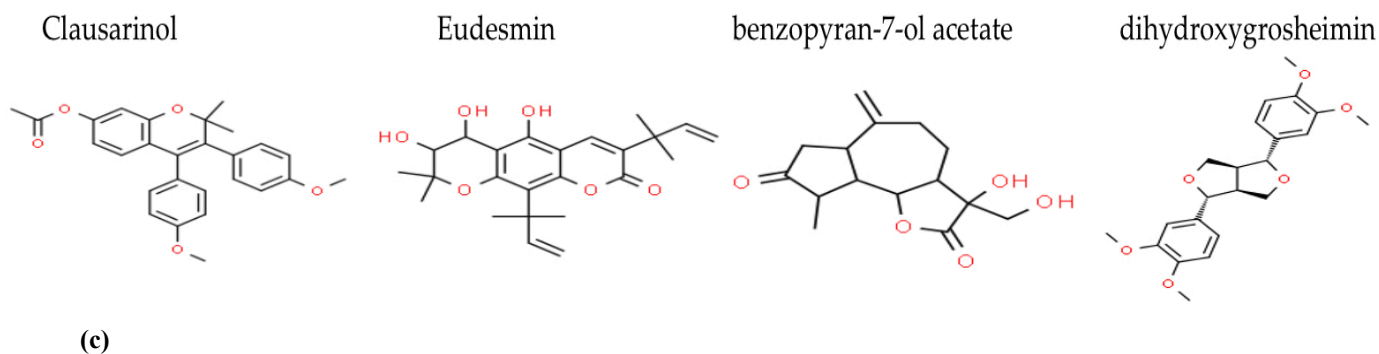
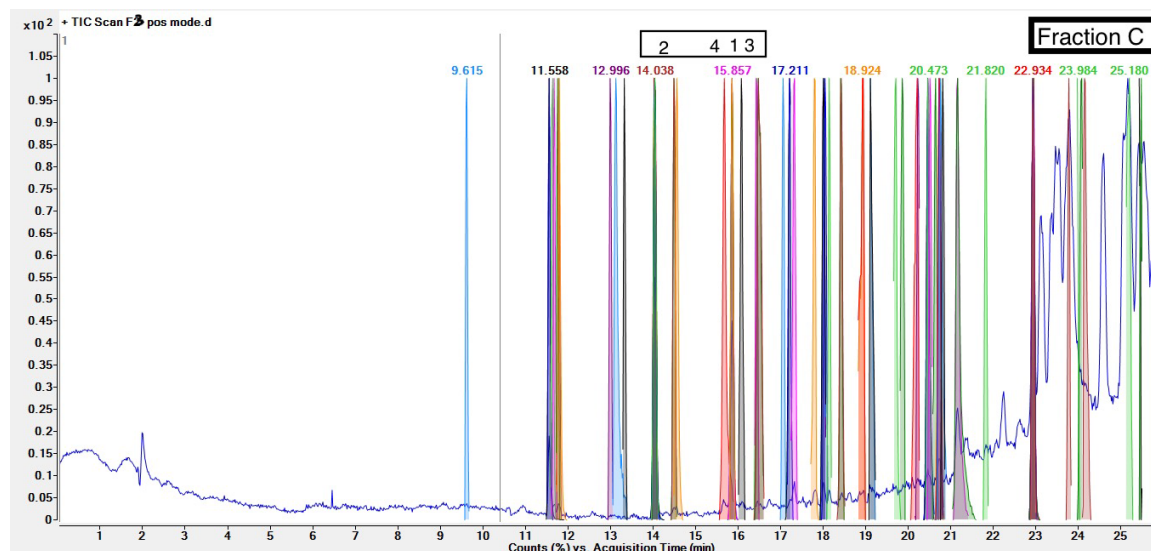


Figure 3. Phenolic compounds LC-QTOF/MS analysis in fractions of *G.inodorum* extract, operated under the following parameters: ionization mode [positive], ionization source [Dual AJS ESI], and collision energy [0]. Mass measurements were conducted with high resolution and accuracy 1.3 Da, 3ppm respectively, ensuring precise determination of molecular masses (a) LC-QTOF/MS analysis of fraction A phenolic compounds; (b) LC-QTOF/MS analysis of fraction B phenolic compounds; (c) LC-QTOF/MS analysis of fraction C phenolic compounds.

3.2 Discussion

This investigation studied different methods for determining the antioxidant potential, screening phenolic compounds, and analyzing the gymnemic acid level using other compound extraction laboratory techniques. Column chromatography and thin-layer chromatography have been used to detect and separate active compounds in the extract (Abeyrathne et al., 2022). Our results suggest that column separation is a good technique for obtaining the active compound in the extract. Interestingly, preliminary thin-layer chromatography results were similar for column separation, which

facilitated selecting the solvents for the best separation of fractions.

Various phenolic classes of Apocynaceae family have attracted significant interest due to their physiological roles, encompassing activities such as scavenging free radicals, preventing mutations, inhibiting carcinogenesis, and reducing inflammation (Brand-Williams et al., 1995). The antioxidative efficacy of phenolic compounds is primarily due to their redox properties, enabling them to function as reducing agents, hydrogen donors, quenchers of singlet oxygen, and potential metal chelators (L. Yu et al., 2002).

The column chromatographic fractionation of *G. inodorum* leaf extract has successfully resolved the critical limitations associated with raw, unfractionated crude extract. Standard crude extractions, such as ethanol reflux, yield highly complex mixtures where pigments and lipids remain co-extracted (Wu et al., 2006), (Cianciosi et al., 2018). These compounds form a dense matrix that physically encapsulates active phenolics, thereby limiting their kinetic rate of free radical scavenging (Nuchuchua et al., 2024). Further, based on the results, a notably elevated concentration of phenolic compounds was detected in the fractions of column separation reflux extract obtained from the leaves of *G. inodorum*. This finding could provide a scientific basis for the extensive historical utilization of this plant in traditional folk medicine practices as well as evidence for high antioxidant activity.

The antioxidant activities of the phytochemical compounds are essential to scavenging free radicals and have a beneficial effect against oxidative-related disorders (Benzie & Strain, 1996). Many studies have reported that plant extracts' bioactive compound possesses a strong antioxidative capacity, possibly enhancing human health (Huang et al., 2005). The DPPH assay has been employed for radical scavenging activity that revealed the antioxidant activity of each fraction. This assay revealed that the separation of fractions via column chromatography showed increased radical scavenging activity of the DPPH radical, which may indicate an increased ability of purified compounds in fractions to donate hydrogen ions (Nwozo et al., 2023). This kinetic pathway is highly favored by less polar, highly mobile phenolic acids and lipophilic flavonoids, which were concentrated in Fraction A. Therefore, it may be postulated that fraction A of *G. inodorum* leaf extract contains higher potent DPPH and ABTS radical scavenging activity due to abundant purified phytochemicals that reduce the radical due to their hydrogen-reducing ability. However, fractions B and C have lower scavenging of DPPH and ABTS radicals, which may be due to the compound's light affinity, temperature, and

solubility of extracts in the solvent system, which were reported factors for affecting the capacity of compounds to quench the radical (Srinuanchai et al., 2019).

The antioxidant capacity test by ABTS assay (Haideri et al., 2024) revealed that fraction A has high ABTS+ radical scavenging, the same as the standard at the highest 1 mg/mL concentration. This indicated the capability of active compounds in our extract to scavenge free radicals, indicating the potential presence of specific phenolic compounds within our extract with in vitro antioxidant activity relevant to oxidative damage associated with free radicals, pending confirmation of therapeutic efficacy in vivo.

The FRAP assay measures the antioxidative capacity of an antioxidant by its reaction with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex, resulting in the formation of a colored ferrous tripyridyltriazine (Fe^{2+} -TPTZ) product (L.-H. Yu et al., 2004). FRAP assay does not involve free radicals but measures the reduction of a ferric tripyridyltriazine complex to its ferrous form via single electron transfer mechanism. Based on the FRAP assay results, conversely, fraction C has reported the highest antioxidant activity than fractions A and B, opposite to DPPH and ABTS results. This divergence likely reflects the different reaction chemistries of the assays: DPPH and ABTS are radical-scavenging assays that respond preferentially to the lipophilic, hydrogen-donating phenolics and flavonoids concentrated in the less polar Fraction A, whereas FRAP measures single-electron-transfer reducing power, which favors the more polar, hydrophilic constituents enriched in Fraction C - consistent with Fraction C also showing the highest TFC and gymnemic acid content in this study (Table 1 and Table 2). FRAP involves the reduction of a ferric ion to its ferrous form by electron donation from these reducing agents, rather than the hydrogen-atom or electron transfer to a stable free radical measured by DPPH/ABTS (L.-H. Yu et al., 2004). Our results are supported by a previous study on *G. inodorum* for high antioxidant activity in crude extract (Wang et al., 2020).

The gymnemic acid levels quantified across the three fractions (Table 2) were markedly higher than the 0.0194 mg/g reported in a previous study, irrespective of extraction method (Srinuanchai et al., 2019), supporting the applied method for separation of active compounds in *G. inodorum* leaf extract. Furthermore, the extraction method's efficiency is bolstered by the determination of gymnemic acid levels.

The current study has shown that the fractions of *G. inodorum* leaf extract contains high concentrations of phenols and flavonoids as active compounds; thus, it is inferred that these active compounds are responsible for high antioxidant activity (Montoro et al., 2018). The correlation between antioxidant activity and total phenolic contents of *G. inodorum* extract is consistent in several reports. The antioxidant activity of fractions revealed that the fractions have higher activity as compared to crude extract. The previous study (Barker, 2019) has published 0.13506 mg TE/g as the highest DPPH antioxidant activity for *Gymnema inodorum* leaf extract, which is lower in comparison to the activity of our fractionated extracts. Furthermore, the fractionation showed better activity for FRAP and ABTS as compared to crude extract. Moreover the results showed the connection between the total phenolic contents of the fractions of *G. inodorum* leaf extract and the antioxidant activity as assessed by the DPPH or ABTS assay. The findings demonstrated a direct relationship between total phenolic levels and either DPPH or ABTS radical scavenging activity. Both the DPPH and ABTS tests indicated the high antioxidant capacity of the fractions with high total phenolic contents. Therefore, the fractions of *G. inodorum* leaf extract have considerable phenolic compounds with antioxidant properties. Compared with previously reported crude *G. inodorum* extracts, the chromatographic fractions demonstrated enhanced antioxidant activity, suggesting enrichment of active phenolic constituents through fractionation.

Interestingly, LC-QTOF/MS analysis results supported that fractions with higher phenolic

compound contents showed greater antioxidant activity. There may be a relationship between phenolic compounds and bioactivity, particularly in terms of scavenging free radicals, as evidenced by the higher quantities of these compounds found in fractions with increased antioxidant activity. Nonetheless, a thorough statistical analysis that definitively establishes this association can support the study's conclusions even more and call for further investigation in the future. The difference in concentration of phenolics and flavonoids was reported by other *Gymnema* studies (Haideri et al., 2024). Moreover, the screened active phenolic compound, Schisanhenol B was found in *Schisandra chinensis* and magnolia vine or five-flavor berry, which were the new active phenolic compounds in *G. inodorum* leaf extract, an aspect not reported in the previous study. These active compounds have been shown to have an antioxidative effect in human low-density lipoprotein (Montoro et al., 2018). Among the identified phenolic compounds, Zhebeiresinol a phenolic lignan has potential health benefits, including antioxidant and anti-inflammatory properties (Barker, 2019). The compound Fluocinolone has been studied for treating localized psoriasis (L.-H. Yu et al., 2004). Furthermore Eudesmin is found in LC-QTOF/MS result, which is natural phenolic lignan having specific antioxidant and anti-inflammatory potential (Barker, 2019), while Clausarinol, Chamuvaritin and Tomentolide A seemed to be a new compounds with limited or no prior study. The identification of Tomentolide A in fraction B is considered tentative because the matching score (79.90%) is comparatively lower than other compounds. The consumption of this plant may play a vital role due to active phenolic compounds for various disease treatments such as diabetes, hyperglycemia, and premature aging. Nevertheless, additional antioxidant potential in vivo models and various antioxidant mechanisms should be comprehensively studied.

Pearson and Spearman correlations were computed across the three fraction-level means (TPC, TFC, DPPH, ABTS, FRAP, and

gymnemic acid; Figure 4). With only $n = 3$ fractions, none of these correlations reach statistical significance (all $p > 0.1$) and the coefficients should be read as exploratory only. The strongest associations were between TFC

and gymnemic acid (Pearson $r = 0.97$) and between ABTS and gymnemic acid ($r = -0.98$), while TPC and DPPH showed a moderate positive association ($r = 0.69$), consistent with the positive association reported in the Abstract.

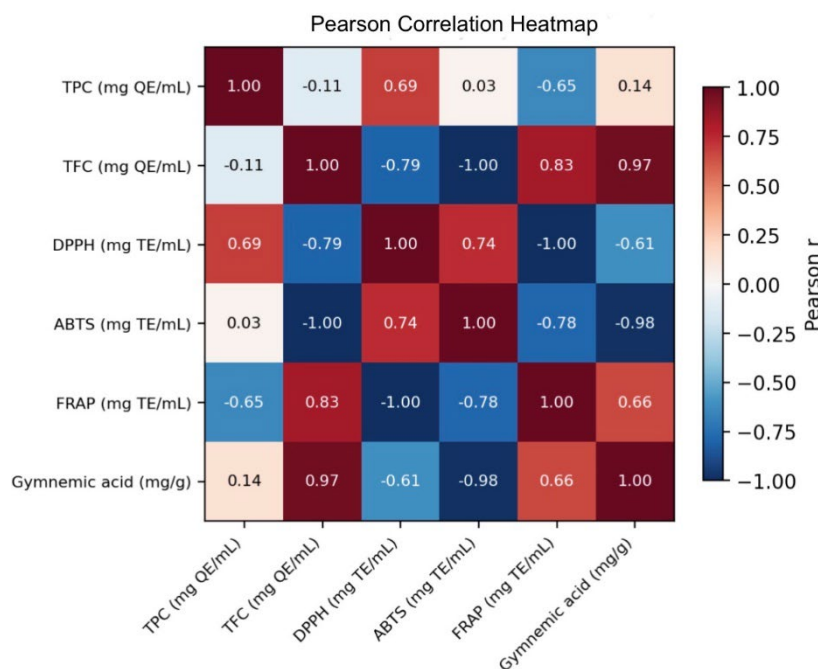


Figure 4. Pearson correlation heatmap across TPC, TFC, DPPH, ABTS, FRAP, and gymnemic acid, computed from the three fraction replicates level means ($n = 9$). using true Trolox/quercetin-equivalent concentrations.

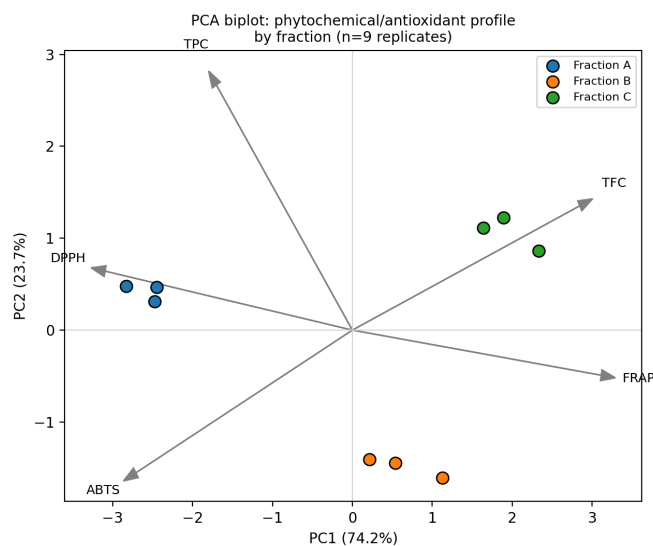


Figure 5. PCA biplot of TPC, TFC, DPPH, ABTS, and FRAP (standardized, $n=9$ replicate readings). Points are individual replicates colored by fraction; arrows show variable loadings

Principal Component Analysis (PCA) on the same five standardized variables (Figure 5) shows the three fractions separating cleanly along two components explaining 98% of total variance (PC1 = 74.2%, PC2 = 23.7%): PC1 is driven by TFC/FRAP loading positively against DPPH/ABTS/TPC loading negatively, separating Fraction C (high TFC/FRAP) from Fractions A and B (high TPC/DPPH/ABTS).

4. Conclusion

In conclusion, the fractions of extract of the plant *G. inodorum* showed significant antioxidant activity and phenolic compounds. This suggests the presence of additional beneficial active phenolic compounds within the plant extract, indicating further potential effects that could be beneficial from its use. The different level of antioxidant activity observed from each fraction are probably due to variation of active phytochemicals. Fraction A exerted the highest radical scavenging capacity as well as highest total phenolic content. The study supported that phenolic compounds may be responsible to antioxidant capacity of *G. inodorum* leaf extract. Our findings further indicate that the column chromatography technique is capable of providing effective purification for these active compounds. Moreover, the results of this study have promising implications across various fields.

Primarily, the endorsement of conventional medical approaches is substantiated by the identification of numerous phenolic components in the leaf extract of *G. inodorum*, coupled with their correlation to potent antioxidant activity. This implies that *G. inodorum* may have therapeutic relevance for oxidative stress-related disorders, pending confirmation in vivo. Moreover, this investigation illuminates neglected phytochemical compounds, prompting additional re-search to unveil the concealed therapeutic potential of this locally edible plant. Utilizing column chromatography for their purification offers promise in isolating and studying these valuable compounds. Nonetheless, future research focusing on characterization and identification of active compounds, and its health effect especially

through in-vivo models and clinical studies, is imperative to translate these findings into practical applications for human health.

5. References

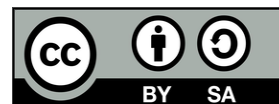
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*Research article*

EFFECTS OF INLET TEMPERATURE AND WALL MATERIAL ON ENCAPSULATION YIELD, MORPHOLOGY AND CHEMICAL PROPERTIES OF SPRAY-DRIED *C. TERNATEA* FLOWER

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ABSTRACT

C. ternatea, commonly known as blue pea flower, is a rich source of natural anthocyanins and other bioactive compounds with potential applications as natural colourants in food products. However, anthocyanins are sensitive to environmental and processing conditions, necessitating suitable protection during processing. This study investigated the effects of inlet temperature and wall material composition on the encapsulation yield, total anthocyanin content, morphology, and chemical profile of *C. ternatea* extract encapsulated by spray drying. Maltodextrin, arabic gum, and a combination of both wall materials were evaluated at inlet temperatures of 160, 180, and 200 °C. Total anthocyanin content was quantified using the pH differential method, particle morphology was examined using Field Emission Scanning Electron Microscopy (FESEM), and selected volatile and semi-volatile compounds were qualitatively identified using Gas Chromatography-Mass Spectrometry (GC-MS). The highest encapsulation yield (46.07%) and total anthocyanin content (27.47 mg/100 g encapsulated powder) were obtained using 10% maltodextrin at 160 °C. FESEM observations showed that wall material composition influenced particle morphology, while GC-MS analysis demonstrated differences in the chemical profiles among the formulations. Overall, the findings indicate that inlet temperature and wall material composition influence powder recovery, anthocyanin content, particle morphology, and chemical composition of spray-dried *C. ternatea* powder. Among the investigated conditions, 10% maltodextrin at 160 °C provided the most favourable combination of encapsulation yield and anthocyanin content

1. Introduction

The increasing demand for natural food colourants has intensified research interest in plant-derived pigments as alternatives to synthetic additives, driven by consumer concerns regarding food safety and regulatory restrictions. Anthocyanins are among the most widely studied natural pigments due to their attractive colour properties and associated antioxidant activity, which make them suitable for application in functional foods and beverages (Khoo et al., 2017). Nevertheless, anthocyanins are inherently unstable and susceptible to degradation during thermal processing and exposure to light, oxygen, and pH variations, limiting their direct incorporation into food systems (Laleh et al., 2006).

C. ternatea flower, commonly known as blue pea flower, is recognised as a rich source of polyacylated anthocyanins that impart an intense blue colour and demonstrate relatively higher colour stability compared to non-acylated anthocyanins from other plant sources (Suppadit, 2011; Muhammad Ezzudin & Rabeta, 2018). In addition to anthocyanins, *C. ternatea* flowers contain various bioactive compounds such as flavonoids, phenolic acids, and antioxidant-related constituents, contributing to their potential use as natural colourants with added functional value (Goh, 2022). Despite these advantages, the application of *C. ternatea* extracts in food processing remains challenging due to the thermal sensitivity and oxidative degradation of its bioactive components.

Microencapsulation has been widely employed to improve the stability, handling, and shelf life of thermally sensitive bioactive compounds. Among the available encapsulation techniques, spray drying is one of the most commonly used methods in the food industry due to its cost efficiency, continuous operation, and suitability for large-scale production (Haque et al., 2015). The effectiveness of spray drying is strongly influenced by processing parameters such as inlet temperature and the selection of encapsulating agents, which can affect powder

recovery, particle characteristics, and the chemical stability of the encapsulated compounds (Pui & Saleena, 2022). Maltodextrin and arabic gum are frequently used as wall materials because of their favourable solubility, film-forming ability, and compatibility with food applications. Maltodextrin has been reported to reduce stickiness and improve powder recovery during spray drying, whereas arabic gum provides favourable emulsification and encapsulation properties for bioactive compounds (Robert & Fredes, 2015). Several studies have investigated the spray drying of *C. ternatea* extracts, particularly in relation to physicochemical stability and antioxidant properties (Hamzah et al., 2013; Liew et al., 2020). However, fewer studies have simultaneously evaluated the effects of inlet temperature and wall material composition on multiple powder characteristics, including encapsulation yield, anthocyanin content, particle morphology, particle size distribution, and chemical profile. Particle morphology and size are important characteristics of spray-dried powders because they can influence properties such as flowability, rehydration behaviour, and susceptibility to oxidation, which are relevant to their subsequent application in food systems (Rezende et al., 2018). In addition to physical characterization, qualitative chemical profiling can provide complementary information on volatile and semi-volatile compounds present after spray drying. Gas Chromatography-Mass Spectrometry (GC-MS) has been used as a qualitative analytical technique for identifying such compounds in plant-derived materials, including *C. ternatea* extracts (Thakur et al., 2018).

Therefore, this study aimed to investigate the effects of inlet temperature and wall material composition on the yield, morphology, and chemical properties of spray-dried *C. ternatea* powder. Encapsulation yield and total anthocyanin content were determined to assess powder recovery and anthocyanin concentration, respectively. Particle morphology was examined using Field Emission Scanning Electron Microscopy

(FESEM), while particle size and size distribution were evaluated using dynamic light scattering (DLS). Selected volatile and semi-volatile compounds were qualitatively identified using GC-MS. By comparing maltodextrin, arabic gum, and their combination at different inlet temperatures, this study provides insight into how processing conditions influence powder recovery, anthocyanin content, particle characteristics, and the chemical profile of spray-dried *C. ternatea* powder.

2. Materials and methods

2.1. Materials

C. ternatea flower used in this study were obtained from a market in Klang Valley, Malaysia and stored at room temperature until usage. The encapsulating agents used are maltodextrin and arabic gum supplied by R&M, Malaysia.

2.2. Methods

2.2.1. Preparation of *C. ternatea* extract

The extraction procedure was adapted from the method described by Netravati et al. (2022), with some modifications. The present study employed a simplified distilled water extraction to preserve the natural profile of water-soluble anthocyanins and maintain food grade compatibility. The dried petals are coarsely shredded to increase the surface area for extraction. Approximately 10 g of dried petals (5% w/v) were mixed with 200 mL of distilled water in a Thermomix (Vorwerk, Malaysia) apparatus. The mixture was stirred at 60°C, 100 rpm, for 30 minutes to promote diffusion of anthocyanins into the solvent. After the extraction period, the mixture was passed through a clean filter cloth to remove insoluble plant material. The resulting filtrate, containing the anthocyanin-rich aqueous extract, was collected in amber bottles to minimise light exposure and stored at 4°C for 24 hours.

2.2.2 Preparation of carrier blend

The wall materials used in this study consisted of maltodextrin (MD) and arabic gum (AG) at three different formulations: MD 10%

(w/v), AG 10% (w/v), and MD 7.5% + AG 2.5% (w/v). Each formulation was prepared by accurately weighing the required amount of MD and AG and dissolving them in distilled water under continuous stirring at room temperature. Stirring was maintained for at least 30 minutes to ensure complete hydration of the arabic gum and full dissolution of the maltodextrin. After complete dissolution, the extract was mixed with the wall material solution at a fixed extract to encapsulating agent ratio of 1:2 (w/w). The mixture was homogenized using a high-speed homogenizer at approximately 10,000 - 12,000 rpm for 5 min to ensure uniform dispersion of anthocyanins within the carrier matrix and to obtain a stable feed emulsion suitable for spray drying. The feed mixture was filtered through a fine mesh ($\leq 150 \mu\text{m}$) to remove particulates that could block the spray dryer nozzle.

2.2.3. Encapsulation process by spray drying

Spray drying was carried out using a laboratory-scale spray dryer (SD-Basic, Lab Plant, UK), following the method described by Ramya et al. (2025) with slight modifications. The feed mixtures were spray-dried at three inlet air temperatures of 160, 180, and 200 °C, with a constant feed rate of 10 mL/min. The resulting powders were collected from the cyclone separator and immediately stored in airtight, opaque containers to minimise exposure to light, moisture, and oxygen prior to further analysis.

2.2.4. Encapsulation yield

The encapsulation yield expressed as a percentage of mass (% w/w) was determined by dividing the weight of the obtained microcapsule powder by the total weight of the primary solid matter. Encapsulation yields were calculated according to Equation (1).

$$\text{Encapsulation Yield} = \frac{\text{TW of Sample}}{\text{TW of Mixture}} \times 100\% \quad (1)$$

2.2.5 Determination of total anthocyanin content

The total anthocyanin content (TAC) of the spray-dried *C. ternatea* powder was determined using the pH differential method. The method is based on the reversible structural transformation of anthocyanins at different pH values, resulting in differences in absorbance between pH 1.0 and pH 4.5. Aliquots of each sample were separately mixed with 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). The absorbance of each solution was measured at 510 nm and 700 nm using a UV-Vis spectrophotometer.

The absorbance difference was calculated using Equation (2) and the monomeric anthocyanin concentration was calculated as cyanidin-3-glucoside equivalents using Equation (3).

$$A = (A_{510} - A_{700})_{pH1} - (A_{510} - A_{700})_{pH4.5} \quad (2)$$

$$\text{Anthocyanins} \left(\frac{\text{mg}}{100 \text{ g}} \right) = \frac{A \times MW_{C3G} \times DF}{\epsilon} \times 100. \quad (3)$$

Where:

MW is the molecular weight of cyanidin-3-glucoside (449.2), ϵ is the molar absorptivity (26,900), and DF is the dilution factor.

2.2.6 Morphological characterisation by Field Emission Scanning Electron Microscope

The morphology of microparticles produced with various encapsulating agents was examined using a cold field emission scanning electron microscope (FESEM, HITACHI SU8010). The FESEM was employed at acceleration voltages ranging from 0.1kV to 30kV. The experiments employed a working distance of 1.5 mm – 30 mm, a resolution of 1.0nm, and a minimum calibration time of 10 nm. For the average particle size and particle size distribution studies, 100-200 microparticles were quantified individually from SEM micrographs. FESEM procedure was adapted from (Rezende et al., 2018).

2.2.7 Particle size and size distribution analysis

The particle size and size distribution of the spray-dried *C. ternatea* powder were determined using dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), with a measurement range of 0.3 nm to 10.0 μm . Sample preparation was adapted from Lee et al. (2016). Approximately 3 mg of spray-dried powder was dispersed in 20 mL of distilled water and vortexed for 20 s. The resulting suspension was filtered through a 0.45 μm syringe filter according to Sadiq et al. (2023) and allowed to equilibrate for 1 h prior to analysis. Measurements were performed at room temperature using refractive indices of 1.38 for the sample and 1.33 for the dispersant. Each sample was measured in triplicate. The mean particle size was expressed as the Z-average diameter, while particle size distribution was represented by the polydispersity index (PDI) (Lee et al., 2016).

2.2.8. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis was performed to qualitatively identify volatile and semi-volatile compounds present in selected spray-dried *C. ternatea* powder samples. The analysis was used for qualitative compound identification and was not intended as a direct measurement of anthocyanin concentration. Sample preparation was adapted from Thakur et al. (2018). For each sample, 200 mg of spray-dried powder was dissolved in 2 mL of methanol and filtered through a 0.22 μm syringe filter prior to analysis. The GC-MS procedure was adapted from Ashaari et al. (2021). An aliquot of 1 μL of the prepared sample was injected into the GC-MS system using splitless injection mode. Separation was carried out using an HP-5MS capillary column (30 m $\times$ 0.25 mm internal diameter $\times$ 0.25 μm film thickness), with helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature was programmed from 50 $^{\circ}\text{C}$ to 280 $^{\circ}\text{C}$ at a heating rate of 10 $^{\circ}\text{C}/\text{min}$. The mass spectrometer was operated in electron-impact ionisation mode at 70 eV. The inlet/transfer line and ion source

temperatures were maintained at 280 °C and 220 °C, respectively.

2.2.8. Statistical analysis

All measurements were performed in triplicate, where applicable, and the results were expressed as mean $\pm$ standard deviation. A two-way analysis of variance (ANOVA) was performed to determine the effects of inlet temperature and wall material formulation, as well as their interaction, on encapsulation yield and total anthocyanin content. When significant differences were detected, Tukey's HSD test was used for multiple comparisons among treatment combinations. Differences were considered statistically significant at $p \leq 0.05$.

3. Results and discussions

3.1. Encapsulation yield

Encapsulation yield reflects the efficiency of powder recovery during the spray drying process and is strongly influenced by both drying temperature and wall material composition. The encapsulation yields obtained under different spray drying conditions are summarised in Table 1. Statistical analysis using two-way ANOVA showed that inlet temperature had a significant effect on encapsulation yield ($p < 0.001$). Wall material formulation also significantly affected encapsulation yield ($p < 0.001$). A significant interaction between inlet temperature and wall material formulation was also observed ($p = 0.005$) indicating that the effect of drying temperature on encapsulation yield depended on the formulation of wall material.

Table 1. Encapsulation yield and total anthocyanin content of *C. ternatea* powder

Sample	Temperature (°C)	Ratio	Encapsulation Yield (%)	Total Anthocyanin Content (mg/100 g)
S1	160	10% MD	46.07 $\pm$ 2.02 ^a	27.47 $\pm$ 4.04 ^a
S2	160	10% AG	37.38 $\pm$ 3.96 ^{ab}	25.38 $\pm$ 1.82 ^a
S3	160	7.5% MD + 2.5% AG	32.78 $\pm$ 5.67 ^{bc}	18.45 $\pm$ 2.69 ^{ab}
S4	180	10% MD	41.59 $\pm$ 0.67 ^{ab}	9.09 $\pm$ 5.89 ^{bc}
S5	180	10% AG	42.76 $\pm$ 6.04 ^{ab}	3.34 $\pm$ 3.46
S6	180	7.5% MD + 2.5% AG	25.06 $\pm$ 5.68 ^c	9.54 $\pm$ 1.21 ^{bc}
S7	200	10% MD	25.05 $\pm$ 2.71 ^c	14.03 $\pm$ 1.99 ^b
S8	200	10% AG	32.51 $\pm$ 3.94 ^{bc}	9.35 $\pm$ 3.71 ^{bc}
S9	200	7.5% MD + 2.5% AG	24.19 $\pm$ 2.74 ^c	8.60 $\pm$ 4.00 ^{bc}

Values are expressed as mean $\pm$ standard deviation ($n = 3$). Different superscript letters indicate significant differences among treatment combinations according to Tukey's HSD test at $p < 0.05$

At an inlet temperature of 160 °C, the highest encapsulation yield was achieved using 10% maltodextrin, as shown by Sample S1 (46.07 $\pm$ 2.02%), followed by 10% arabic gum in Sample S2 (37.38 $\pm$ 3.96%) and the mixed wall material formulation in Sample S3 (32.78 $\pm$ 5.67%). The superior performance of maltodextrin at lower inlet temperature may be attributed to its low viscosity, high solubility, and effective film-forming ability, which promote rapid crust formation around droplets

and reduce material loss due to wall deposition during spray drying. The encapsulation performance and morphological characteristics observed in this study are generally consistent with previously reported findings on spray-dried anthocyanin systems. The higher encapsulation yield obtained using maltodextrin at moderate inlet temperature is in agreement with earlier studies reporting improved powder recovery and reduced wall deposition when maltodextrin is used as the

primary encapsulating agent (Robert & Fredes, 2015; Pettinato et al., 2017). Maltodextrin has been widely reported to promote rapid film formation and reduce stickiness during spray drying, which supports the higher encapsulation yield observed in the present study.

Increasing the inlet temperature to 180 °C resulted in a slightly lower encapsulation yield for the maltodextrin-based sample, while the arabic gum formulation exhibited a comparable yield of $42.76 \pm 6.04\%$. At higher temperatures, increased thermal stress may enhance surface stickiness and particle rupture, particularly for formulations containing mixed wall materials, as reflected by the significantly lower yield observed for Sample S6 ($25.06 \pm 5.68\%$). At 200 °C, a further decrease in encapsulation yield was observed for most formulations, particularly S7 and S9, which showed significantly lower yields compared with S1. High inlet temperatures are known to accelerate moisture evaporation, which may cause particle deformation, surface cracking, and adhesion to the drying chamber wall, thereby reducing powder recovery. These findings indicate that moderate inlet temperature combined with maltodextrin as the encapsulating agent provides favourable conditions for achieving higher encapsulation yield.

3.2. Total anthocyanin content (TAC)

The total anthocyanin content (TAC) of the spray-dried *C. ternatea* powders was significantly affected by both inlet temperature and wall material formulation. As depicted in Table 1, the highest TAC was recorded for Sample S1 (27.47 ± 4.04 mg/100 g), whereas the lowest value was observed for Sample S5 (3.34 ± 3.46 mg/100 g). Overall, samples produced at 160 °C showed higher TAC than those produced at 180 and 200 °C. Two-way ANOVA confirmed that inlet temperature

significantly affected TAC ($p < 0.001$), and wall material formulation also showed a significant effect ($p < 0.05$). Meanwhile, the interaction between inlet temperature and wall material formulation was also significant ($p = 0.05$). Tukey's HSD test further showed that S1 and S2 were among the treatment combinations with the highest TAC.

The strong effect of inlet temperature is consistent with the thermal sensitivity of anthocyanins. Trirahayu et al. (2025) similarly reported that increasing inlet temperature during spray drying reduced the total anthocyanin content of *C. ternatea* powder. Higher thermal exposure can accelerate anthocyanin degradation through structural transformation and oxidation, resulting in lower measurable pigment content. Wall material formulation also influenced TAC, which agrees with previous findings that carrier composition affects anthocyanin stability. Lourith and Kanlayavattanukul (2020) reported improved stability of *C. ternatea* anthocyanins when protected by biopolymeric wall systems containing maltodextrin and gum arabic. Multisona et al. (2023) also highlighted the importance of maltodextrin, arabic gum, and mixed carrier systems in preserving *C. ternatea* anthocyanins during encapsulation. In addition, Mariano et al. (2025) showed that different proportions of maltodextrin and gum arabic influenced the physicochemical and bioactive properties of spray-dried *C. ternatea* powder.

3.3. Morphological characteristics

The morphological characteristics of the spray-dried *C. ternatea* powders were examined using Field Emission Scanning Electron Microscopy (FESEM) to evaluate the influence of wall material composition on particle structure and surface properties. Representative FESEM micrographs of samples produced at an inlet temperature of 160 °C are shown in Figure 1.

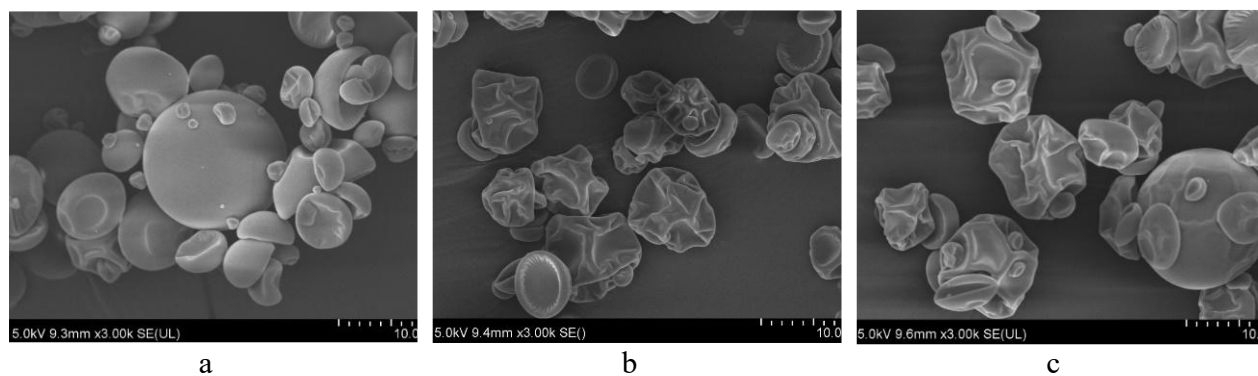


Figure 1. FESEM image of (a) Sample S1, (b) Sample S2, (c) Sample S3, of spray-dried *C. ternatea* powders at 160 °C with 3000X magnification

Particles encapsulated with 10% maltodextrin (Sample S1) exhibited predominantly spherical to near-spherical shapes with smooth and continuous surfaces. This morphology suggests effective encapsulation, where rapid film formation of maltodextrin around the droplets resulted in structurally stable particles. Smooth particle surfaces are generally associated with improved flowability and reduced exposure of bioactive compounds to external oxidative conditions. The spherical particle morphology with smooth surface characteristics observed for maltodextrin-encapsulated powders is also consistent with FESEM observations reported for spray-dried plant extracts and anthocyanin-rich materials (Rezende et al., 2018; Balci-Torun & Ozdemir, 2021).

In contrast, particles encapsulated with 10% arabic gum (Sample S2) displayed noticeable surface shrinkage and irregular deformation. The observed morphological collapse may be attributed to slower crust formation and higher hygroscopicity of arabic gum, which can lead to structural contraction during moisture removal. Such surface irregularities may increase susceptibility to oxidation and reduce powder stability during storage. The surface shrinkage and particle deformation associated with arabic gum-based formulations have been previously attributed to slower crust formation and higher hygroscopicity (Pui & Saleena, 2022), which aligns with the morphological features observed in this study. For the mixed wall

material formulation with 7.5% MD + 2.5% AG (Sample S3), particles exhibited intermediate morphological characteristics, showing partially smooth surfaces with slight shrinkage. This indicates that the presence of maltodextrin contributed to improved structural rigidity, while arabic gum influenced the drying behaviour and particle contraction.

The FESEM analysis demonstrates that maltodextrin produced more uniform and structurally intact microcapsules compared to arabic gum-based formulations. These morphological features are consistent with the higher encapsulation yield observed for maltodextrin and support its suitability as an encapsulating agent for producing stable anthocyanin powders.

3.4. Particle size and size distribution

The mean particle size and polydispersity index (PDI) of the spray-dried *C. ternatea* powders varied with inlet temperature and wall material formulation as shown in Figure 2. The smallest particle size was observed for S1 containing 10% maltodextrin at 160 °C (35.47 nm), whereas S7 containing the same wall material at 200 °C exhibited the largest particle size (188.79 nm). A marked increase in particle size with increasing inlet temperature was particularly apparent for the maltodextrin formulation, while the arabic gum formulations showed comparatively smaller variations across the investigated temperatures.

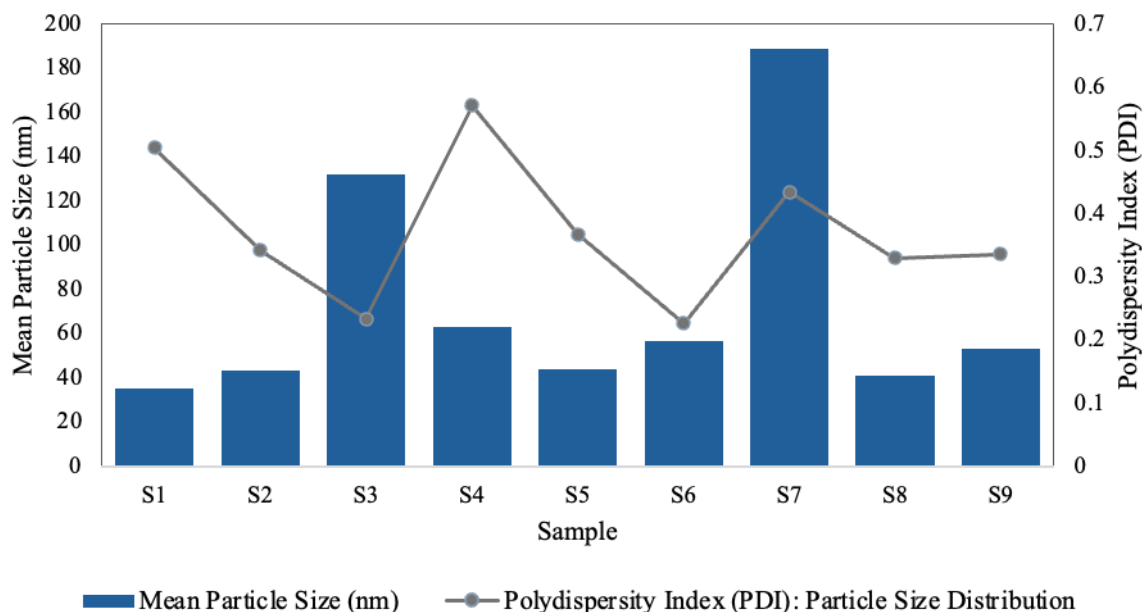


Figure 2. Mean particle size (Z-average) and polydispersity index (PDI) of spray-dried *C. ternatea* powders under different inlet temperatures and wall material formulations

The effect of carrier composition on the particle size of spray-dried *C. ternatea* powder has also been reported by Mariano et al. (2025). They observed particle sizes ranging from approximately 121 to 199 nm and reported smaller particles in formulations containing higher proportions of maltodextrin. The authors attributed this behaviour partly to the lower viscosity of maltodextrin-containing feed solutions, which may favour the formation of smaller droplets during atomisation. Previous studies on microencapsulated *C. ternatea* have similarly demonstrated that the type and proportion of coating material can influence particle size and morphological characteristics (Multisona et al., 2023).

In this study, the increase in particle size of the maltodextrin formulation from 160 to 200 °C suggests that inlet temperature also influenced particle formation. Higher drying temperatures can accelerate moisture evaporation and promote rapid crust formation, which may alter particle expansion, shrinkage, and aggregation during drying. Similar effects of processing conditions and carrier

composition on the particle characteristics of spray-dried anthocyanin-rich powders have been reported for other plant materials (Hoang et al., 2024; Hay et al., 2025).

The polydispersity index (PDI) ranged from 0.227 to 0.572, indicating differences in the width of particle size distribution among the formulations. The lowest PDI was observed for S6 (0.227), followed closely by S3 (0.234), both containing the mixed maltodextrin–arabic gum formulation. This suggests a comparatively narrower particle size distribution for these samples. In contrast, S4 exhibited the highest PDI (0.572), indicating a broader and more heterogeneous particle population. The DLS results indicate that both processing temperature and wall material composition contributed to differences in particle size and size distribution of the reconstituted spray-dried powders. The variation observed among formulations further supports the importance of carrier selection and drying conditions in determining the physical characteristics of spray-dried *C. ternatea* powder.

3.5. Chemical profile by GC-MS

GC-MS analysis was used to qualitatively identify volatile and semi-volatile compounds present in the spray-dried *C. ternatea* powder prepared using different wall material formulations. The comparison presented in

Table 2 and Figure 3 focused on Samples S1, S2, and S3 produced at an inlet temperature of 160 °C, as this temperature produced the highest total anthocyanin contents among the investigated drying conditions.

Table 2. Gas Chromatography Mass Spectrometry (GCMS) analysis for Sample S1, S2 and S3 of spray-dried *C. ternatea* powder at 160 °C

Compound Name (Formula)	S1 Component Area	S1 Area %	S2 Component Area	S2 Area %	S3 Component Area	S3 Area %
2-Furanmethanol (C ₅ H ₆ O ₂)	22947051.68	14.16	ND	ND	81500625.71	15.79
1,2-Cyclopentanedione (C ₅ H ₆ O ₂)	7664022.25	4.73	1630232.57	5.20	15887108.84	3.08
Maltol (C ₆ H ₆ O ₃)	2257618.17	1.39	ND	ND	12814765.12	2.48
4H-Pyran-4-one, 2,3- dihydro-3,5-dihydroxy-6- methyl- (C ₆ H ₈ O ₄)	9944055.81	6.14	422573.70	1.35	29713226.43	5.76
5-Hydroxymethylfurfural (C ₆ H ₆ O ₃)	11087894.88	6.84	ND	ND	54553965.17	10.57
Tetradecanoic acid (C ₁₄ H ₂₈ O ₂)	2013610.15	1.24	ND	ND	1293264.01	0.25
Tris(1,3- dichloroisopropyl)phosphate (C ₉ H ₁₅ Cl ₆ O ₄ P)	2251598.64	1.39	1161433.30	3.71	4516628.97	0.88
Dimethylsulfoxonium formylmethylide (C ₄ H ₈ O ₂ S)	ND	ND	2639058.82	8.42	ND	ND
2-Hydroxy-gamma- butyrolactone (C ₄ H ₆ O ₃)	ND	ND	1096376.55	3.49	ND	ND
Pentanal (C ₅ H ₁₀ O)	ND	ND	3529846.33	11.26	ND	ND
2(3H)-Furanone, dihydro-4- hydroxy- (C ₄ H ₆ O ₃)	ND	ND	531464.96	1.69	ND	ND
4-Vinylphenol (C ₈ H ₈ O)	ND	ND	1001998.58	3.19	ND	ND
2,4-Di-tert-butylphenol (C ₁₄ H ₂₂ O)	ND	ND	ND	ND	3419335.35	0.66

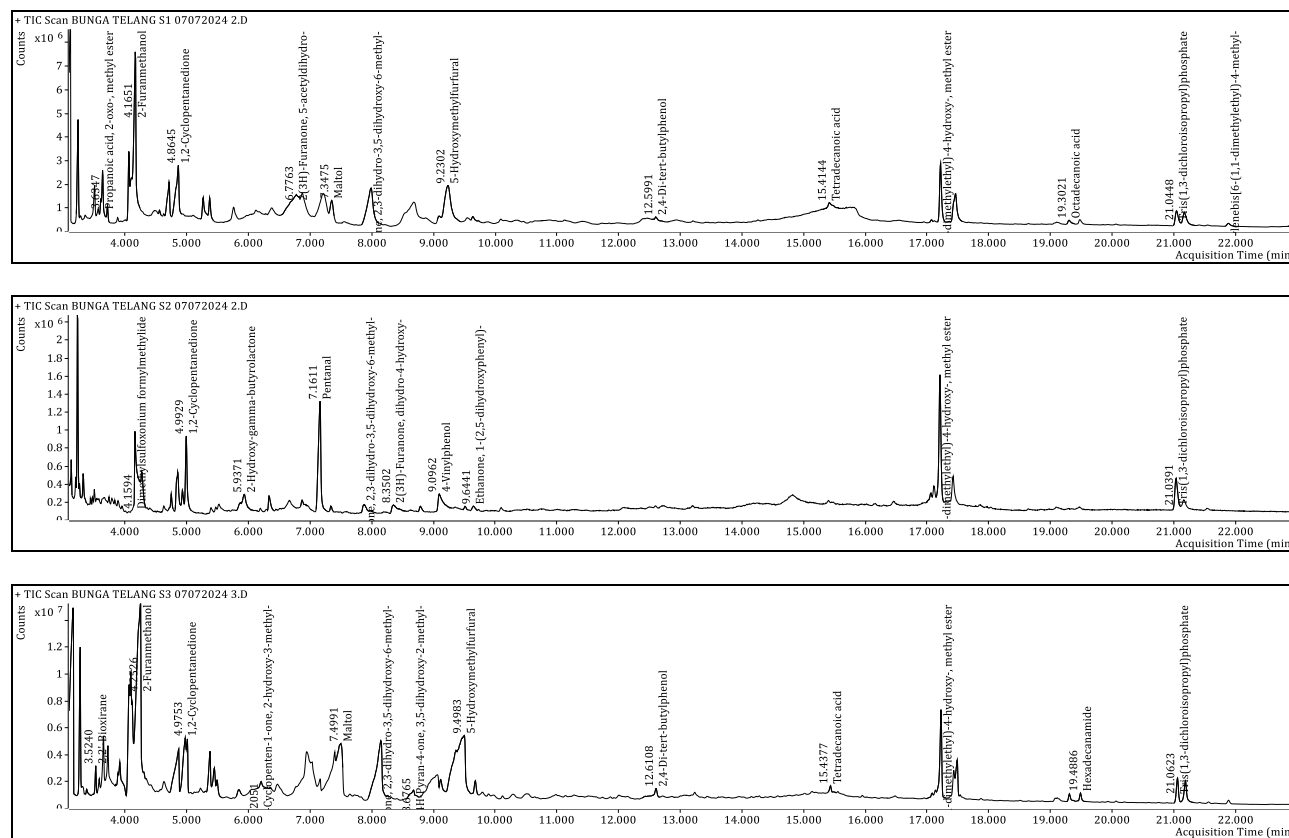


Figure 3. Chromatography diagram for (a) Sample S1, (b) Sample S2, (c) Sample S3 of spray-dried *C. ternatea* powders

Sample S1 showed several identifiable compounds, including 2-furanmethanol, 1,2-cyclopentanedione, maltol, 4H-pyran-4-one derivatives, 5-hydroxymethylfurfural, tetradecanoic acid, and tris(1,3-dichloroisopropyl) phosphate. Several of these compounds, particularly furans and pyranone derivatives, may originate from thermal transformation or degradation reactions involving carbohydrates during spray drying rather than representing intact anthocyanins. The presence of maltol, hydroxymethylfurfural, and related compounds may therefore reflect thermal processing of the extract and carrier matrix.

In contrast, Sample S2 exhibited a different chemical profile, with compounds including pentanal, dimethylsulfoxonium

formylmethylide, 1,2-cyclopentanedione, 4-vinylphenol, and 2(3H)-furanone, dihydro-4-hydroxy-. The occurrence of aldehydes and furan-related compounds may be associated with oxidation and thermal transformation pathways during drying. Differences in the relative abundance and type of detected compounds among the formulations suggest that wall material composition may influence the chemical environment of the spray-dried matrix.

Sample S3 showed a profile containing several compounds also detected in S1, including 2-furanmethanol, 1,2-cyclopentanedione, maltol, 4H-pyran-4-one derivatives, 5-hydroxymethylfurfural, tetradecanoic acid, and 2,4-di-tert-butylphenol. The similarity in several detected compounds

between S1 and S3 may be related to the presence of maltodextrin in both formulations, although the qualitative nature of the GC-MS analysis does not permit conclusions regarding compound retention or degradation kinetics.

The GC-MS results demonstrated that the spray-dried powders contained different qualitative profiles of volatile and semi-volatile compounds depending on the wall material formulation. These findings complement the total anthocyanin content analysis and FESEM observations by providing information on the chemical composition of the powders after spray drying. However, GC-MS was not used to quantify anthocyanins or to establish anthocyanin retention, and the detected compounds should be interpreted primarily as part of the broader chemical profile of the spray-dried powders.

4. Conclusions

This study demonstrated that inlet temperature and wall material composition influenced the properties of spray-dried *C. ternatea* powder. The highest encapsulation yield and total anthocyanin content were obtained at 160 °C, with the 10% maltodextrin formulation showing the highest values for both parameters. Statistical analysis confirmed that inlet temperature and wall material significantly affected encapsulation yield and total anthocyanin content. FESEM observations showed differences in particle morphology among the wall material formulations, with maltodextrin producing relatively smoother and more uniform particles at 160 °C. Dynamic light scattering analysis further demonstrated variation in particle size and size distribution across the drying conditions and formulations. GC-MS analysis identified differences in the qualitative profiles of volatile and semi-volatile compounds among the samples, although these results were interpreted as chemical profiling rather than direct evidence of anthocyanin retention or biological activity. Overall, an inlet temperature of 160 °C provided the most favourable condition for maintaining higher anthocyanin content and powder recovery. Among the investigated formulations,

maltodextrin showed favourable performance in terms of encapsulation yield, anthocyanin content, and particle morphology. These findings provide useful information for the development of spray-dried *C. ternatea* powder for potential food applications, although further evaluation of colour stability, antioxidant activity, storage stability, and other functional properties is recommended.

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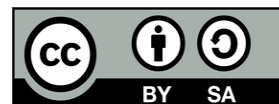
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*Research article*

FORMULATION AND EVALUATION OF NUTRITIOUS BISCUITS: A COMPARATIVE STUDY OF COMPOSITE FLOUR BLENDS WITH JACKFRUIT SEEDS (*Artocarpus heterophyllus*), KITHUL PALM FLOUR (*Caryota urens*), AND PUMPKIN SEEDS (*Cucurbita maxima*) AGAINST WHEAT FLOUR

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ABSTRACT

Nutritional deficiencies remain a major global health concern particularly in developing countries where monotonous diets fail to provide adequate nutrients. In Sri Lanka, biscuits manufacturing heavy reliance on imported wheat flour (WF) limits dietary diversity and reduces nutritional quality. This study aimed to formulate and evaluate nutritious biscuits using composite flour (CF) consisting of jackfruit seed (*Artocarpus heterophyllus*), kithul palm (*Caryota urens*), and pumpkin seed (*Cucurbita maxima*) flours in a 1:1:1 ratio. Four flour blends were prepared: T1 (100% WF, control), T2 (85% WF + 15% CF), T3 (70% WF + 30% CF), and T4 (55% WF + 45% CF). Techno functional properties of flours, sensory attributes of the biscuits using 48 untrained panelists (9-point hedonic scale), total phenolic content (TPC) and total antioxidant capacity (TAC) of the biscuits were evaluated for all formulations. Proximate composition, shelf-life analyses and physical properties were conducted only for the selected T2 formulation. The T2 flour blend exhibited the highest water absorption, bulk density, emulsion activity and stability, and the lowest gelatinization temperature and gelation concentration. T2 biscuits achieved the highest scores for taste, aroma, and overall acceptability. Proximate analysis of T2 biscuits (per 100 g) showed 3.11 g moisture, 1.56 g ash, 20 g fat, 0.61 g fibre, 7.85 g protein, 66.87 g carbohydrate, and 478.88 kcal energy. The total bacterial count after one week without preservatives (1.9×10^2 CFU/g) remained within safe limits (1×10^4 CFU/g). All composite biscuits showed significantly higher ($p < 0.05$) TPC and TAC compared to wheat flour

biscuit. These findings highlight that substituting 15% of wheat flour by developed composite flour produced nutritionally rich and consumer acceptable biscuits, supporting food security and economic sustainability through local resource utilization.

1. Introduction

Non communicable diseases (NCDs) are chronic conditions resulting as a combination of behavioral, environmental, genetic, and physiological determinants. Most common NCDs such as obesity, diabetes, cancers, and cardiovascular diseases that are prevalent among all age groups are significantly influenced by bad dietary habits. Inadequate intake of fiber, vitamins, and minerals is considered a leading dietary risk factor (World Health Organization, 2024). Notably, NCDs are accounting for approximately 75% of global deaths unrelated to pandemics (World Health Organization, 2024), while the rising incidence of NCDs imposes a substantial economic burden on both developed and developing countries (Okunogbe *et al.*, 2022). Modifying dietary and nutritional behaviors is widely recognized as one of the most effective preventive strategies against these diseases (Budreviciute *et al.*, 2020). In this context, popular foods consumed among all age groups present an opportunity to deliver nutrients often deficient in modern diets (Wang *et al.*, 2023).

Biscuits are classified as fast-moving consumer goods, are widely consumed ready to eat snack across all demographics, all age groups since they are affordable, have appealing sensory qualities, variety, and extended shelf life (Eugenia Ramashia *et al.*, 2024). Traditional biscuit formulations consist of wheat flour, fat, and sugar. However, the nutritional quality of biscuits is limited by the refined wheat flour used as the main raw material. During milling, the bran which contains most of the bioactive compounds, fiber, vitamins, and minerals is partially or completely removed. As a result, refined wheat flour-based products have lower nutritional value compared to composite flour alternatives. Wheat protein is inferior in quality due to low levels of lysine, methionine, and threonine (Cheng *et al.*, 2021).

To address the nutritional limitations of wheat flour, biscuit quality can be enhanced either through fortification with minerals, vitamins, and fiber or by exploring alternative flours (Davidson, 2019). Encouraging the use of composite flour is one of the cost-effective methods to produce nutritionally rich biscuits. Composite flour is a blend of various flours, starches, and other ingredients designed to partially or completely replace wheat flour. This approach offers additional advantages for developing countries, such as reducing reliance on imported wheat, promoting the use of domestic crops, and valorizing agricultural byproducts. Previous research indicates that biscuits made from composite flours can achieve good nutritional, physical, and sensory quality, with characteristics similar to traditional wheat-flour biscuits (Goubgou *et al.*, 2021).

The jackfruit (*Artocarpus heterophyllus*), is originated in the rainforests of Western Ghats of India. Various parts of the jackfruit tree, including fruit pulp, leaves, seeds, skin, and wood provide a range of advantages in industrial processing, trade commodities, medicinal purposes, fuel and construction material (Karunakaran *et al.*, 2025). Jackfruit seed flour, is an underutilized agricultural byproduct suitable for composite flour formulations. It is rich in carbohydrates (73.87 g/100 g), proteins (14 g/100 g), potassium (768.63 mg/100 g), calcium (132 mg/100 g), and iron (8.94 mg/100 g), making it a nutritious food source (Sévérin *et al.*, 2024). The flour's antioxidant properties, derived from polyphenolic compounds help extend product shelf-life. Furthermore, its functional properties, including high bulk density gelation and oil absorption capacity, enhance its suitability as a wheat flour substitute (Paula *et al.*, 2025).

Pumpkin (*Cucurbita maxima*) seed flour is another nutrient dense agriculture byproduct that can be utilized for composite flour

formulation (Kaur and Sharma, 2017). These olive-green kernels are buttery in texture and in flavor. It is a good source of calories, protein, carotenoids, minerals (iron, zinc, magnesium, calcium), fiber, and phytosterols (Syed, 2019). The flour is also a valuable source of unsaturated fatty acids, particularly omega-6 (37.63–51.74%) and omega-9 (28.64–37.45%), which are contributing to cardiovascular, cognitive, and dermal health (Prommaban *et al.*, 2021). Studies have shown that incorporating pumpkin seed flour into wheat-based products significantly boosts protein content and antioxidant activity without negatively affecting sensory appeal (Galenko *et al.*, 2024).

Tropical Asia's native rainforest species, *Caryota urens*, is referred to as the Kithul in Sri Lanka. Additionally, it is among the most prevalent trees in Sri Lanka's highland perennial forest gardens (Malki *et al.*, 2025). A traditional starch source with increasing potential for use in composite flour applications is Kithul flour, which is made from the pith of the *Caryota urens* palm. The inflorescences are reportedly used in traditional medicine to cure rheumatic swellings, migraine headaches, gastric ulcers, and snake bites. Genuine Kitul products are thought to be appropriate for diabetic patients due to their antidiabetic qualities, which may entail the regulation of inflammatory pathways and oxidative damage caused by hyperglycemia (Andrade *et al.*, 2023).

Although these plant-based ingredients have been studied individually for their nutritional and functional properties, limited research has examined their combined use in a single composite flour. This multi-ingredient approach is expected to provide combined benefits, with jackfruit seed flour contributing starch-based functionality, pumpkin seed flour enhancing protein and lipid content, and Kithul flour improving dietary fibre, thereby enhancing both the nutritional value and the techno functional properties of biscuits. Based on this background, the current study was designed to formulate a composite flour blend incorporating jackfruit seed flour, pumpkin

seed flour, and kithul flour to substitute varying proportions of wheat flour in biscuit production. The study involved the preparation of composite flour blends at different substitution levels. The suitable biscuit in this study was determined by the sensory analyses and functional properties. Furthermore, the proximate composition, microbial shelf life, and colour characteristics were analysed for the most consumer accepted biscuit formulation. In addition, TPC and TAC were determined for all the biscuit formulations.

2. Materials and methods

2.1. Materials

2.1.1. Plant material and sample preparation

The Jackfruit seeds, pumpkin seeds, Kithul flour and all-purpose wheat flour and additional ingredients like vegetable oil, sugar, baking soda, baking powder and salt were procured from the local market in Wellawatta, Sri Lanka. Clean mature pumpkin seeds were obtained and washed to eliminate sand and soil. Followed by washing with tap water, seeds were oven dried (Meditry DHG-9053A, China) at 70°C for 30 minutes. Then they were milled and sieved to separate the particles below 500µm (Syam *et al.*, 2023).

Jackfruit seeds were boiled at 100°C for 45 minutes and the arils were removed. Lye peeling was done by soaking in 0.05% NaOH for 3 minutes, then the seeds were washed using distilled water. The spermoderms were removed and were soaked in 0.2% sodium metabisulfite for 30 minutes. Finally, wet milling was done and dried at 70°C for 12 hours, and it was ground and sieved to obtain flour particles below 500µm (Jayampathi *et al.*, 2025). All four types of flour (all-purpose wheat flour, Kithul flour, jackfruit seed flour, and pumpkin seed flour) were stored at 4°C in sealed polyethylene bags for further use.

2.1.2. Chemical and reagents

All chemicals used were of analytical reagent grade, including, Absolute ethanol, Ammonium molybdate, Ascorbic acid, Distilled water, Folin-Ciocalteu reagent, Gallic acid, Sodium carbonate, Sodium hydroxide,

Sodium metabisulfite, Sulfuric acid, Tryptone Soya Agar (TSA) and Yeast extract.

2.2. Methods

A completely randomized design was conducted in this study. Four biscuit formulations (T1-T4) containing different levels of composite flour substitution were prepared and evaluated with control formulation of 100% wheat flour. The optimal formulation was selected based on the sensory evaluation scores, supported by the functional property analyses. Subsequently, the selected formulation was subjected to further analyses including the proximate and microbial shelf-life.

2.2.1. Formulation of composite flour

Three composite flour blends and a control were prepared using jackfruit seed flour, pumpkin seed flour, Kithul flour, and wheat

flour. The composite flour was formulated by blending jackfruit seed flour, pumpkin seed flour, and Kithul flour in equal proportions (1:1:1) to enable a balanced evaluation of their combined effects following the approach reported by Chandra, Singh, and Kumari (2015) (Table 1).

2.2.2. Determination of functional properties of the flours

Oil absorption capacity (OAC), water absorption capacity (WAC), emulsion activity (EA), emulsion stability (ES), bulk density (BD), foaming capacity (FC), water solubility (WS) and swelling power (SP), dispersibility, gelatinization temperature (GT), and least gelation concentration (LGC) were determined in triplicate for the four developed composite flour treatments using the methods described by Chandra, Singh and Kumari (2015).

Table 1. Composition of the composite flour preparations

Treatment	Wheat flour %	Composite flour		
		Jackfruit seed flour %	Kithul flour %	Pumpkin seed flour %
T1	100	0	0	0
T2	85	5	5	5
T3	70	10	10	10
T4	55	15	15	15

2.2.3. Preparation of biscuits

Sugar and butter were mixed using a beater (Philips, China) until the sugar was dissolved. Then, milk powder, wheat flour, composite flour, baking powder, milk, and vanilla essence were added to the creamy mixture and were blended to achieve a homogeneous consistency (Table 2). The obtained dough was piped into circular shapes

(5 cm in diameter) and baked at $170 \pm 2^\circ\text{C}$ for 20 minutes in a preheated bakery oven (Panasonic, Japan). After baking, the biscuits were allowed to cool and were then stored in airtight containers at room temperature for subsequent analysis and sensory evaluation (Figure 1).

Table 2. Formulation of the ingredients

Ingredient	Weight
Wheat flour/ composite flour	166 g
Butter	100 g
Powdered sugar	100 g
Milk	66 mL
Milk powder	16 g
Baking powder	1.5 teaspoon
Vanilla essence	1 tsp



Figure 1. Biscuits made from developed composite flour types. T1 - biscuit made from 100% wheat flour, T2 - biscuit made from 85% wheat flour + 5% each of other flours, T3 - biscuit made from 70% wheat flour + 10% each of other flours, T4 - 55% wheat flour + 15% each of other flours

2.2.4. Sensory evaluation

The sensory attributes of color, aroma, taste, texture, aftertaste, and overall acceptability were evaluated by a panel of 48 untrained panelists. All participants were informed about the purpose and procedures of the study, and the informed consent was obtained before their participation. Panelists were randomly selected from the student population (age 18-25 years) at the BMS campus in Colombo, Sri Lanka. This might restrict the findings to the general consumer population therefore; future research should consider more diverse age groups. The sensory evaluation procedure was approved by the Ethics Committee of Northumbria University, UK (Protocol code 2024-7357-7464).

Samples were presented in a randomized order, with each assessor receiving one full biscuit under same laboratory environmental conditions. Assessments were conducted using a 9-point hedonic scale (1 = Dislike extremely; 2 = Dislike very much; 3 = Dislike moderately;

4 = Dislike slightly; 5 = Neither like nor dislike; 6 = Like slightly; 7 = Like moderately; 8 = Like very much; 9 = Like extremely).

Based on the highest overall acceptability scores from the sensory analysis, the optimal biscuit formula was selected for further investigation. This selected formula then underwent proximate analysis and total plate count testing (Plustea *et al.*, 2024).

2.2.5. Proximate analysis of composite biscuit

According to the standard analytical methods the moisture content, ash content, crude fiber, crude protein, and crude fat of the developed T2 composite biscuits (85% wheat, 15% composite flour) were analyzed according to standard AOAC analytical methods. All measurements were conducted in triplicate, and the results are reported on a dry weight basis as mean $\pm$ standard deviation (SD) (Twinomuhwezi, Awuchi and Rachael, 2020).

2.2.6. Total plate count

Total plate count of the selected biscuit (T2: 15% composite flour) was evaluated after

a 1-week storage period. Biscuits were stored in airtight containers at ambient room temperature. 10 g sample of the ground biscuit was aseptically transferred into a sterile stomacher bag containing 90 mL of 0.1% peptone water. The bag was homogenized in a stomacher (Seward Stomacher, UK) for 2 minutes to create a 10^{-1} initial homogenate biscuit extract. Then the pour plate technique was employed as per the method described by Kumari *et al.* (2021). Serial dilutions (10^{-1} to 10^{-6}) were prepared in triplicate from the initial homogenate using sterile diluent. From each dilution, 1 mL aliquot was transferred into a sterile petri dish. Approximately 15 mL of molten Tryptone Soya Agar (TSA), supplemented with yeast extract at 45°C, was then poured into each plate. Plates were rotated gently clockwise and anticlockwise on a level surface. The solidified plates were incubated at 30°C for 48 hours. Following incubation, bacterial colonies on plates containing 30-300 colonies were counted. The colony-forming units per gram of biscuit (CFU/g) were calculated using the standard formula (Ramashia *et al.*, 2024):

$$\text{CFU/g} = \frac{\text{Number of colonies counted}}{\text{Dilution factor} \times \text{Volume plated (mL)} \times \text{Sample weight (g)}} \quad (1)$$

2.2.7. Texture profile analysis

The textural properties of the selected biscuit formula (T2: 15% composite flour) were determined using a Texture Analyzer (TX 700 Shimadzu, Japan) to perform a two-cycle compression test. Measurements were conducted in triplicate. A biscuit (4 cm diameter) was placed centrally on the stationary base plate. This was performed using a flat cylindrical probe (P/36) and following parameters were used; 3.0 mm/s pre-test speed, 1.0 mm/s test speed, 2.0 mm/s post-test speed, 70% strain target mode, 0.1N trigger force and 3 seconds pause between cycle. Hardness (N), Cohesiveness (ratio), Gumminess (N), and Chewiness (mJ) were calculated automatically by the instrument's software from the resultant force-time curve.

2.2.8. Colour measurement

The surface color of the selected biscuit formula (T2: 15% composite flour) was measured using a Hunter Lab colorimeter (CR-400, Konica Minolta Inc., Japan). The instrument was calibrated using a standard white calibration plate prior to analysis. Color measurements were taken directly on the top surface of intact biscuits. For each sample, measurements were performed at three distinct, non-overlapping locations. L^* , a^* , and b^* values were recorded in triplicate using different biscuits (Bakare *et al.*, 2020). Then Chroma (C^*) was calculated using the equation: $C^* = \sqrt{(a^{*2} + b^{*2})}$.

2.2.9. Preparation of sample extract

Exactly 1.5 mL of distilled water at 45°C was added with 0.2 g of the ground samples in a centrifuge tube while being swirled for five minutes. Samples were centrifuged (Gemmyco PLC-025, Taiwan) for 10 minutes at 4000 rpm after being rested for 30 minutes at 4°C. The supernatant was transferred to another fresh Falcon tube. The residue was reconstituted with 1 mL water at 40°C and centrifuged again under the same conditions. The resulting supernatant was combined with the first aqueous extract for each sample. These pooled extracts were used for total phenolic content and total antioxidant capacity analysis (Conforti and Patrignani, 2021).

2.2.10. Total Phenolic Content (TPC)

A stock solution containing 1 mg/mL of gallic acid was prepared in order to create the TPC standard and a range of working standard values, from 10 to 100 mg/mL were prepared for all four treatment biscuit extracts (Najjar *et al.*, 2022). Each biscuit sample had three extracts made, and each extract was made in triplicate. An aliquot of 300 µL of sample, each standard solution or blank (distilled water) were mixed with 1.2 mL of diluted Folin-Ciocalteu reagent. The test tubes were then left in the dark for five minutes at room temperature (RT). Each test tube was then filled with 1.5 mL of 7.5% Na_2CO_3 , and the tubes were left in the dark for an hour at RT. A UV-visible spectrophotometer (JENWAY 6305, United Kingdom) was used to measure

the absorbance at 765 nm, and the standard curve was used to calculate the unknown amounts. TPC was expressed as grams of gallic acid equivalents per gram of the sample (g GAE/g) (Ferradji *et al.*, 2024).

2.2.11. Testing for Total Antioxidant Capacity (TAC)

The standard for TAC was made by preparing a 1 mg/mL ascorbic stock solution. A series of working standard concentrations, 25 to 200 mg/mL, was prepared by dilution for all four treatment biscuit extracts (Batista *et al.*, 2021). Three extracts per sample were prepared, and for each extract, a triplicate was performed. Phosphomolybdenum reagent was prepared with 2.3% (w/v) Na₃PO₄, 0.392% (w/v) ammonium molybdate, and 8 mL of concentrated H₂SO₄. In each test tube 2.5 mL of the prepared reagent was added, along with 250 µL of the sample, the blank (distilled water) and the working standard solutions. For 90 minutes, the tubes were incubated at 95°C in the dark. A UV-visible spectrophotometer (JENWAY 6305, United Kingdom) was used to measure the absorbance at 695 nm, and the standard curve was used to calculate the unknown amounts. TAC was expressed as grams of ascorbic acid equivalents per gram of the sample (g AAE/g) (Korese *et al.*, 2021).

2.2.12. Data analysis

For statistical analysis, IBM SPSS Statistics Version 29 Windows (v29.0.2.0) was used. The parametric data were assessed using analysis of variance (ANOVA), and testing was conducted using one-way ANOVA. Mean significant differences of triplicate data were separated using Tukey's Honestly Significant Difference (HSD), where a *p*-value of less than 0.05 was considered statistically significant. Microsoft Excel (version 2506) was used to generate graphs, and values are presented as mean ± SD.

3. Results and discussions

3.1. The functional properties of different composite flour formulations

The functional characteristics of flour play a crucial role in determining the physicochemical behavior of dough during processing, the structural and sensory qualities of the final baked product. Replacing wheat flour with composite flours (T2, T3, and T4) resulted in significant changes in these functional properties as shown in Table 3. Attributes such as Water Absorption Capacity (WAC), Oil Absorption Capacity (OAC), Emulsion Stability (ES), Bulk Density (BD), Swelling Power (SP), Foaming Capacity (FC), and Water Solubility (WS) directly influence the texture, structure, and crispness of biscuits.

The developed T2 flour blend (85% wheat, 15% composite flour) showed a particularly advantageous hydration property balance in this study. Although it was statistically similar to wheat flour (T1), its Water Absorption Capacity (WAC) was significantly greater than that of T3 and T4 blends. This indicates that hydrophilic elements, such as the globulin proteins from pumpkin seeds and the soluble dietary fibers from Kithul flour, provide extra water binding sites at a 15% substitution level without significantly altering the natural starch-gluten matrix (Giannoutsos *et al.*, 2025). Significant reduction in WAC at higher substitution levels (T3 at 30% and T4 at 45%) suggests that once a threshold limit is exceeded, the increased concentration of insoluble fiber and non-gluten proteins impedes water absorption and starch swelling, while simultaneously weakening the gluten matrix responsible for retaining moisture (Li *et al.*, 2023). In contrast, the stable WAC observed in T2 likely supported better dough structure and handling, resulting in biscuits with less crumbly, more desirable texture according to sensory analysis. A significant increase in the Oil Absorption Capacity (OAC) was observed in all composite flour blends, with the highest value recorded in the T3 formulation. This increase is likely due to the dense and porous structure of jackfruit seed fiber, which physically traps oil, and the high levels of

hydrophobic amino acids (leucine and alanine) in pumpkin seed protein, which provide strong non-polar sites for binding fat molecules (Hasmadi *et al.*, 2020). As the proportion of composite flours increased the oil absorption capacity (OAC) increased. This could be due to differences in non-polar side chains in the proteins, which are responsible for binding oil through their hydrophobic nature. In a study by Aremu *et al.* (2007), on composite flours showed OAC values ranging from about 130% to 156%. They observed that higher OAC was linked to increased levels of legume and tuber flour. This was explained by the presence of more non-polar (hydrophobic) amino acids in proteins, which improve the ability of flour to bind oil. Based on this, the moderate OAC value of T2 (160.00), moderate oil absorption is often suitable for many baked items, because

very high OAC can make products too greasy and negatively affect texture. In addition, the T2 blend showed a significantly higher Emulsion Stability (ES), more than twice that of the control sample. This suggests that the balance of soluble fibers and amphiphilic proteins in T2 creates a viscoelastic, mechanically robust interfacial film at the oil-water interface. This film helps stop oil droplets from merging during the intense mixing of biscuit dough (Liu *et al.*, 2016; Qiu, Zhao & McClements, 2015). As a result, the fat is more evenly distributed throughout the dough. Also, T2 had the highest Bulk Density (BD) among all treatments, while T3 and T4 showed the lowest values. This suggests that the flours in T3 and T4 are more porous and less compact compared to refined wheat flour (T1) and T2.

Table 3. The mean functional scores for the four flour compositions.

Functional properties	Wheat flour	Composite flours		
	T1	T2	T3	T4
OAC %	137.33±21.33 ^a	160.00±10.00 ^{ac}	246.67±3.33 ^b	200.00±0.00 ^{bc}
WAC %	133.30±6.67 ^a	146.70 ^a ±3.33 ^a	56.67±3.33 ^b	67.35±17.35 ^b
EA %	46.03 ±1.59	49.35 ±5.96	43.95 ±0.36	44.06 ±0.68
ES %	38.00 ±1.15 ^a	91.96±4.39 ^b	79.3 ±0.67 ^c	74.8±0.47 ^c
BD (g/cm ³)	0.70±0.01 ^a	0.74±0.00 ^b	0.53±0.00 ^c	0.52±0.00 ^c
FC %	11.11 ±2.22 ^a	7.41 ±0.00 ^{ab}	7.41 ±0.00 ^{ab}	3.85 ±0.00 ^b
WS %	14.37±1.42 ^a	15.43 ± 2.36 ^{ab}	24.30 ±3.24 ^b	18.17± 0.98 ^{ab}
SP %	572.55±5.90 ^a	428.76±37.93 ^b	473.70±31.71 ^{ab}	411.56±32.96 ^b
D %	52.00±0.00 ^a	48.00±0.00 ^b	44.00 ±0.00 ^c	41.00±0.00 ^d
GT (°C)	60.00 ±0.00 ^a	68.00 ±0.00 ^a	75.00 ±0.00 ^a	84.00 ±0.00 ^a
LGC (g/ml)	10.00 ±0.00	12.00 ±0.00	14.00 ±0.00	18.00 ±0.00

Values are expressed as mean ± standard deviation (SD) of triplicate determinations. Means within the same row followed by different superscript letters are significantly different ($p < 0.05$). T1-100% wheat flour, T2 - 85% wheat flour + 5% each of other flours, T3 - 70% wheat flour + 10% each of other flours, T4 - 55% wheat flour + 15% each of other flours, OAC- Oil absorption capacity, WAC- Water absorption capacity, EA- Emulsion activity, ES- emulsion stability, BD- Bulk density, FC- Foaming capacity, WS- Water solubility, SP- Swelling power, D- Dispersibility, GT- Gelatinization temperature, LGC- Least gelation concentration.

A higher bulk density, which is often associated with better packing and finer particles, is desirable in food processing because it allows for more consistent and predictable dough yield (Jima, Abera, & Kuyu, 2025). The developed composite flours (T2 and T4) have lower SP indicating that their starch granules retain less water during heating compared to refined wheat flour. This is advantageous for biscuits, as it helps achieve a crisp texture with reduced gumminess (Devi, Sindhu, & Khatkar, 2018). The simultaneous rise in water solubility (WS), especially in T3, suggests higher amounts of other soluble components or soluble amylose. This may lead to greater spread of the dough during baking and could result in a firmer texture once cooled if the fat content is not properly balanced (Xiong *et al.*, 2023). Foaming Capacity (FC) decreased with increasing composite blends, T4 showing the lowest value (3.85%). According to Dusabumuremyi *et al.* (2025), a lower FC is beneficial for biscuit quality, as it indicates during mixing the composite flours are less likely to form big, unstable air bubbles. This allows the fat and chemical leavening agents to produce a uniform, fine, and tender crumb structure. Emulsion Stability (ES) was significantly higher in the composite flours compared to wheat flour. Although biscuits are not emulsion-based, increased ES illustrates the presence of surface-active compounds (as proteins from non-wheat ingredients). These components can help create a more stable dough, contribute to a more uniform texture, improve fat distribution, and potentially extend shelf life by decreasing the spread of moisture (Chandra, Singh, and Kumari, 2014).

Dispersibility and Emulsion Activity (EA) are crucial for dough handling and mixing. Dispersibility gradually decreased from T1 to T4, indicating that the composite flour does not easily form a uniform suspension in water. As a result, T3 and T4 require longer mixing times to develop a homogeneous dough. Among the developed composite flours, T2 remains the most advantageous in this instance. On the other hand, EA showed no significant differences between the treatments, suggesting that variations in dough handling are unlikely to be originated by this property (Nawaz *et al.*, 2021). Gelatinization Temperature (GT) and Least Gelation Concentration (LGC) affect the thermal behavior of flours during baking. GT in this study, increased considerably from T1 (60°C) to T4 (84°C), meaning that starch in the composite flours requires more heat to gelatinize and form the biscuit structure. This can delay the setting of the biscuit in the oven, allowing the dough to spread more and produce thinner biscuits, particularly in T3 and T4. Additionally, LGC increased from T1 (10%) to T4 (18%), which is favorable for biscuit quality, as greater LGC illustrates weaker gel-forming ability. As gelation leads to hardness, the elevated LGC from T2-T4 suggests that biscuits made from these composites are likely to be less hard and more tender compared to the 100% wheat control (Kristanti & Setiaboma, 2022). Among all treatments, T2 exhibited the best overall functional properties. It recorded the highest WAC, EA, ES, and BD, indicating superior water-binding ability, emulsification performance, and packing characteristics compared to the other treatments.

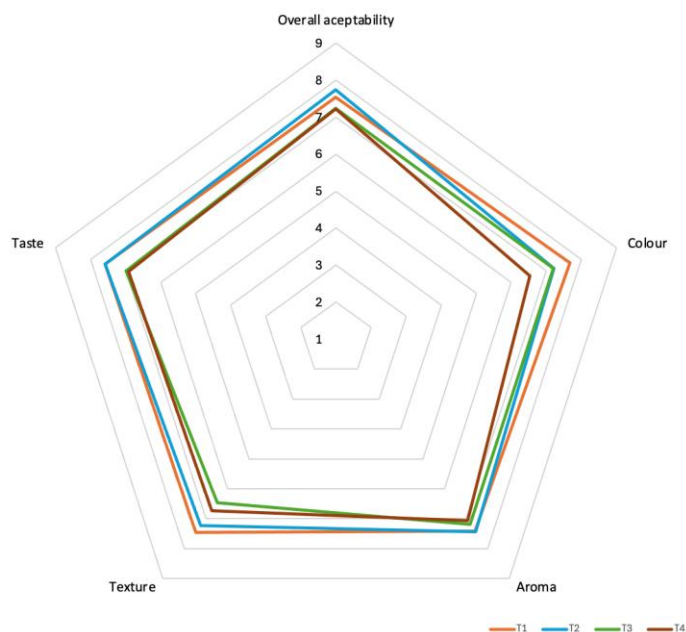


Figure 2. Radar chart of mean scores obtained for the sensory attributes of developed biscuits from composite flour (T1-100% wheat flour, T2 - 85% wheat flour + 5% each of other flours, T3 - 70% wheat flour + 10% each of other flours, T4 - 55% wheat flour + 15% each of other flours). Each attribute is rated on a scale from 1 to 9 (Scale: 9- Like extremely, 1- Dislike extremely). Values are presented as mean $\pm$ SD. The sensory data were statistically analysed using one-way ANOVA followed by Tukey's HSD test, with statistical significance considered at $p < 0.05$.

Table 4. Mean sensory scores of developed biscuits from composite flour.

Sensory attributes	T1	T2	T3	T4
Overall acceptability	7.54 $\pm$ 1.41 ^a	7.75 $\pm$ 1.14 ^a	7.25 $\pm$ 1.55 ^a	7.23 $\pm$ 1.33 ^a
Colour	7.67 $\pm$ 1.43 ^a	7.21 $\pm$ 1.41 ^{ab}	7.19 $\pm$ 1.33 ^{ab}	6.52 $\pm$ 1.60 ^b
Aroma	7.42 $\pm$ 1.35 ^a	7.44 $\pm$ 1.22 ^a	7.19 $\pm$ 1.51 ^a	7.06 $\pm$ 1.47 ^a
Texture	7.46 $\pm$ 1.90 ^a	7.23 $\pm$ 1.49 ^{ab}	6.46 $\pm$ 1.86 ^b	6.73 $\pm$ 1.85 ^{ab}
Taste	7.58 $\pm$ 1.53 ^a	7.58 $\pm$ 1.15 ^a	6.98 $\pm$ 1.51 ^a	6.92 $\pm$ 1.49 ^a

Values are expressed as mean $\pm$ SD (n=48). Means within the same row followed by different superscript letters are significantly different ($p < 0.05$). Sensory attributes were rated on a 9-point hedonic scale (Scale: 9- Like extremely, 1- Dislike extremely). T1-100% wheat flour, T2 - 85% wheat flour + 5% each of other flours, T3 - 70% wheat flour + 10% each of other flours, T4 - 55% wheat flour + 15% each of other flours.

3.2. Sensory Evaluation of Biscuits Formulated with Composite Flours

The sensory evaluation data (Figure 2, Table 4) reveals that moderate substitution with composite flours yielded biscuits that were identical from the wheat flour control in terms of consumer liking. For texture, the biscuit made from T3 (30% substitution) received a score (6.46) that was significantly lower ($p < 0.05$) than the T1 control (7.46). The texture scores for T2 and T4 were not statistically

different from T1. This result is likely due to the reduced WAC (56.67%) and SP (47.70%) of the T3 composite flour. However, T2 composite flour, with a WAC (146.70%) and SP (428.76%) closer to the control, avoided this textural defect. Similarly, for colour, only biscuits made from T4 flour (45% substitution) were rated significantly lower than those made from refined wheat flour (T1). This is likely due to altered Maillard browning at high levels of non-wheat components (Antonyrasan,

Pushparaja and Vasantharuba, 2025). However, T2 and T3, at lower substitution levels, avoided this significant colour rejection. For aroma and taste, no significant differences were found among any of the formulations. This indicates that the composite flours did not introduce off-flavors or detract from the pleasant taste profile at any substitution level. Overall, although the treatments showed relatively minor differences among the tested parameters, T2 obtained the highest overall acceptability score, therefore it was identified as most suitable formulation based on combined evaluation of sensory and functional analysis.

3.3. Proximate analysis

The proximate composition of the biscuit made from T2 (85% wheat, 15% composite flour) is mentioned in Table 5. The analysis reveals the product contain favorable macronutrient profile modulated by the inclusion of Kithul, Jackfruit seed, and Pumpkin seed flours. The moisture content of the biscuit was determined to be 3.11g in 100g

of biscuit (on a dry matter basis). This value is critically below the 5% threshold widely recommended for biscuits to ensure microbial stability, prevent rancidity, and maintain texture during storage (El-Sayed *et al.*, 2023). The crude fat content was determined to be 20g in 100g of biscuit. This substantial level is the combined result of two sources: butter added as a standard ingredient in the biscuit formula, and the intrinsic lipids contributed by the lipid-rich Jackfruit seed and Pumpkin seed flours. While the added fat increases energy density, the fat from the composite flours is important because it may contain unsaturated fatty acids such as oleic and linoleic acids which are predominant in these seeds (Prommaban *et al.*, 2021). Therefore, compared to a traditional biscuit made with wheat flour and the same amount of added shortening, the T2 biscuit delivers a higher total fat content with a potentially improved fatty acid composition, shifting the lipid profile towards more health beneficial unsaturated fats.

Table 5. Proximate analysis of biscuits developed from T2 composite flour (on dry matter basis).

Biscuits made from T2	
Moisture (g/ 100g)	3.11 ± 0.82
Crude Protein (g/ 100g)	7.85 ± 0.45
Crude Fat (g/ 100g)	20.00 ± 1.13
Ash (g/ 100g)	1.56 ± 0.62
Crude Fibre (g/ 100g)	0.61 ± 0.11
Carbohydrate (g/ 100g)	66.87 ± 14.01
Energy content (kcal/100 g)	478.88 ± 18.25

Composition of T2 - 85% wheat flour, 5% Jackfruit seed flour, 5% Kithul flour, 5% Pumpkin seed flour. Values are presented as mean ± SD. T2 was the selected formulation for further characterization; therefore, comparison with T1, T3, and T4 was not performed for these parameters.

The developed T2 biscuit had a crude protein content of 7.85%, which is lower than the exclusive wheat biscuit reported in other studies (11.70%) (Ramashia *et al.*, 2024). This low protein level may be due to the small amount of protein rich ingredients, and the composition of the formulation, which may naturally contain less protein than commercial biscuit. In comparison, some studies on biscuits made with composite flours often report higher protein levels (12–18%) because they include substantial amounts of protein-rich flours, such

as soybean or legume flours, which enhance both amino acid composition and overall protein content. These findings suggest that T2 biscuit provides some protein, and by increasing the proportion of protein-rich composite flours the content of protein could be increased to surpass commercial and experimental standards (Victor-Aduloju, 2024).

In terms of crude fiber, the T2 biscuit value of 0.61% is slightly higher than the 0.27% crude fiber found in the wheat biscuit reported by Ramashia *et al.* (2024). The

relatively low crude fiber observed can be due to the limited substitution levels of the composite flour, where wheat flour had the higher composition. This dilution effect may have reduced the overall fiber contribution from jackfruit seeds and pumpkin seed flours. However, composite flour biscuits in the literature that include high-fiber ingredients such as pulses, hulls, or mushroom typically exhibit greater crude fibre levels than both wheat-only and T2. For instance, biscuits produced from wheat supplemented with pulses such as African yam bean and tigernut showed crude fibre in the range of 3.96–7.43%, reflecting substantial increases due to fibre-rich legume contributions. These studies collectively indicate that although T2 crude fiber content modestly exceeds the commercial wheat biscuit, yet it remains lower than other composite formulations enriched with fibrous non-wheat flours, which tend to significantly increase total dietary fiber in the final product (Bello, Olufwamukomi and Enujiugha, 2017).

The carbohydrate content of the developed biscuit (66.87%) was slightly higher than the 65.46% reported for a commercial wheat biscuit in Ramashia *et al.* (2024), reflecting the high proportion of starch-rich ingredients typical of cereal-based bakery products. In studies on composite flour biscuits, carbohydrate values often vary depending on the nature and level of substitution; for example, biscuits from blends of wheat, soy okara and tigernut residue flours showed carbohydrate contents in the range of 56.9–59.2%, which were lower than both the commercial wheat and the current formulation, likely due to the higher protein and fat contributions of the non-wheat ingredients reducing the relative carbohydrate proportion. Overall, the carbohydrate content of the current biscuit is slightly higher than wheat biscuit but tends to exceed a few other composite biscuit formulations where substitution with non-starchy ingredients lowers carbohydrate proportion (Agu, Ihionu and Mba, 2023).

3.4. General microbial quality assessment

The aerobic bacterial count in the biscuits made from T2 flour increases from $0.3 \pm 0.2 \times 10^2$ CFU/g at the date of manufactured to $1.9 \pm 1 \times 10^2$ CFU/g at 7 days, indicating growth over time under ambient storage conditions ($27 \pm 2^\circ\text{C}$). The total aerobic bacterial count remained below 1×10^4 CFU/g, which is commonly accepted limits for biscuits in terms of general microbial quality (Koh *et al.*, 2023). However, since pathogen specific tests for *Salmonella*, *E. coli*, and *S. aureus* were not conducted, microbiological safety cannot be confirmed.

3.5. Texture analysis

The texture profile analysis (Table 6) explains the sensory panel's favorable perception of the T2 biscuit. The combination of moderate hardness, low chewiness and gumminess, and low cohesiveness is characteristic of a tender and crisp biscuit, which aligns with quality attributes reported for acceptable biscuit products. The hardness value of 46.7 N falls within the range 20 N and 80 N commonly reported for biscuits and composite-flour biscuits, where hardness is strongly influenced by formulation and moisture content (Chauhan *et al.*, 2016). Such moderate hardness indicates a product that is crisp yet easy to bite. This textural behavior can be attributed to the functional properties of the T2 flour blend, where balanced water absorption capacity and swelling power promote adequate starch gelatinization and protein hydration during baking, resulting in a continuous but non-dense matrix (Masri *et al.*, 2026). Similar observations were reported by Oyeyinka *et al.* (2018), who found that biscuits produced from composite flours exhibited improved crispness and desirable textural properties compared to control wheat biscuits. The low values for chewiness (8.55 N) and gumminess (8.57 N) confirm the product's non-chewy texture. Moreover, low cohesiveness (0.18) is an indicator of the biscuit crumbles easily rather than deforming plastically. The dilution of wheat gluten by the 15% substitution with Kithul, Jackfruit seed, and pumpkin seed flours

disrupts the formation of a strong, elastic gluten network. While this is detrimental in bread, it is highly desirable in biscuits, where a short, tender texture is preferred. This aligns

with the work of Gbenga *et al.* (2018), who noted that partial substitution with non-gluten flours significantly reduced dough elasticity and final product cohesiveness.

Table 6. Texture properties of the biscuits made from T2 composite flour

Texture attributes	Biscuits made from T2
Hardness (N)	46.7 ± 3.49
Chewiness (N)	8.55 ± 2.22
Gumminess (N)	8.57 ± 2.64
Cohesiveness	0.18 ± 0.04

Composition of T2 - 85% wheat flour, 5% Jackfruit seed flour, 5% Kithul flour, 5% Pumpkin seed flour. Values are presented as mean ± SD. T2 was the selected formulation for further characterization; therefore, comparison with T1, T3, and T4 was not performed for these parameters.

3.6. Colour analysis

The colorimetric analysis of the T2 biscuit (Table 7) reveals a product with a moderately dark, reddish-yellow hue, which aligns with expectations for baked goods containing Jackfruit seed, Kithul, and Pumpkin seed flours. The measured L* value of 53.4 ± 0.7 indicates a product of medium lightness. This is lower than values reported for biscuits made from 100% refined wheat flour, which typically exhibit L* values ranging from 60 to 75 (Bortlíková, Kolarič & Šimko, 2019; Jan *et al.*, 2018). This reduction in lightness is a consequence of incorporating non-wheat ingredients. For instance, studies enriching biscuits with jackfruit seed flour (Noor *et al.*,

2021) and pumpkin seed meal (Mbaeyi-Nwaoha & Umeh, 2016) have reported similar decreases in L* values, attributable to intrinsic pigments like carotenoids and higher fiber content that reduce light reflectance. Moreover, the a* value of 8.70 ± 0.3 exhibits a pronounced red tone. This is notably higher than the a* values of 2.0 to 6.0 commonly reported for wheat biscuits (Bortlíková *et al.*, 2019). The increase in redness can be attributed due to an altered Maillard reaction during baking. Similarly, the b* value of 23.5 ± 0.9 reflects a strong yellow component. While this falls within the typical range for baked goods (20-30) (Pathare, Opara and Al-Said, 2013).

Table 7. Colorimetric properties of the biscuits made from T2 composite flour

Colour parameter	Biscuits made from T2	Description
L*	53.4 ± 0.7	0 - Black, 100 - White
a*	8.70 ± 0.3	+a* - Red, -a* - Green
b*	23.5 ± 0.9	+b* = Yellow, -b* = Blue
Chroma (C*)	25.1 ± 1.0	Color Saturation

Composition of T2 - 85% wheat flour, 5% Jackfruit seed flour, 5% Kithul flour, 5% Pumpkin seed flour. Values are presented as mean ± SD. T2 was the selected formulation for further characterization; therefore, comparison with T1, T3, and T4 was not performed for these parameters.

3.4. Antioxidant properties of biscuits made from composite flours

The analysis of TPC and TAC (Table 8) shows that incorporating Jackfruit seed, Kithul, and Pumpkin seed flours significantly enhances

the bioactive profile of biscuits. A significant ($p < 0.05$), and progressive increase in both TPC and TAC was observed with increasing levels of composite flour substitution. The TPC rose from 0.95 g GAE/100g in T1 to 2.25 g

GAE/100g in T4, representing a 137% increase at the highest substitution level. Similarly, TAC increased from 2.97 g AAE/100g in T1 to 4.14 g AAE/100g in T4, a 39% increase. This confirms that Jackfruit seed, Kithul, and Pumpkin seed flours are rich sources of phenolic compounds and other antioxidants, which were successfully transferred to the final baked product. These findings align with the

literature on the high antioxidant potential of these underutilized ingredients. For instance, jackfruit seeds are known for their gallic acid and ellagic acid content, while pumpkin seeds contain phenolic acids and flavonoids, all of which contribute to the measured TPC and TAC (Peng *et al.*, 2021, Rodríguez *et al.*, 2021).

Table 8. Comparison of Total Phenolic Content (TPC) and Total Antioxidant Capacity (TAC) in biscuits made from different composite flours.

Type of flour use to make biscuits	TPC (g GAE/100g)	TAC (g AAE/100g)
T1	0.95 ± 0.01 ^a	2.97 ± 0.01 ^a
T2	1.42 ± 0.11 ^b	3.38 ± 0.03 ^{ac}
T3	1.95 ± 0.05 ^c	3.74 ± 0.04 ^{bc}
T4	2.25 ± 0.06 ^c	4.14 ± 0.00 ^b

Values are expressed as mean ± SD. Means within the same column followed by different superscript letters are significantly different ($p < 0.05$). T1-100% wheat flour, T2 - 85% wheat flour + 5% each of other flours, T3 - 70% wheat flour + 10% each of other flours, T4 - 55% wheat flour + 15% each of other flours.

4. Conclusion

This study successfully developed, characterized, and optimized nutritionally enhanced biscuit through the strategic substitution of wheat flour with a composite of Jackfruit seed, Kithul, and Pumpkin seed flours. All four formulations were initially evaluated and compared based on their functional and sensory properties. The investigation revealed that the functional properties of the flours; specially increased oil absorption, emulsion stability, and altered hydration dynamics directly affect the physicochemical and sensory quality of T2. Sensorially, T2 (15% total substitution) was statistically equivalent to the 100% wheat biscuit across all key attributes, avoiding the significant textural and color defects observed at higher substitution levels (T3, T4). Based on these results, T2 was identified as the most acceptable formulation and selected for further detailed characterization including proximate composition and microbial analysis. This

approach is commonly used in product development where comprehensive analyses are done on the optimized formulation. The incorporation of the novel ingredients significantly improved the TPC, TAC. This formulation successfully transforms a conventional biscuit into a product with an improved nutritional profile. It provides a model for enhancing the nutritional quality of staple foods without compromising the sensory characteristics essential for consumer acceptance and market success. The absence of full compositional analysis and extended microbial shelf-life assessment for all formulations represents a limitation of the present study. Future research should address this limitation by conducting these analyses across all formulations, thereby enabling a more comprehensive comparison of their nutritional and storage characteristics.

5. References

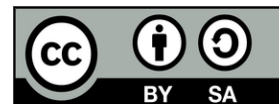
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Research Article

AI-LONG: DETECTION OF FOOD SPOILAGE IN COMMON CARINDERIA DISHES USING AN ELECTRONIC NOSE SYSTEM BASED ON SUPPORT VECTOR MACHINE

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Abstract

Food safety remains a global concern, particularly because conventional approaches, such as visual inspection and sensory evaluation, have limitations in detecting food spoilage and preventing foodborne illnesses. Recent advances in sensing technology, including the E-Knows electronic-nose system, provide promising tools for real-time food-quality monitoring. This study integrated E-Knows olfactory sensors, gas sensors, pH measurements, and temperature monitoring to detect spoilage in selected foods served by PUP Lagoon concessionaires. Samples comprised coconut-milk-based, vinegar-based, and pasta dishes. Sensor responses to spoilage-related gases, including methane, hydrogen, carbon dioxide, hydrogen sulfide, and ammonia, were classified using a Support Vector Machine (SVM) model. The E-Knows system demonstrated high classification accuracy and complemented pH and temperature measurements, thereby strengthening the overall assessment of food quality. These findings may support the development of a food safety management system for PUP Lagoon food stalls and contribute to improved monitoring and food-handling practices among vendors.

1.Introduction

Foodborne diseases and spoilage are significant global health concerns, both contributing to public health risks and economic losses. According to a study, from January 2005 to September 2021 in the Philippines, there were 63 foodborne disease outbreaks (FBDOs)

affecting school-aged learners and teachers, resulting in 4,354 hospitalizations, 635 instances of home medication, and 38 deaths (Limon et al., 2022). Food spoilage, primarily caused by microbial activity, leads to changes in the appearance, taste, or smell of food. However,

not all contaminants exhibit visible signs, which makes early detection of spoilage particularly challenging (Deng et al., 2023). Effective food safety and management practices are essential in minimizing foodborne diseases and spoilage. Such practices also contribute to sustainable development by supporting global sustainability goals (SDGs 12 and 13), reducing food waste, and offering both economic and environmental benefits.

The limitations in traditional sensory evaluations have driven the development of advanced detection systems such as electronic noses (e-noses). These systems, integrated with machine learning algorithms such as Support Vector Machine (SVM), can detect food spoilage with 78.33% accuracy by monitoring gases released during microbial activity, such as hydrogen sulfide (H_2S) and ammonia (NH_3) (Yuan et al., 2020; Saini et al., 2021). This study aims to validate the results and readings of an e-nose system by analyzing a range of foods available at the Polytechnic University of the Philippines (PUP) Lagoon, including items with varying ingredients, such as pasta, vinegar-based dishes, non-vinegar dishes, and foods prepared with coconut milk (gata).

The general objective is to integrate the e-nose as a tool for assessing the safety and quality of foods sold at the Polytechnic University of the Philippines Lagoon, ultimately contributing to the development of university food safety guidelines informed by the findings of this study.

A. Food Spoilage

Food spoilage is a biochemical reaction caused by microorganisms like bacteria and fungi, which alter food's color, texture, taste, and odor. The primary factors contributing to deterioration of food quality are microbiological and chemical changes, such as protein modifications and lipid separation. Bacteria, temperature, relative humidity, and storage conditions can significantly affect the freshness of food in containers or refrigerators. While the underlying mechanisms remain unclear, sensory assessment is the primary method for spoilage detection, with advanced techniques measuring

even low contaminant concentrations (USDA, 2024).

1. Public Health Risks Associated with Food Spoilage

Food spoilage poses important public health issues, primarily due to the proliferation of harmful microorganisms that may grow when food is improperly stored or handled. Spoiled food could contain hazardous bacteria at "Danger Zone" temperatures (40°F to 140°F) where bacteria can multiply every 20 minutes and generate toxins that cause foodborne diseases (Preethichandra et al., 2022).

2. Polytechnic University of the Philippines Lagoon

The Polytechnic University of the Philippines (PUP) Lagoon, a man-made feature on campus, functions as a central location for student dining and socialization. Several food concessionaires operate within this area, offering a diverse selection of food items, such as rice meals, street foods, sandwiches, and beverages. The PUP Lagoon Food Concessionaires' operating hours, generally from 8:00 AM to 6:00 PM, align with university class schedules, allowing students easy access to affordable food options throughout the academic day.

B. Role of Gases in Food Spoilage

Gases play an important role in food spoilage, with microorganisms producing harmful gases such as hydrogen sulfide (H_2S), carbon monoxide (CO), ammonia (NH_3), methane (CH_4), and volatile organic compounds (VOCs). H_2S is released by bacteria such as *Desulfovibrio* during protein breakdown (Djenane & Roncales, 2018). On the other hand, CO is produced by clostridium species in anaerobic conditions [8], while Ammonia and VOCs, including aldehydes, contribute to the foul odors and degradation associated with spoiled food (Saini et al., 2021; Sinharoy et al., 2019).

1.1. Aldehydes

Aldehydes are organic compounds that are often found in the air and in foods. When present in excessive amounts, they contribute to undesirable smells and flavors. These

substances are related with lactic acid bacteria, *Saccharomyces*, and enterobacteria, which contributes to food spoiling (El-Maghrabey et al., 2021). Moreover, aldehydes can increase lipid oxidation, resulting in rancidity and off-flavors in food (Jung et al., 2021). It also causes color changes in food. Acetaldehyde, benzaldehyde, and hexanal are the most frequent aldehydes associated with food spoilage. With which Acetaldehyde is a highly reactive and hazardous compound found in fruits and vegetables that causes cellular and chromosomal damages (NCBI, 2024). While Benzaldehyde, a clear to yellow liquid found naturally in almonds and cherries, may produce an intolerable bitter taste when consumed excessively (Zhu et al., 2022).

1.2.Ketones

Ketones, along with other volatile organic compounds (VOCs) such as alcohols, esters, and sulfides, are common in spoiled foods and are linked to microbial spoilage metabolism (Yin et al., 2023). Bacteria break down food components, producing ketones and other byproducts, which impact food quality by altering taste, smell, and acceptability. Studies show that spoilage in apples results in VOC emissions, including ketones, detectable through gas sensors and chemometrics (Cauchie et al., 2020), and similar findings are observed in minced pork to assess decomposition and shelf life (Zhou et al., 2022). Understanding ketones' role in spoilage is crucial for improving food preservation and safety.

1.3.Alcohol and Ester

Alcohol is produced during the spoilage of various foods, including fruits and meats, with ethanol content in grapes increasing over time as a spoilage indicator (Parlapani et al., 2023). Bacteria, such as *Pseudomonas* species in spoiled fish, produce alcohols and other VOCs, correlating with advanced spoilage (Cui et al., 2022). These compounds contribute to off-flavors, odors, and health risks due to pathogenic microorganisms. Alcohol emissions are also common in food waste (Sourri et al., 2022), while ester production during microbial

fermentation further deteriorates food quality (karanth et al., 2023). Environmental factors, such as temperature, pH, and acidity, influence microbial metabolism, exacerbating spoilage (Marconkowska & Jeleń, 2022).

1.4.Sulfur Compounds

Volatile sulfur compounds are crucial in determining the odor and flavor of food. Marcinkowska and Jeleń highlight that these compounds are often responsible for specific food aromas (Chevron Phillips Chemical, n.d.). For instance, dimethyl sulfide, a methyl sulfide naturally produced by marine algae, is associated with cooked vegetables and certain seafood, resulting from the breakdown of sulfur-containing amino acids (Deng et al., 2023). Another sulfur compound, hydrogen sulfide, is produced by bacteria in anaerobic environments (Awosika & Connolly, 2023). Additionally, mercaptans, sulfur-containing compounds known for their strong and unpleasant odor, are primarily used as odorants in natural and fuel gases. These compounds can be produced by both spoilage and beneficial microorganisms (Utah State University, 2023).

C. Role of Humidity in Food Spoilage

The study of Utah State University (2023), suggested that the humidity in the storage environments such as food stalls should be at an exact or enough percentage (%), notably 50%-60% to keep the food safe (URS Testing Laboratory, 2024). Based on the study of URS Testing Laboratory (2024), Humidity is known as the silent spoiler because high humidity in soup based food and dried foods can lead to mold growth that can increase the activity of microbial organisms which leads to food spoilage (Chang et al., 2022). On the other hand, low humidity can cause the food to dry out, losing its taste and texture.

D. Role of Temperature in Food Spoilage

Temperature plays an important role in metabolic rates and growth of food-spoiling microorganisms. The chance of these microorganisms to survive are practically zero below freezing, however, the chance rises along with the temperature, as the chemical reactions that keep living things alive occur more slowly

in the cold (Bastiaan Molleman, et al., 2022). Taking into account that microorganisms require time to multiply, when small amounts of microorganisms are present, the risk is usually low, but extended time under the right conditions will enable the microorganisms to multiply, thus increasing the chance of contamination.

E. Gas Readings

The gas sensors employed in this study are designed to detect various gases, including ammonia (NH₃), hydrogen sulfide (H₂S), carbon dioxide (CO₂), methane (CH₄), and volatile organic compounds (VOCs) at specific concentrations, measured in parts per million (ppm). Ammonia (NH₃) at 4.5% gas concentration has a 0.1 to 1 ppm value in room temperature and 5 ppm at 175°C, whereas at 20% of the gas concentration has a value of 0.2 ppm or higher (Ricci & Gregory, 2021; Shen et al., 2020; Hingangavkar et al., 2023). The hydrogen sulfide (H₂S) at 0.51% gas concentration yields a 1 ppm measurement at room temperature, while at 39.19% it yields a 100 ppm reading (Shwetha et al., 2022). Carbon dioxide (CO₂) has a reading of 552 to 4751 ppm at gas concentrations less than 5%, while methane (CH₄) has a reading of 300 ppm at low concentrations and more than 300 ppm at high concentrations (Sandhya et al., 2023; Pranav et al., 2021).

2. Materials and methods

2.1. Raw Materials

2.1.1. Device Calibration

The electronic nose (e-nose) system consisted of five gas sensors: MQ-5, MQ-8, MQ-135, MQ-136, and MQ-137. These sensors were used to detect changes in volatile compounds potentially associated with food spoilage, including methane, hydrogen, carbon dioxide and other air-quality-related gases, hydrogen sulfide, and ammonia. Before data collection, the sensors were allowed to warm up and were calibrated under controlled conditions using known gas concentrations or reference values. Calibration was performed to establish the baseline response of each sensor and

improve the consistency of measurements during the experiment.

2.2. Sample Preparation

The study evaluated six common carindaria dishes grouped according to their major ingredients or preparation methods:

- Coconut milk (gata) dishes: Bicol Express and Ginataang Kalabasa
- Vinegar-based dishes: Paksiw and Pork Adobo
- Noodle dishes: Palabok and Pancit Canton with Bihon

One prepared batch of each dish was obtained from the PUP Lagoon Food Concessionaires. One sample unit was collected from each batch and placed in a designated container for e-nose monitoring. Therefore, the experiment included six food samples representing six independently prepared dishes.

Each sample was monitored once every hour for six hours, from 11:00 AM to 4:00 PM. This produced six time-point readings per food sample. With six dishes and six readings per dish, the dataset consisted of:

$$6 \text{ food samples} \times 6 \text{ hourly readings} = 36 \text{ total observations} \quad (1)$$

Each observation contained readings from the five gas sensors, together with the corresponding pH and temperature measurements. Thus, the dataset contained 180 individual gas-sensor values:

$$36 \text{ observations} \times 5 \text{ gas sensors} = 180 \text{ sensor values} \quad (2)$$

No technical or biological replicates were conducted; therefore, each hourly measurement represented one observation for each dish.

2.3. Data Collection

Data collection was conducted over a six-hour period corresponding to the operating hours of the PUP Lagoon Food Concessionaires. Measurements were obtained at six hourly time

points: 11:00 AM, 12:00 PM, 1:00 PM, 2:00 PM, 3:00 PM, and 4:00 PM.

At every time point, the e-nose system recorded the responses of the MQ-5, MQ-8, MQ-135, MQ-136, and MQ-137 sensors for each of the six food samples. A pH meter equipped with a temperature probe was also used to measure the pH and temperature of each sample. Accordingly, six observations were obtained per dish, resulting in 36 total observations.

The hourly measurements were used to monitor changes in sensor responses, pH, and temperature throughout the observation period under the environmental conditions encountered at the concessionaire site.

2.4. Data Analysis

The collected data were organized so that each observation represented one food sample at one hourly time point. The input variables for the classification model included the responses of the five gas sensors, while pH, temperature, observation time, and food type were recorded as supporting variables.

Before model development, the sensor data were checked for incomplete or invalid measurements and normalized to minimize differences in scale among the sensors. A Support Vector Machine (SVM) algorithm was then used to classify the spoilage condition of the food samples.

The dataset consisted of 36 observations derived from six dishes measured at six hourly time points. The procedure used to divide the observations into training and testing datasets was documented to ensure that model performance could be reproduced. The resulting SVM classification was compared with the reference spoilage classifications established through pH measurements, temperature measurements, sensory evaluation, and visual inspection. Relationships between sensor responses, pH, and temperature were also examined. The SVM model achieved an overall classification accuracy of 78.33%

2.5. Validation

The classification performance of the SVM model was evaluated using accuracy, precision, recall, and F1 score. A confusion matrix was also generated to show the number of correctly and incorrectly classified observations for each spoilage category.

The classifications generated by the e-nose system were compared with the reference assessments obtained through pH and temperature measurements, sensory evaluation, and visual inspection. In addition to classification performance, the operational quality of the developed system was evaluated based on selected ISO/IEC 25010 characteristics, including reliability, portability, and maintainability.

Because the dataset contained only 36 observations from one batch per dish without independent replicates, the reported accuracy should be interpreted as preliminary. Additional batches and independent replicates are recommended to evaluate the model's generalizability and reliability under varying preparation and storage conditions.

3. Results and Discussion

3.1. Device and Tools

The instruments used in the study were the AI-Long electronic nose (e-nose) prototype and a digital pH meter equipped with a temperature sensor.

The AI-Long device is an e-nose system designed to detect volatile compounds associated with food spoilage. It integrates multiple gas sensors with a Support Vector Machine (SVM) algorithm to classify the spoilage levels of the selected food samples. The prototype was originally developed during the initial phase of the AI-Long research project and was subsequently used in the present study for data collection and spoilage classification.



Figure 1. Actual Device Prototype

A digital pH meter with an integrated temperature sensor was also used to measure the pH and temperature of each food sample. The pH measurements provided information on changes in acidity or alkalinity that may occur during spoilage, while the temperature measurements were used to monitor the thermal condition of the samples during the observation period. Because pH readings can be affected by temperature, the integrated temperature sensor supported more accurate interpretation and, where applicable, temperature compensation of the pH measurements. Both pH and temperature values were recorded alongside the e-nose sensor readings at each hourly sampling point.

3.2. Data Set

The dataset was collected using the E-Knows electronic nose system, which consists of an array of five MQ gas sensors: MQ-5, MQ-8, MQ-135, MQ-136, and MQ-137. A digital pH meter with an integrated temperature sensor was also used to record the pH and temperature of each food sample. The E-Knows device was programmed to collect gas-sensor readings at two-hour intervals.

Six dishes commonly served in carinderias were included in the study. These were grouped according to their primary characteristics:

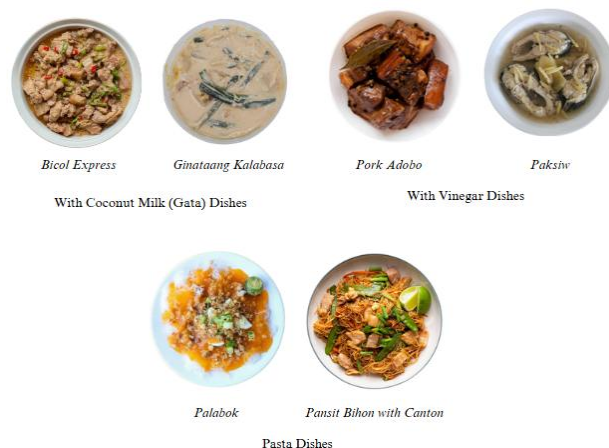


Figure 2. Data Set for Food Testing

At each testing interval, the readings from the five gas sensors were recorded together with the corresponding pH and temperature measurements. These measurements were used as input variables for analyzing changes associated with food spoilage.

Paksiw and Pork Adobo were classified as vinegar-based dishes because vinegar is a major ingredient in their preparation. The E-Knows device was used to evaluate the condition of the samples by detecting changes in the volatile compounds they emitted. The readings from the MQ gas-sensor array were processed using a Support Vector Machine (SVM) algorithm, which classified the samples according to their spoilage condition.

For the Paksiw sample, Tests 1–3 were classified as “good,” whereas Tests 4–6 were classified as “spoiled.” These findings indicate that the pattern of volatile compounds changed as the observation period progressed.

Although pH can provide useful information about food quality, it should not be treated as a definitive indicator of spoilage. Paksiw and Pork Adobo are naturally acidic because they contain vinegar. Their initially low pH values may therefore reflect the dishes’ ingredients rather than their spoilage condition. Moreover, acidity may inhibit some microorganisms without preventing all forms of microbial growth or chemical deterioration. Consequently, pH measurements alone may not

reliably distinguish good samples from spoiled ones in vinegar-based dishes.

The E-Knows system provided an additional basis for assessment by analyzing the combined responses of the gas sensors. However, its results should be described as complementary to the pH and temperature measurements rather than inherently more accurate unless this is demonstrated through comparison with an established reference method, such as microbial analysis.

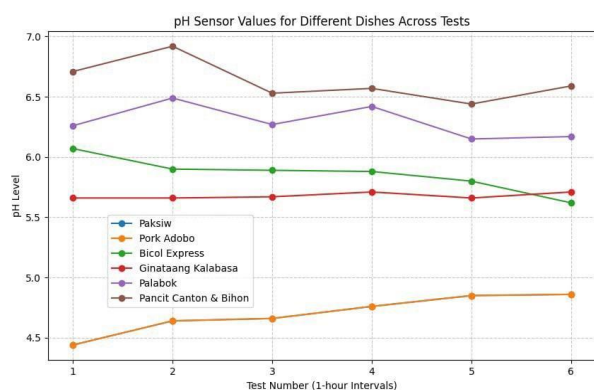


Figure 3. pH Meter Result

The samples were maintained at approximately 30–31 °C during testing. This temperature should not be described as a safe storage range because it falls within the temperature danger zone in which many foodborne microorganisms can multiply rapidly. Prolonged exposure to these conditions may therefore accelerate microbial activity and food deterioration.

The two-hour guideline provides a practical food-safety reference for potentially hazardous foods kept at room temperature. Nevertheless, the exact rate of spoilage may differ among dishes because of variations in ingredients, moisture, acidity, preparation methods, initial microbial load, and environmental exposure. Temperature measurements were therefore used to document the samples' storage conditions and support the interpretation of the e-nose and pH results.

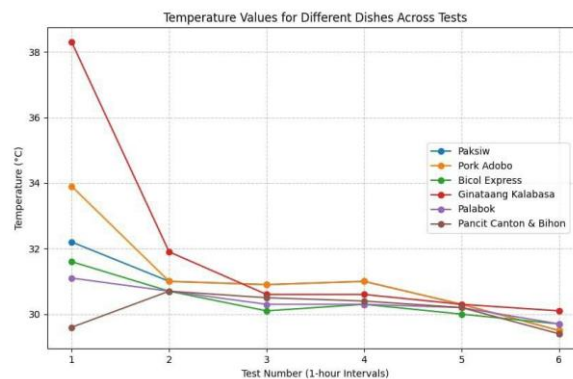


Figure 4. Temperature Result

The relationships among pH, temperature, and the E-Knows classifications were not consistent across all dishes. This result suggests that food spoilage cannot be reliably evaluated using a single indicator. Instead, the combined effects of storage time, temperature, acidity, ingredients, and volatile-compound production should be considered.

For Bicol Express, the pH remained within the expected range during some testing intervals even though the E-Knows system classified the sample as spoiled. This disagreement suggests that changes in volatile compounds may occur without a substantial change in pH. The dish's ingredients and the specific microorganisms or chemical reactions involved in deterioration may also have influenced the results.

For Ginataang Kalabasa, changes in pH occurred alongside classifications of spoilage by the E-Knows system. Because the sample was also exposed to approximately 30–31 °C, temperature and storage duration may have contributed to its deterioration. However, without microbial testing or another reference method, the individual effects of pH, temperature, and time cannot be conclusively determined.

For Palabok and Pancit Canton with Bihon, the E-Knows system identified changes associated with spoilage. These classifications were accompanied by increased responses from several gas sensors, particularly MQ-5, MQ-8, and MQ-135. This finding suggests that these sensors responded to changes in the mixtures of volatile compounds emitted as the noodle dishes deteriorated. However, the individual

compounds responsible for the responses were not identified.

Temperature and testing duration should also be considered when interpreting these findings. Because the samples were exposed to room temperatures of approximately 30–31 °C and tested at two-hour intervals, elapsed time may have influenced both volatile-compound production and the resulting SVM classifications. The findings demonstrate the potential of an MQ sensor-based electronic nose for supporting food-quality assessment. Further research involving larger datasets, independent sample replicates, microbial analysis, sensor calibration, and controlled storage conditions is needed to determine the system's sensitivity, specificity, and reliability.

3.3. Device Performance

The temperature sensor and pH meter were placed on the same device. In the food testing, foods with vinegar such as paksiw and pork adobo showed a slight increase in their pH over the time of testing. The food with coconut milk such as bicol express and ginataang kalabasa developed a slightly sour odor and discoloration over the testing period. The food with pasta such as palabok and pancit canton with bihon showed an increased pH and developed an off odor over the testing period. This result from pH meters was also caused by the temperature. Temperature can alter the reactions and build up microorganisms inside the food.

In correlation of the pH meters and temperature to the MQ gas sensors (MQ-5, MQ-8, MQ-135, MQ-136, and MQ-137) result. Gas sensors detect the emitted gasses that the spoiled food releases. Temperature with a high result can accelerate microbial growth, leading to increased VOC production and faster food spoilage. In general, higher temperatures can lead to higher gas sensor readings, as increased microbial activity that alters the pH results in greater VOC emission.

Fig. 5-10 indicates the result for gas sensors (MQ-5, MQ-8, MQ-135, MQ-136, and MQ-137). This graph shows that the first 3 testing commonly resulted to a good food quality and

the last 3 (4-6) testing commonly result to a spoiled food quality.

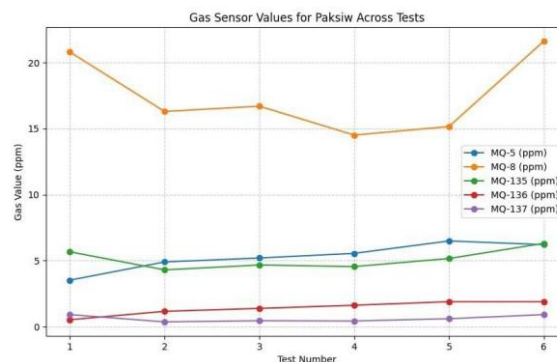


Figure 5. Gas Sensor Values Paksiw

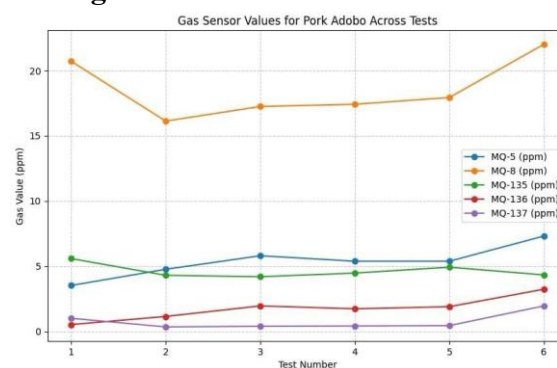


Figure 6. Gas Sensor Values Pork Adobo

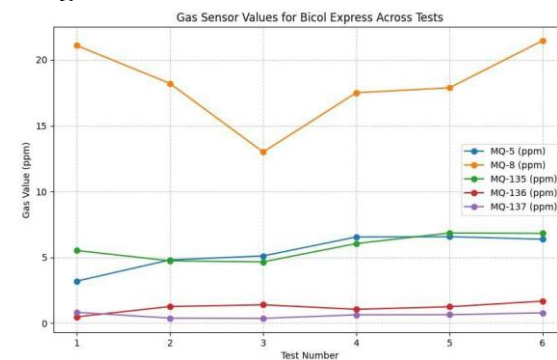


Figure 7. Gas Sensor Values Bicol Express

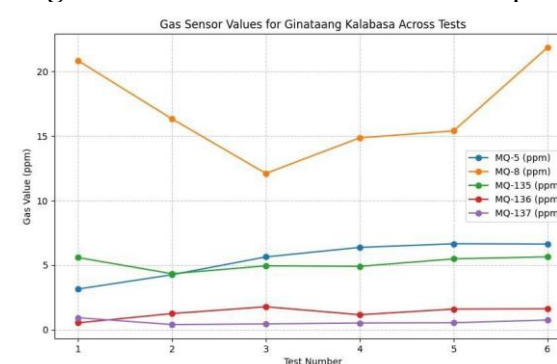


Figure 8. Gas Sensor Values Ginataang Kalabasa

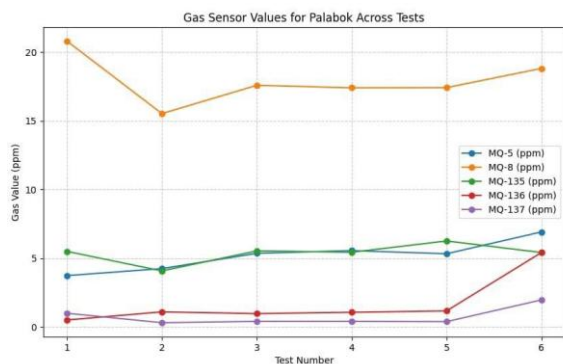


Figure 9. Gas Sensor Values Palabok

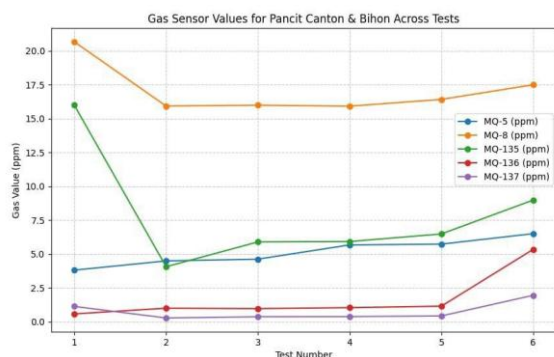


Figure 10. Gas Sensor Values Pancit Canton & Bihon

4. Conclusions

In conclusion, this study demonstrates the potential of integrating electronic-nose technology with pH and temperature measurements to assess food spoilage. The E-Knows sensor array provided useful information on changes in the volatile compounds emitted by different food categories, including vinegar-based dishes, coconut-milk-based dishes, and pasta dishes. Its results suggest that olfactory sensor data can complement conventional measurements when evaluating food quality.

The pH measurements were less indicative of spoilage in acidic foods such as Paksiw and Pork Adobo because their vinegar content maintained an acidic environment that may have obscured spoilage-related changes. In contrast, variations in the pH of Ginataang Kalabasa showed a clearer association with the spoilage classifications generated by the system. These findings indicate that pH should not be used as the sole basis for determining food quality.

Temperature was also an important factor. Samples stored at approximately 30–31 °C were exposed to conditions favorable to microbial

growth and food deterioration. Longer exposure at these temperatures was generally associated with changes in pH, increased gas-sensor responses, and spoilage classifications from the E-Knows system. This finding emphasizes the importance of proper temperature control and limited holding times in reducing food-safety risks.

The MQ-5, MQ-8, MQ-135, MQ-136, and MQ-137 sensors successfully detected changes in gas-response patterns associated with the volatile compounds released during food deterioration. When these responses were evaluated together with pH, temperature, and storage duration, the system provided a more comprehensive assessment than any single measurement alone.

Overall, the findings indicate that the AI-Long prototype, incorporating the E-Knows electronic-nose system, has potential as a supplementary component of a food-safety monitoring system for the food stalls at the PUP Lagoon. However, further testing using a larger and more diverse dataset is necessary. Future studies should compare the system's classifications with microbiological and chemical laboratory analyses, calibrate the sensors under controlled conditions, and evaluate the prototype in actual food-service environments before it is used for operational food-safety decisions.

Recommendation

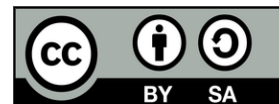
While this study showed a promising result, based on the findings, the following recommendations are proposed:

1. Conduct study on the microbial growth of carindaria dishes counterpart to further enhance the concept of AI-Long
2. Consider combining the AI-Long with other sensors such as colorimeter and moisture level of the food to create a more comprehensive and reliable food quality monitoring system.

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Research Article

IMPACT OF SELECTED INDUSTRIAL AND LABORATORY PROCESSING TECHNIQUES ON THE PHYTIC ACID CONTENT IN BARLEY FLOUR: A COMPARATIVE APPROACH

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Megazyme assay kit.

Abstract

Barley is mainly utilized in brewing, and for processing breakfast cereals and fermented bakery products. Phytic acid (PA) is the major storage form of phosphorus in barley. It is considered an antinutrient because it chelates polyvalent mineral cations, hereby reducing their bioavailability in humans and animals. Therefore, the main aim of the study was to apply malting and roasting as treatments in order to understand enzymatic and thermal effects on reducing PA level. Four industrially processed barley flour types—whole or raw barley (RB), malted barley (MB), and malted barley subjected to both mild (MB-l) and intense (MB-i) roasting—along with two raw flours subjected to mild (R-l) and intense (R-i) roasting in the laboratory, were analysed in quadruplicates for their PA contents using the Megazyme® phytic acid assay kit. Data were analysed by one way ANOVA and Tukey HSD tests using SPSS. The PA content of whole raw barley was 0.86 ± 0.03 g/100 g d.b. All malted flours—MB, MB-l, MB-i—contained significantly less ($P < 0.05$) PA than raw flour but treated flours did not differ significantly from one another ($P > 0.05$). Similarly, roasted barley in the laboratory—R-l and R-i—contained significantly ($P < 0.05$) less PA compared to RB. The free phosphorus content of industrial barley flour increased as follows ($P < 0.05$): RB < MB < MB-l < MB-i. Similarly, among the roasted flours, R-i contained the highest free phosphorus level. Endogenous phytase activity during malting was likely the primary factor responsible for phytate degradation. Additionally, it can be suggested that the reduction of PA during roasting in the laboratory, was due to thermal degradation of phytic acid. Among all treatments, intense roasting of raw barley decreased phytic acid content by 32.56% suggesting that as the most promising strategy for reducing PA levels in barley-based food ingredients.

1.Introduction

Phytic acid (PA), which is scientifically abbreviated as InsP6 or IP6, is chemically defined as *myo*-inositol-1,2,3,4,5,6-

hexakisphosphate (Chen and Xu, 2023; Marolt and Kolar, 2021). According to its molecular formula ($C_6H_{18}O_{24}P_6$), there is a *myo*-inositol ring that is bounded with six phosphate groups

(Bloot et al., 2023; Doung et al., 2017). This unique chemical structure allows phytic acid (PA) to chelate with mineral cations forming stable complexes at physiological pH levels (Chen and Xu, 2023). This chelation depicts antinutrient effects as it resulted in altering the solubility and digestibility of some essential minerals contained in food such as iron and zinc, ultimately obstructing their absorption to body (Farag, Xiao and Abdallah, 2022). Apart from minerals, PA was found to bind with amino acids either via direct electrostatic interactions or through indirect bridging function of multivalent cations such as zinc, iron and calcium (Chen and Xu, 2023). Moreover, the presence of PA in higher concentrations negatively affected the digestion and absorption of starch since the formation of interactions among phosphate bonds in PAs and digestive enzymes (salivary and pancreatic amylases) (Kumar et al., 2022).

Barley has been ranked as the fourth mostly produced cereal crop in the world while it is prominently utilized in brewery and animal feed sectors (Van de Velde et al., 2025). Beyond that, fermented bakery products and breakfast cereals are produced from barley malt extracts and syrups (Arif et al., 2011). As a recent growing trend, barley flour is used to partially or completely substitute wheat flour in unleavened bakery products (Lukinac and Jukic, 2022). PA content of some widely consumed cereals such as rice, oat, barley and rye was in the range 4.5-7.5 mg g⁻¹, which was lower compared to that of in pseudocereals such as millet, sorghum and corn with a PA range of 7.5-10 mg g⁻¹ (Wu et al., 2009). When concerned about whole barley grains, more than 80% of PA is available in the aleurone layer, a cellular layer located between endosperm and hull, and PAs mostly exist in association with protein globoids (Dai et al., 2007; Mayer et al., 2023).

Both wet and dry processing techniques can be applied to reduce naturally occurring PA levels in cereals (Feizollahi et al., 2021). Application of some dry techniques such as mechanical de-branning with pearling via friction and abrasion caused a reduction of 50%

of inositol phosphorus, while conventional storage of barley kernels at 41 °C and 75% RH for 3 months resulted in a slight decrease of PA content (4-9%) (Mayer et al., 2023; Feizollahi et al., 2021). When concerned about wet processing techniques, malting, which is a controlled germination process combined with kilning caused for decreasing phytic acid content by 33.50%, whereas treatment of barley bran suspension in water with high intensity ultrasound waves (24 kHz), decreased phytic acid content by 39% (Arif et al., 2011; Grgic et al., 2023).

Quantification of PA in barley using some classical methods such as Huag and Lantzsch method, and Vaintraub and Lapteva method cause for slight inaccuracies since the non-selectivity of Fe(III) ion for PA, its dephosphorylated analogues and other inorganic polyphosphates, and also due to the stoichiometric inconsistency between iron(III) and PA (Raboy et al., 2017; Kahrman et al., 2020; Marolt and Kolar, 2021; Wu et al., 2009). Compared to those, AOAC method is based on separation of PAs using anion exchange method and quantification by colour development by complexation of phosphorus with molybdate reagent (Kahrman et al., 2020). However, an overestimation of PA content can happen since the inability to differentiate between phytic acid (IP6) and its dephosphorylated forms like IP4 and IP5 (Wu et al., 2009). In addition, some advanced techniques including high performance ion exchange chromatography coupled with UV detection were applied to determine the contents of PA and other inositol isomers (Schemmer, Spillner and Sudekum, 2020). However, application of those techniques can be less practical during analysis of a larger set of samples on a routine basis due to their low throughput and time consuming nature (McKie and McCleary, 2016).

Although previous studies have examined influence of single processing techniques on phytic acid content of barley, there is limited evidence on exploring combined effect of several techniques on the phytic acid content. Most studies have investigated the effect on PA

content when processed under controlled laboratory conditions. However, previous studies have not made a comparative approach to determine phytic acid content of barley that was processed within industrial and laboratory conditions. In addition, there are no previous studies conducted to explore the roasting impact of barley at different levels on phytic acid content. Moreover, limited studies have employed highly specific enzyme assay methods such as Megazyme method (enzymatic dephosphorylation method), as a common analytical technique for quantification of phytic acid content of barley that was processed under both experimental and industrial conditions.

2. Materials and Methods

2.1. Materials

Four types of industrially processed barley samples: Raw barley or whole barley flour (RB) malted barley (MB), malted barley subjected to mild roasting (MB-l) and malted barley subjected to intense roasting (MB-i) were provided by a cereal products processing company in United Kingdom.

Concentrated sulphuric acid (Sigma cat. no. 258105), ascorbic acid (Sigma cat. no. 95210), trichloroacetic acid (Sigma cat. no. T6399), ammonium molybdate (Sigma cat. no. 277908), Sodium hydroxide (Sigma cat. no. 795429), hydrochloric acid (Sigma cat. no. 258148) were used as analytical grade chemicals. Phytic acid assay kit was purchased from Megazyme Ltd.

2.2. Sample preparation

In addition to the four industrially processed flour types, two portions of raw barley flour were ground and roasted to two different levels by using an electric oven (SQ-4870) in the laboratory. One portion (R-l) was roasted at a temperature range between 120 °C to 150 °C for 30-40 minutes, while the other portion (R-i) was roasted at a temperature range between 170-210 °C for 40-50 minutes. Colour of flour samples were measured time to time by using a CIE Lab* space colourimeter (SISCO-CM-CR8) until colour profiles of R-l and R-i samples approximately reached to colour profiles of MB-

The present study aimed to determine and compare phytic acid content of industrially processed barley flour types; whole, malted, and roasted combined to malting, alongside barley samples roasted to varying extents under controlled laboratory conditions. The current study has made a novel approach by employing a common analytical technique to comparatively explore impact of distinct processing pathways on the phytic acid content of barley flour. Through this research, it is expected to enrich food scientists and manufacturers with actionable insights to optimize barley processing for improved nutritional quality.

l and MB-i respectively (table 2). However, temperatures were maintained within above stated ranges and roasting was carried out for specified time lengths until achieving approximately similar colours. The colourimeter was calibrated against standard white reference prior to the colour measurement, and such measured colours were recorded with respect to L* (lightness), a* (red-green axis) and b* (yellow-blue axis).

2.3. Sample extraction

Each sample was extracted in duplicate. Initially 1 g of flour sample was measured into a 75 ml glass beaker. Then 20 mL of 0.66 M HCl was added. After that the beaker was covered with foil and vigorously stirred on a magnetic stirrer (Dlab MS7-H550-Pro) overnight at room temperature.

Quantification of phytic acid content and free phosphorus content

Phytic acid content of barley samples was quantified via phytic acid assay kit by following the method and calculations as described by McKie and McCleary (2016).

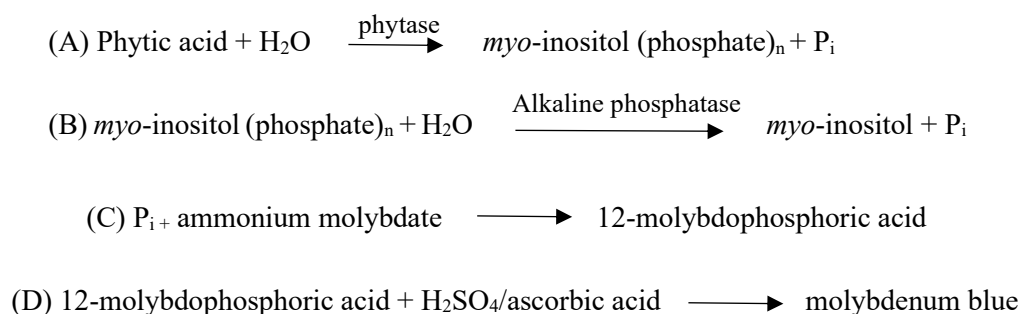
Initial free phosphorus content of each sample was determined as a pre-requisite to calculate the phytic acid content. This was included in the method described by McKie and McCleary (2016).

The principle and applicability of phytic acid assay kit

Colorimetric technique was applied to determine total phosphorus and PA content. This method is underpinned by the principle of measuring phosphate released from PA and lower inositol phosphates (InsP₁₋₅) due to the action of phytase and alkaline phosphatase enzymes in the assay kit (McKie and McCleary, 2016). Such enzymatically released phosphates were measured by a modified colorimetric molybdenum blue assay (McKie and McCleary, 2016). The same assumption of AOAC Official

Method 986.11, which presumed that the total amount of phosphorus quantified, is solely released from phytic acid instead of *myo*-inositol phosphates and other phosphate esters, was used during calculations (McKie and McCleary, 2016).

The set of reactions that involve during phytic acid determination procedure can be explained in accordance with the reaction scheme that was explained by McKie and McCleary (2016).



The assay kit measures total phosphorus content available in the samples because phytase enzyme has a broader range of specificity for lower *myo*-inositol phosphate compounds (IP₂-IP₅) in addition to phytic acid, while the action of alkaline phosphatase results in releasing last phosphate group from *myo*-inositol phosphate (McKie and McCleary, 2016). Therefore, theoretically it was presumed that the total phosphorus content of samples should fall within a narrow range since the processing involves only in altering the form of phosphorus (McKie and McCleary, 2016). Hence, for calculating PA content, the difference between free phosphorus and total phosphorus contents becomes a crucial value. Therefore, free phosphorus content is an important measurement which reflects the degradation of phytic acid when exposed to different processing conditions.

2.4.Data analysis

For each sample, 4 replicates were performed. Normality and homogeneity of variance were tested prior to conducting

statistical analysis. Mean values and standard deviations were calculated using SPSS software (version 29). Data were analysed to determine significant differences between sample means using SPSS software (version 29) by following one way ANOVA and Turkey HSD tests.

3. Results and Discussion

3.1.Phytic acid content of raw barley

Phytic acid content for two batches of raw or whole barley (RB), malted barley (MB), malted barley subjected to mild roasting (MB-l), malted barley subjected to intense roasting (MB-i), ground barley subjected to mild roasting (R-l) and ground barley subjected to intense roasting (R-i), are depicted in the table 1. Additionally, the table presents free phosphorus contents for each sample.

PA content of the two batches of raw barley samples was not significantly different ($P < 0.05$), and it fell within the range from 3.85 mg g⁻¹ to 9.85 mg g⁻¹ (0.385 – 0.985 g/100 g) and was also comparable with the mean value of 7.01 mg g⁻¹ (0.701 g/100 g) for phytic acid content according to the findings of Dai et al.

(2007). However, a non-mutant, wild type barley grains contained a greater level of PA content (1.02% on dry basis) compared to PA

content of raw barley that was determined in the present study (Ockenden et al., 2004).

Table 1. Phytic acid content and free phosphorus content of raw and processed barley samples under industrial and laboratory conditions

Barley sample	Phytic acid content (g/100g) d.b.	Free phosphorus content (g/100 g) d.b.
<i>Barley samples processed under industrial conditions</i>		
RB (Batch I)	0.859 ± 0.027 ^a	0.034 ± 0.001 ^A
MB	0.664 ± 0.021 ^b	0.054 ± 0.008 ^B
MB-I	0.712 ± 0.025 ^b	0.079 ± 0.005 ^C
MB-i	0.685 ± 0.029 ^b	0.110 ± 0.007 ^D
<i>Barley samples processed under laboratory conditions</i>		
RB (Batch II)	0.863 ± 0.027 ^a	0.045 ± 0.004 ^P
R-I	0.745 ± 0.074 ^b	0.052 ± 0.004 ^{PQ}
R-i	0.582 ± 0.003 ^c	0.060 ± 0.002 ^Q

Values are means ± SD expressed in dry basis; values with same superscript letter for each column are not significantly different (P<0.05).

Based on those previous literature findings, authors of the current study suggest that the naturally present phytic acid content in raw, unprocessed barley in current study might have been influenced by both intrinsic factors and extrinsic factors (Bloot et al., 2023). Genetic effect was determined to be the prominent intrinsic factor according to the findings of Kumar et al. (2021), who reported that Indian barley varieties cultivated at similar environmental conditions were detected with varying PA levels that fell within a significantly broader range (0.6-2.0 g/100 g).

Apart from genetic factors, phytic acid biosynthesis pathway is influenced by external environmental factors such as climate, geographical location, soil, fertilizer usage and irrigation conditions (Bloot et al., 2023). According to a presentation shared by the UK based cereal processing company (2025), 85-

90% of barley is obtained from East Anglia region. This region is well known for its light sandy soil that is naturally having a high level of phosphate, dry weather with less rainfall and more hours of sunlight per year that provide the ideal conditions for barley cultivation (<https://crispmalt.com/>). As revealed by previous studies, phytic acid content of spring barley correlated negatively with temperature, whereas it correlated positively with precipitation and application of different doses of phosphorus fertilizer (Korge et al., 2025; Reineke and Steffens, 2022).

3.2. Phytic acid content of malted barley

Malted barley sample showed a significantly (P<0.05) lower PA content compared to raw barley flour sample (figure 1). This finding reflects that PA content was subjected to a significant decrease (P<0.05) upon germination

step. This treatment accounted for reducing PA content by 22.70%, which was the second highest reduction compared to other processing conditions employed in the study (figure 3). Findings of Velde et al. (2025) revealed an approximately similar percentage reduction of PA (27.77%), which was corresponding to the fall of PA contents from 0.72 ± 0.07 g/100 g (d.b.) to 0.52 ± 0.00 g/100 g (d.b.) when subjected to germination at 20 °C for 95.5 hours. However, another study showed that PA content decreased linearly to a minimum value which ranged between $0.12 - 0.36$ mg g⁻¹ ($0.012 - 0.036$ g/100 g d.b.) from an initial PA content that fell between $4.22 - 4.74$ mg g⁻¹ ($0.422 - 0.474$ g/100 g d.b.) following germination for a period of 12 days (Bouajila et al., 2020).

The activity of phytase enzyme which is also termed as *myo*- inositol hexaphosphate hydrolase, plays a crucial role in determining the extent of PA reduction since the enzymatic action hydrolyses PA into inorganic phosphate (Bouajila et al., 2020). Major class of phytases in barley is known as purple acid phosphatase (PAP) (Madsen and Brinch-Pedersen, 2019). This enzyme results in the formation of *myo*-inositol compounds and inorganic free phosphates due to the removal of at least a single phosphate group from PA (Li et al., 2024). Enzymatic action is evident in the current study due to the presence of a significantly higher content ($P < 0.05$) of free phosphorus in MB sample compared to RB sample (table 1).

Barley cultivar influenced for varying phytase activity since there are two main genes namely PAPHy_a, which is expressed during grain filling stage, and PAPHy_b that is expressed during germination stage (Bouajila et al., 2019; Madsen and Brinch-Pedersen, 2019). Apart from intrinsic factors such as genetic makeup, extent of PA reduction was influenced by some extrinsic factors like germination conditions employed (Haraldsson et al., 2004). In the current study, the MB sample was commercially processed in diastatic form. As the initial step of malting process, barley grains were steeped in water for two days until the moisture content get increased up to 45%

causing to initiate germination. Following germination for 4-6 days, barley was subjected to a kilning process for 1-2 days to obtain malted barley (UK malted grain processor, 2025).

Absorption of water into barley grains results in the activation of phytase enzyme (Bouajila et al., 2019). Degradation of PA was positively correlated with germination time, while phytase activity was greater at 25 °C compared to lower temperatures (Li et al., 2024; Bouajila et al., 2019). In addition, findings of another study revealed that maximum phytase activity was observed after four days of germination at 20-25 °C, while the optimum phytase activity was observed at 50-55 °C and within the pH range of 5-6 (Sung et al., 2005).

3.3. Phytic acid content of malted and roasted barley

Two types of non-diastatic malted flours that were lightly roasted (MB-l) and intensely roasted (MB-i) at industrial processing conditions, were analysed in the current study. The extent of roasting varied based on temperature that ranged from 120 to 170 °C and time duration that ranged within 90 to 240 minutes (UK malted grain processor, 2025). According to findings of the present study, phytic acid content of MB, MB-l and MB-i samples did not show a significant difference ($P > 0.05$), and the PA content varied within a narrow range from 0.640 to 0.740 g 100 g⁻¹ (table 1). Those outcomes highlighted that malting followed by roasting did not cause further decrease in PA content, while malting effect was prominent over roasting for decreasing PA level. Roasting step resulted in producing flours that are enzymatically inactive since extreme temperatures beyond 55 °C terminate endogenous phytase enzyme activity (Frontela et al., 2008). However, application of processing techniques including malting alone and malting combined with roasting at two different intensities resulted in decreasing PA contents of MB, MB-l and MB-i samples significantly ($P < 0.05$) compared to RB sample (figure 1).

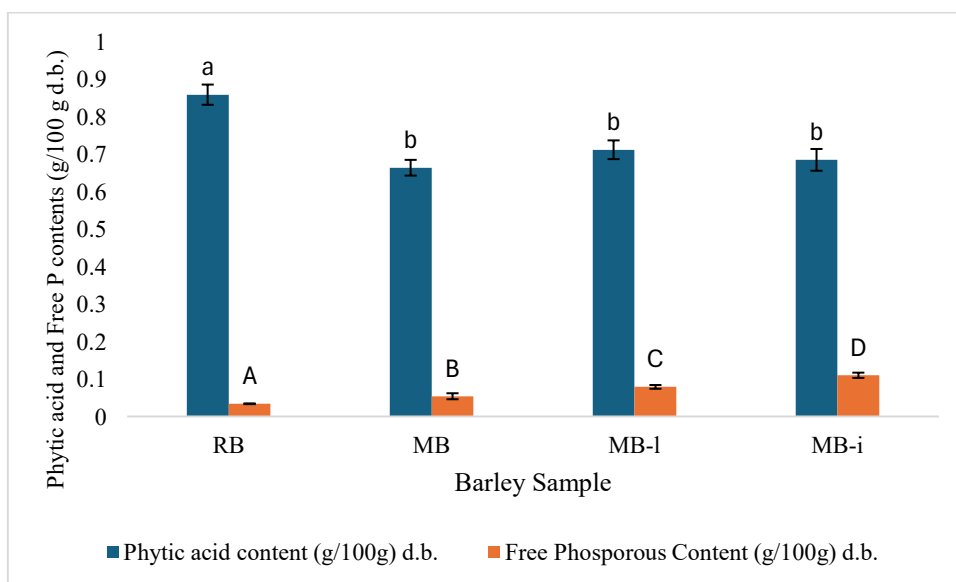


Figure 1. Phytic acid content (g/100g) and free phosphorus content (g/100 g) expressed on dry basis.

Legend: different simple letters in bar chart indicate significant differences ($P < 0.05$) between the samples with respect to phytic acid content. Different capital letters in line chart indicate significant differences between samples with respect to free phosphorus content. Samples: raw barley (RB), malted barley (MB), malted barley with light roasting (MB-l), malted barley with intense roasting (MB-i).

Content of phosphorus that existed in unbound or free form was minute compared to corresponding PA content (figure 1) of each sample. However, it showed a significant difference ($P < 0.05$) among raw (batch I) and industrially processed samples of barley and varied within a broader range from 0.033 g/100 g to 0.117 g/100 g d.b. As shown in the figure 1, processing conditions including malting alone and roasting combined with malting, caused for releasing significant levels of phosphorus ($P < 0.05$). More specifically, findings suggested that, along with intensifying roasting conditions free phosphorus content get increased. Therefore, free phosphorus content of the three samples can be arranged in ascending order as: MB, MB-l and MB-i (figure 1). It is noteworthy that both mild and intense roasting did not result in decreasing the PA content, although it increased free phosphorus content. Based on this, it can be hypothesized that malting prior to roasting caused for generating a pool of lower inositol intermediate compounds, which get dissociated further due to heat applied during roasting, while releasing free phosphorus.

Furthermore, it can be presumed that difference between thermal stabilities of phytic acid and its lower inositol phosphate compounds, is likely the main reason behind this. However, additional studies are required to investigate those mechanisms.

3.4. Phytic acid content of barley roasted in laboratory conditions

MB-l and R-l barley samples showed no significant difference ($P > 0.05$) with respect to a^* , b^* and chroma values as depicted in table 2. In addition, a^* values of MB-i and R-i samples were not significantly different ($P > 0.05$), while the percentage decrease of L values between RB and MB-l samples (12.7%) were approximately closer to that of between RB and R-l samples (14.6%). Similarly, the percentage reduction of L values among RB and MB-i samples (40.5%) were approximately closer to that of among RB and R-i samples (42.6%). Therefore, it is evident that roasting of ground barley in the laboratory had been conducted in a controlled manner in order to achieve a similar degree of roasting compared to industrially roasted samples.

Table 2. Colour parameters (L, a*, b* chroma and hue values) of raw and roasted barley flour samples

Parameter	RB	MB-l	MB-i	R-l	R-i
L	84.33 ± 0.50 ^a	73.60 ± 0.46 ^b	50.20 ± 0.10 ^c	72.03 ± 0.40 ^d	48.37 ± 0.75 ^e
a*	2.77 ± 0.32 ^a	6.34 ± 0.21 ^b	10.50 ± 0.36 ^c	4.93 ± 0.38 ^b	10.23 ± 1.06 ^c
b*	7.80 ± 0.61 ^a	17.90 ± 0.20 ^{bc}	20.83 ± 0.70 ^c	18.26 ± 0.32 ^{bc}	13.40 ± 3.99 ^b
C	8.30 ± 0.69 ^a	18.97 ± 0.25 ^b	23.37 ± 0.75 ^c	18.90 ± 0.45 ^b	17.00 ± 3.05 ^b
h	70.27 ± 0.76 ^a	70.43 ± 0.38 ^a	63.27 ± 0.15 ^b	74.90 ± 0.96 ^c	57.14 ± 2.16 ^d

Values are means ± SD. Values with same subscript letter for each row are not significantly different ($P < 0.05$). Colour expressed for samples: Raw barley (RB), malted barley with light roasting (MB-l), malted barley with intense roasting (MB-i), raw barley with light roasting (R-l), raw barley with intense roasting (R-i)

As illustrated in figure 2, PA content was significantly decreased ($P < 0.05$) when ground barley samples were subjected to both light and intense roasting. Moreover, intense roasting accounted for the highest percentage reduction (32.56%) of PA content among all treatment types as depicted by the figure 3. In contrast, an increasing trend was observed with respect to the free phosphorus content when roasting conditions were intensified (figure 2). Those outcomes aligned with the findings of Frontela et al. (2008), which revealed a significant reduction of inositol hexaphosphate (IP6) content from 639.0 ± 2.1 mg/100 g to 257.7 ± 9.0 mg/100 g (0.639 g/100 g to 0.257 g/100 g) when barley flour was roasted at 120 °C for 30 minutes. However, the corresponding percentage reduction (59.7%) achieved in the study conducted by Frontela et al. (2008), is significantly greater compared to the percentage reduction of PA achieved by roasting in the current study. It can be hypothesized that this significant difference is attributable to the type of analytical techniques employed, barley variety and some pre-processing parameters employed including milling size.

In addition, Li et al. (2023) highlighted that main possible reason behind the increase of iron and calcium contents in oven roasted cereal flours, was the elimination of antinutritional factors including phytic acid due to applied heat. Therefore, the possibility to degrade PA in samples that are in solid state due to heat can be justified based on those findings. Moreover, determination of PA content of roasted cereal flour can be considered as an important analytical test when designing infant food formulations. It is useful to maintain the molar ratio between PA and respective mineral within acceptable safe limits, while determining the content of minerals that should be added externally to cereal-based infant formulations. Apart from that, PA content of raw barley decreased when different processing conditions were applied in the current study and therefore, it can be presumed that, it results in decreasing the molar ratio (phytic acid to mineral molar ratio) below critical levels with respect to each mineral. So that, there is no effect on hindering mineral absorption and bioavailability (Abdulwaliyu et al., 2019).

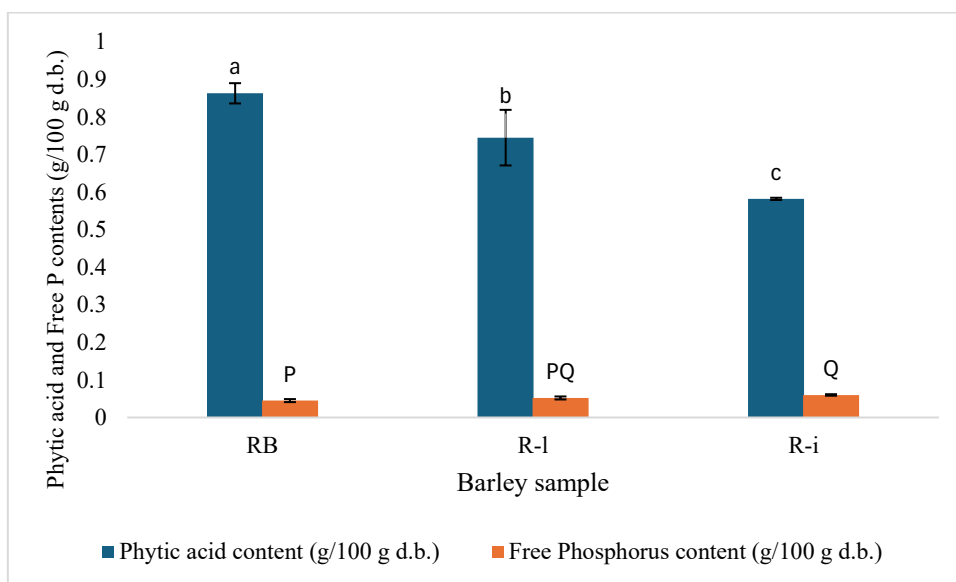


Figure 2. Phytic acid content (g/100g) and free phosphorus content expressed in dry basis.

Legend: Different simple letters in bar graph indicate significant differences ($P < 0.05$) between the samples with respect to the phytic acid content. Different capital letters in the line graph indicate significant differences ($P < 0.05$) with respect to the free phosphorus content. Samples: raw barley (RB), raw barley with light roasting (R-l), raw barley with intense roasting (R-i).

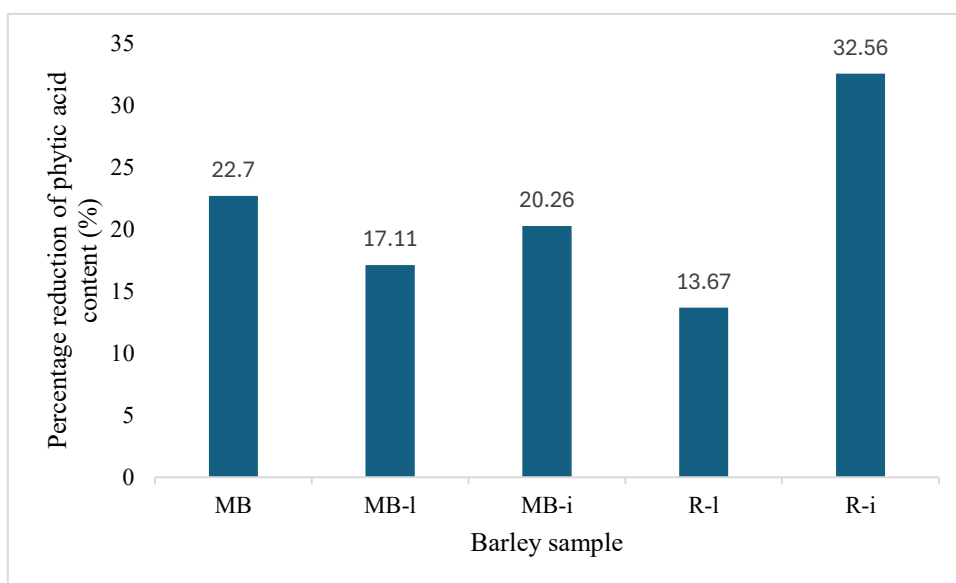


Figure 3. Bar graph demonstrates the percentage reduction of phytic acid content of barley when processed at different conditions.

Legend: MB (malted), MB-l (malted and lightly roasted), MB-i (malted and intensely roasted), R-l (lightly roasted barley), R-i (intensely roasted).

Significant decrease in PA concentration along with a simultaneous increase in orthophosphate (free phosphorus) concentration upon heating of a solid phytate sample at 60 °C for one day as revealed by Doolette and Smernik (2017), provided evidence for facile

decomposition of pure phytate due to heat. Formation of intermediate molecules such as inositol phosphates (IP_n), orthophosphate and pyrophosphate, when a phytate sample was heated at 85 °C for 36 hours, provided further evidence for thermal degradation of PA

(Watson, Smernik and Doolette, 2019). In the current study, free phosphorus content of barley samples depicted a rising trend when roasting conditions were intensified (figure 2). Therefore, decomposition of phytate in a solid sample due to heat was indicated by the increase of free phosphorus content (Doolette and Smernik, 2017).

Myo-inositol pentakisphosphates that were produced due to releasing of an orthophosphate, can exist in four different isomers namely Ins (1,2,3,4,6)P₅, DL-Ins (1,2,3,4,5)P₅, Ins (1,3,4,5,6)P₅ and DL-Ins (1,2,4,5,6)P₅ (Watson, Smernik and Doolette, 2019). Therefore, the presence of IP₅ as a mixture all four possible isomers, differentiates thermal degradation of phytic acid from its biotic pathways of degradation (Watson, Smernik and Doolette, 2019). Particularly, enzymes that generate Ins (1,3,4,5,6)P₅ and Ins (1,2,3,4,6)P₅ are known to be very rare, and presence of a combination of biota that would produce all IP₅ isomers in measurable quantities, is quite unlikely (Watson, Smernik and Doolette, 2019).

Phytate degradation mechanism at solid state was explained as a process that occurs as a result of facile transfer of a phospho group from phosphate esters to other phosphate groups present, which act as recipients (Doolette and Smernik, 2017). Orthophosphates and phosphate groups of an inositol phosphate act as recipients to generate diphosphates and organic diphosphates respectively (Doolette and Smernik, 2017). Therefore, the same mechanism can be presumed to explain phytate degradation during roasting of ground barley. However, further studies are required to elucidate the mechanism and rate of PA degradation when it is present within a natural food matrix such as ground barley.

4. Limitations of the study

This study has some limitations. Use of Megazyme assay, which specifically quantifies phytic acid (IP₆), did not measure other intermediate products such as PI₅, IP₄ and IP₃ along with other heat generated products. In addition, use of a single analytical technique

prevented cross-validation and may introduced method specific biasness. Only a single barley type was used in the present study, which narrowed down the potential to explore whether processing impacts in reducing phytic acid contents in the same pattern irrespective of the cultivar. In addition, phytase activity was not determined in the current study, which narrowed the ability to understand effects of endogenous phytase alone for reducing the phytic acid content.

5. Future recommendations

Future research should expand the scope of this study by using multiple barley cultivars to explore whether processing effects are consistent across genotypes or cultivar specific. In addition, quantifying phytase activity before and after processing would provide deeper insights into the mechanisms underpinning phytic acid degradation. Further work should also focus on employing a complementary analytical technique such as HPLC, alongside Megazyme assay to capture both phytic acid and lower inositol compounds that generated during processing. Apart from that, it is recommended to measure mineral bioavailability of samples before and after processing, to highlight the practical significance of phytic acid reduction.

6. Conclusions

Phytic acid contents of MB, MB-1 and MB-i samples were significantly ($P < 0.05$) lower compared to RB sample. However, the phytic acid contents of MB, MB-1 and MB-i samples were not significantly ($P > 0.05$) different from each other indicating that malting was the prominent causal factor compared to roasting that resulted in reducing the phytic acid content. Roasting of barley in ground form caused for a significant ($P < 0.05$) reduction of phytic acid content during both R-1 and R-i samples. Among the two roasting treatments, intense roasting showed the greatest reduction of phytic acid content. Overall, these findings provide useful insights into selecting and optimizing processing strategies of barley, while improving nutritional value of barley-based food products.

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Declaration of Interest Statement

The authors declare that they have no conflict of interests.