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**CONTENT**

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# CONTAINER'S INFLUENCE ON THERMAL DEGRADATION OF VEGETABLE OIL

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**ABSTRACT**
The influence of the container on the thermal degradation of different vegetable oils were evaluated. Four oils, namely groundnut, rice bran, soybean and sunflower were heated in glass, copper and iron containers of identical shape, size and thickness at 180°C for 12 hours. The oils were evaluated for conjugated dienes, conjugated trienes and total phenolic content before and after heating. The findings revealed that the container surface had a discernible influence on the formation of conjugated diene and triene in vegetable oil. However, a general trend was not found and the results were dependent on the nature of the oil. Except for groundnut, all other oils showed maximum conjugated diene content when heated in glass. For groundnut oil, maximum conjugated diene was found in copper. However, except rice bran, all other oils showed maximum post-heating conjugated triene content when heated in a copper container. For rice bran oil, the maximum conjugated triene content was observed when heated in a glass container. Soybean oil heating in a glass and copper container produces statically identical values. Thus, it appears that copper containers are more efficient in converting the primary oxidation products into secondary products. The phenolic content of the oils, on the other hand, was found to be stable under the experimental condition and not particularly dependent on the container.

## 1. Introduction

Vegetable oils can be used for a variety of purposes. They are primarily the triesters of glycerol with saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. These fatty acids play a key role in cellular metabolism by storing energy and providing it when needed. In addition to dietary applications, vegetable oils are utilized in industrial applications such as biofuels, soaps, paints, coatings, adhesives, lubricants, and so on(Kumar et al., 2016). Deep frying is a popular cooking technique, which involves emerging food in vegetable oils heated to extremely high temperatures (usually between 160 and 180°C) (Fauziah et al., 2000). Within a few minutes of immersion, several processes occur in food such as dehydration of the food surface, absorption of fat in food, production of flavour compounds, development of surface colour, and so on. The oil also degrades as a result of the harsh conditions used in deep-frying. It undergoes a number of processes, including pyrolysis, oxidation, hydrolysis, polymerization, isomerization, cyclization, and so on (Dobarganes et al., 2000). During this process, oxidation occurs resulting in large amounts of lipid peroxidation products such as conjugated diene hydroperoxides etc. Cleavage of these products generates aldehydes, such as malondialdehyde, which are toxic. To guarantee excellent oil quality, it is critical to check the oxidative condition of the oil on a regular basis. The thermal degradation of oil and fat is affected by a lot of factors. For example, numerous studies have revealed that unsaturated

fatty acids are more sensitive to oxidation during frying than saturated fatty acids (Kim et al., 2010; Liu et al., 2019; Normand et al., 2006; Warner et al., 1994). However, relatively little research was focused on the influence of the surfaces of the frying container on the thermal deterioration of vegetable oil. The topic is especially relevant when traditional cooking utensils made of copper or iron are used. Such metals are widely recognised for their ability to catalyze a wide range of organic reactions (Smith & Notheisz, 1999). Hence, the surface of these containers must influence numerous degradation processes in hot oil. According to one study, when sunflower, coconut, palm, and sesame oils were cooked at different temperatures, iron and copper containers produced much more malondialdehyde than aluminium and glass containers (Doureradjou & Koner, 2008). Another study found that using cast-iron containers increases the production of trans fatty acid 18:1 in groundnut and palm olein oil (Kala et al., 2012).

The conjugated diene and triene content are an effective indicator of the oxidation state of the vegetable oil (Chandran et al., 2017). For example, it was discovered that as microwave heating progressed, the amount of conjugated dienes and trienes steadily rose during heating. Also, it is noted that conjugated dienes were generated at somewhat greater levels than conjugated trienes (Hassanein et al., 2003). During the peroxidation of unsaturated fatty acids, substantial isomerization of double bonds occurs. This isomerization produces conjugated diene. Conjugated dienes can be detected by their strong absorption at 234 nm wavelength (White, 1991). Conjugated trienes are formed as the oxidation products of linolenate or by the dehydration of hydroxyl linoleate. They can be detected by their absorption at 268 nm wavelength (Houhoula et al., 2002). Phenolic antioxidants impart oxidative stability to the oil. Moreover, when consumed with oil they help in elevating oxidative stress in our body. A linear relationship was found between the total phenolic content of the oil and its oxidative stability by Rancimat (Salvador et al., 2001).

The purpose of this study was to determine the effect of containers on the stability of vegetable oils during heating. Four different commonly used oils; groundnut (GN), rice bran (RB), soybean (SO) and sunflower (SU) were chosen for this study. The total phenolic, conjugated diene and triene content of the oils were assessed before and after heating to evaluate the effect of containers on the thermal stability.

## 2. Materials and methods

### 2.1. Materials

Four vegetable oils - Groundnut, Rice bran, Soybean and Sunflower were purchased from the local market of Haryana, India. All the purchased oils had an expiry date exceeding the time period for this investigation. Three Containers made up of glass, copper and iron were also purchased, The containers have the same shape and size and thickness. All the chemicals and materials used were of analytical grade.

### 2.2. Sample Preparation

100 mL of different oils were placed in separate 500 mL containers of glass, copper and iron. The containers were then submerged in a silicone oil bath that was maintained at 180 ± 5 °C. For 12 hours, the oils were heated at this temperature. After that, the oils were allowed to cool to room temperature and kept in amber bottles to reduce oxidation. All of the assays were done within 12 hours of heating.

### 2.3. Statistical Analysis

All analyses were performed in triplicates. The findings were statistically evaluated by ANOVA and Duncan's multiple range tests using the R software programme (version 4.0.2). Statistical significance was defined as a p-value of $< 0.05$. The graphs were created in R using the ggplot package.

## 2.4. Methods

### *2.4.1. Conjugated dienes and trienes content*

The oxidation products of oils and fats have distinct spectra in the UV region. Linoleic hydroperoxide and the conjugated dienes that may emerge from its breakdown exhibit an absorption band at around 232 nm, whereas subsequent products of oxidation, notably ethylenic diketones, have an absorption band at approximately 268 nm. Conjugated trienes exhibit a triple absorption band, with the primary peak at approximately 268nm, a secondary peak at about 278nm, and minima close to the primary peak at about 262 and 274 nm. As a result, determining the absorbance at 232 nm might possibly provide an indication of the oil's condition of oxidation. Furthermore, determining the absorbance at 268 nm would show the existence of subsequent oxidation products and conjugated trienes. Absorbance at 232 and 268 nm were used for determining conjugated dienes and trienes, respectively (Dieffenbacher & Pocklington, 1992). Conjugated dienes give an indication of primary oxidation products and conjugated trienes indicate secondary oxidation products. In brief, 0.2 g of oil sample was dissolved in 10 ml Isooctane. Further, this solution was diluted by adding 150 µL solution in 5 ml isooctane. Then, its spectra were taken against isooctane as a reference.

### *2.4.2. Total Phenol content*

It was determined according to Folin–Ciocalteu's method (Singleton et al., 1999). The standard used was gallic acid, and the findings were expressed as gallic acid equivalents (GAE). The concentration of phenolics was expressed as micrograms of gallic acid equivalents per g of oil (µg GAE/ g) and was calculated using the following linear equation based on the calibration curve:

$$A = 0.41 \times C - 0.0799, R^2 = 0.99$$

where A is absorbance and C is concentration

Some modifications were made to the method (Karakaya & Şimşek, 2011). In 2 g oil, distilled water (3 ml), Folin–reagent Ciocalteu's solution(250µL), and 7 % $Na_2CO_3$ (750 µL) were added and incubated for 8 minutes at room temperature for TP analysis. After that, 950 µL of distilled water was added. The mixture was allowed to stand at room temperature for 2 hours. Similarly, a blank (without an oil sample) was made and allowed to stand for the same time. Then, the absorbance was determined at 765 nm against blank.

## 3. Results and discussions

### 3.1. Conjugated dienes (CD)

At the onset of thermal oxidation, the double bonds present in linoleate (18:2) and other higher polyunsaturated fatty acids (PUFA) change position. This occurs because generally oxidation starts with the loss of hydrogen from the methylene group sandwiched between two double bonds in the original configuration. The resultant pentadienyl radical reacts with oxygen to form conjugated hydroperoxides. These conjugated compounds are absorbed strongly at 232-234 nm. Hence, an increase in absorbance correlates with increased oxidation in these oils (White, 1995).

The results of the current study were shown in Table 1 and Figure 1. Dienes rose in metal containers and reduced in glass containers (GC) for groundnut oil (GN). Copper and iron were found to increase by 152.99% and 196.28% respectively compared to untreated. In the case of oil heated in glass, the value of CD reduced marginally (9.67%). Conjugated dienes were completely destroyed in Rice Bran oil heated in a copper container (99.86% decrease compared to untreated). A less severe decrease was observed in iron containers (30.38%). Glass on the other hand increased the value by 26.68%. The CD of soybean oil rises when it is heated irrespective of the container although the amount of increase depends on the container in which the oil is heated. Glass showed the most increase 422.40%, followed by iron, which caused an increase of 203.67%, while the increase in copper was only 129.50%. The relatively bigger changes in this oil compared to other oils may be

due to the presence of high amounts of PUFA in this oil. It is known that PUFA increases CD (Tekin et al., 2009). However, the sunflower oil shows very little change in CD. There is no statistically significant difference in CD between unheated oil and oil heated in iron. However, the value of glass and copper grew by 41.04% and 11.47%, respectively, with glass experiencing the greatest gain.

**Table 1.** Values of untreated and heated oils (mean ± standard deviation)

| Oil | Treatment | Conjugated dienes by $E^{1\%}$(232 nm) | Conjugated trienes by $E^{1\%}$(268 nm) | Total Phenolic Content |
|---|---|---|---|---|
| GN | UT | 3.153 ± 0.08 | 8.020 ± 0.22 | 20.116 ± 7.98 |
| | GC | 2.848 ± 0.06 | 1.209 ± 0.08 | 19.018 ± 6.58 |
| | CC | 7.978 ± 0.18 | 5.227 ± 0.16 | 14.079 ± 4.95 |
| | IC | 9.343 ± 0.22 | 2.008 ± 0.12 | 13.591 ± 4.34 |
| RB | UT | 7.683 ± 0.38 | 1.171 ± 0.28 | 57.25 ± 9.25 |
| | GC | 9.733 ± 0.28 | 4.513 ± 0.30 | 27.372 ± 4.05 |
| | CC | 0.01 ± 0.01 | 3.394 ± 0.16 | 27.372 ± 5.43 |
| | IC | 5.349 ± 0.21 | 2.600 ± 0.06 | 18.957 ± 3.83 |
| SO | UT | 4.340 ± 0.12 | 0.464 ± 0.07 | 20.116 ± 4.75 |
| | GC | 22.672 ± 0.43 | 5.647 ± 0.19 | 19.628 ± 6.00 |
| | CC | 9.96 ± 0.18 | 5.77 ± 0.27 | 10.908 ± 2.69 |
| | IC | 13.179 ± 0.24 | 3.532 ± 0.26 | 15.603 ± 3.31 |
| SU | UT | 13.242 ± 0.15 | 5.848 ± 0.15 | 13.591 ± 3.80 |
| | GC | 18.677 ± 0.31 | 3.137 ± 0.13 | 11.64 ± 2.80 |
| | CC | 14.762 ± 0.22 | 6.654 ± 0.22 | 10.786 ± 2.61 |
| | IC | 13.251 ± 0.21 | 3.744 ± 0.22 | 10.603 ± 4.25 |

The results show that the observed CDs after heating are dependent on the container for all four oils. However, the relative order among different containers depends on the oil type. Three of the four oils show maximum value in the glass container. When trying to understand these differences it must be kept in mind that CD represents the primary oxidation product. These

products are formed on heating but with time they are also decomposed to secondary oxidation. Thus, the observed values are results of the rate of formation of dienes subtracted by their rate of decomposition. So, a lower CD may be due to the slow formation of dienes but may also be because of the higher decomposition rate. If it is due to a lower rate of oxidation, then the values of CT are also expected to follow the same pattern, because conjugated trienes are formed from the degradation of dienes. However, if the rate of decomposition of dienes is the dominant factor, reversed trends are expected to be observed in CT. For most oils, maximum triene content post-heating was observed in GC. Thus, glass may be more effective in preventing the dienes from decomposing. The different behaviour of GN in GC needs further investigation.

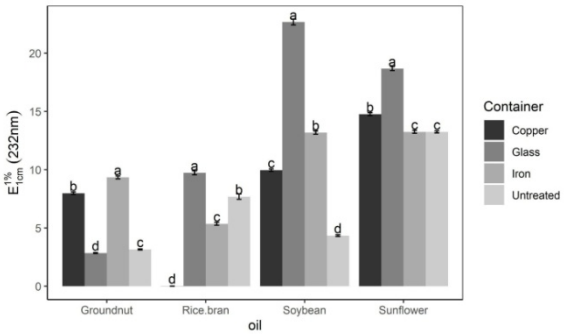


**Figure 1.** Conjugated dienes (CD) in untreated and heated oils. Data with the different superscript letters are significantly different ($p<0.05$)

### 3.2 Conjugated trienes (CT)

These hydroperoxides are unstable and under thermal treatment dehydrated from conjugated trienes which show a strong absorbance in the 268-270 nm region. Hence absorbance in this range is an indication of the formation of secondary products in oils (White, 1995).

The results were shown in Table 1 and Figure 2. The levels of CT in groundnut oil decrease when heated in various containers. The largest drop was 84.91% in the Glass container, followed by 74.95% in the Iron container, and just 34.82% in the Copper container. The CT of Rice Bran and Soybean rises after heating in the order Glass > Copper > Iron. The greatest rise is seen in glass, at 285.41%, followed by copper at 189.89% and iron at 122.09%. Like CD increase in CT on heating in soybean substantially higher than the other oils. Glass and copper containers register around 1100% increase while the increase in the iron container is around 700%. This reflects the poor oxidative stability of the oil. The values in glass and copper are statistically identical and higher than that in the iron container. Copper > untreated > iron > glass is the sequence for sunflower oil. In the case of copper, there is a little rise of 13.78%, whereas CT falls by 35.96% and 46.35% in iron and glass, respectively. Again, it is observed that CTs are dependent on the container. Except for RB, all the other oils register maximum post-heating CT value in CC. So for GN, SO and SU the copper surface is most effective in producing CTs. RB behaves differently from the other 3 oils. Here the GC produces the highest degradation in terms of CD and CT. This observation warrants further investigation. One interesting observation is that it is also the only oil which registers a significant and substantial decrease in phenolic compounds on heating. At this point, it could be postulated that some unique minor ingredients in the oils such as γ-original may be responsible for this.

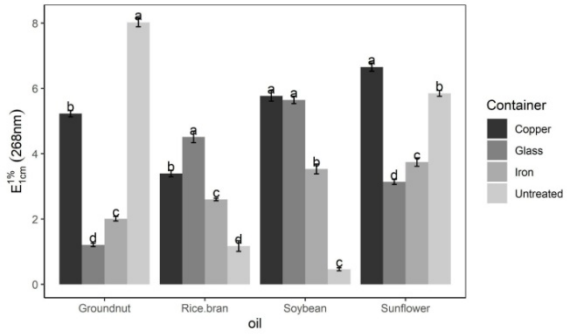


**Figure 2.** Conjugated trienes (CT) in untreated and heated oils. Data with the different superscript letters are significantly different ($p<0.05$)

### 3.3 Total Phenolic Content

Phenolic compounds serve as important antioxidants because of their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates. Hence, they prevent oxidation and the antioxidant activity of oils varies due to changes in phenolic content (Nyam et al., 2009).

So, it was decided to study total phenolic content. The results were shown in Table 1 and Figure 3. Generally, it was observed that heating under the current protocol has little effect on total phenolic content. All of the values in groundnut and sunflower oil are statistically the same. The total phenolic content of Rice Bran oil drops when heated. However, containers do not have any effect on this decrease. Values are statically the same in all containers. In the case of soybeans, the values for untreated and glass containers were statically identical. However, there is a little decline of 45.77% in the case of copper. The value observed in iron is statically identical to both iron and glass containers.

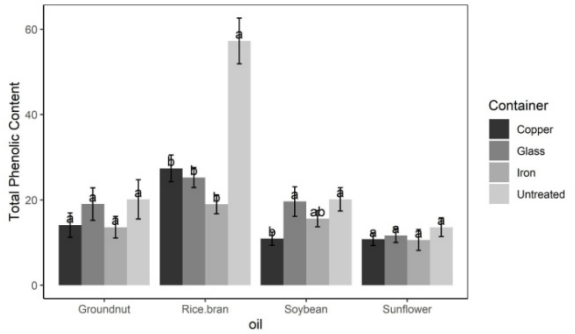


**Figure 3.** Total Phenolic Content of untreated and heated oils. Data with the different superscript letters are significantly different ($p<0.05$)

### 4. Conclusions

The effect of containers on the thermal degradation of vegetable oil was investigated by conjugated dienes and trienes content. The phenolic content of the oils was also studied. The results of the experiments with glass, copper and iron container revealed that the container in which the oils were heated had an observable influence on the conjugated diene and triene content of the vegetable oils. Though which container caused the maximum degradation is dependent on the oil type. Except for GN, all other oils register maximum diene concentration when heated in a glass container. However, in general, maximum conjugated triene values were observed in the copper container. Thus it can be postulated that copper containers promote the decomposition of primary oxidation products into the secondary product. The current heating protocol does not produce appreciable changes in the phenolic content of the oil. Where there is some decrease in the concentration of phenolic compounds; the effect of containers is either nonexistent or minimal. There is a scope for further investigation of the topic with more parameters and oil types. The authors hope this study will initiate a discourse on the effect of the container on the thermal stability of vegetable oil. This is especially important in a time when popular opinion is turning against nonstick cookware.

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# EXTRACTION OF CAROTENOIDS FROM VEGETABLE RESOURCES AND ITS UTILIZATION IN DAIRY PRODUCTS

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**ABSTRACT**
Food colorants will be classified broadly into natural and synthetic colorants. Natural colorants are extracted mostly from plant material. Natural colorants such as lycopene, carotene, anthocyanin, etc. are used to provide colors in food. The aim of the study was to estimate the total amount of carotenoids from naturally available vegetables namely tomato (Lycopersicon esculentum), carrot (Daucus carota), broccoli (Brassica oleracea), beetroot (Beta vulgaricus) and to incorporate the extracted natural colors in processed dairy product e.g. sweetened syrupy cheese ball (rasgulla). Total carotenoid content in tomato, carrot, beetroot and broccoli were found to as 29.34 mg/100g, 37.13 mg/100g, 25.64 mg/100g, 23.33 mg/100g. Moreover, the ascorbic acid contents of tomato, carrot, beetroot and broccoli were found as 20.67 mg/100g, 8.05 mg/100g, 7.65 mg/100g and 15.12 mg/100g respectively. Similarly, the total phenolic contents of tomato, carrot, beetroot, and broccoli were 3.72 mg GAE/g, 2.02 mg GAE/g, 2.12 mg GAE/g and 1.87 mg GAE/g respectively. The antioxidant activity of tomato, carrot, beetroot and broccoli were obtained 40.24%, 38.46%, 56.52% and 42.23%. Sensory analysis data revealed that the rasgulla sample (S1) i.e. added natural colorant extracted from carrot showed better results in terms of color attributes. It was evident from the experimental results that naturally occurring vegetable resources contains significant health benefits as well as nutraceutical components and can suitably contribute the natural color during the preparation of processed food product.

## 1.Introduction

Fruits and vegetables e.g. carrot, beet root, broccoli, tomato, grapefruit, cabbage etc. are good sources of natural colorants and most often, mainly divided into 04 major groups: anthocyanins, betalains, carotenoids and chlorophyll(Rodriguez-Amaya,2019a).Carotenoids are a group of phytochemicals that are responsible for different colors of foods. All carotenoids are tetraterpenoids, meaning that they are produced from 8 isoprene molecules and contain 40 carbon atoms. Carotenoids partially or completely protect intact cells (e.g. human liver cell line HepG2) against oxidant-induced lipid peroxidation, and the protective effect is independent of provitamin A activity.The oxygenated carotenoids which are derivatives of these hydrocarbons known as xanthophylls, examples of these compounds are zeaxanthin and lutein (hydroxy), spirilloxanthin (methoxy), echinenone (oxo), and antheraxanthin (epoxy) (Meshram etal.,2013). Carotene is an orange

photosynthetic pigment important for photosynthesis. They are recognized as playing an important role in the prevention of human diseases and maintaining good health (Rebecca et al.,2014). β-carotene is the most widely studied carotenoid and present in human blood and tissues. Color varies from yellow to orange.Lycopene isan important carotenoid and principally responsible for the characteristics deep-red color of ripe tomato fruits and tomato products. Other sources of lycopene include watermelon, guava, papaya etc. Lycopene undergoes degradation via isomerization and oxidation. Lycopene is the principal hydrocarbon carotenoid in tomatoes with lesser amounts of α-carotene, β-carotene, ϒ-carotene and lutein. Lycopene is very sensitive to light, heat, oxygen and acids in degradation(Shi andMaguer,2000).Lycopene as an effective antioxidant quenches highly reactive singlet oxygen (O2) and traps peroxyl radicals(Shi andMaguer,2000). Lycopene has been shown to protect important bio-molecules, such as lipids, low-density lipoproteins, proteins, and DNA against oxidative damage, which is how lycopene contributes to the prevention of cancers, atherogenesis, and cell proliferation (Rao and Agarwal, 1999; Stahl etal.1998). Betalains are water-soluble nitrogen-containing pigments, which are synthesized from the amino acid tyrosine into two structural groups: the red-violetbetacyanins and the yellow-orange betaxanthins and provides these color shades respectively (Stintzingand Carle,2007). Betalains are natural dyes extracted from different fruits and vegetables. They are largely used as food colorants in food products like yogurts, ice cream and other products. Recent studies have shown that betanines have antioxidant, antimicrobial and antiviral activity (Ravichandran etal.,2013). Beet (*Beta vulgaricus*) is the main source of natural red dye, known as beetroot. The betalains are stable in the pH range 4-6 and their subject to degradation by thermal processing as in canning.Broccoli (Brassica oleracea) has the highest levels of carotenoids in the brassica family. It is particularly rich in lutein and also provides a modest amount of beta-carotene (ShadaksharaswamyandManay,2011). The use of natural colorants can provide technological and bioactive functionalities to those foods in which they are applied, delivering additional value-added properties (Rodriguez-Amaya,2016). Nowadays, single-phase coloring systems such as baking products (solid phase) or drinks (liquid phase) have been successfully assayed with natural colorants such as carotenoids or anthocyanins (Lin etal.,2018). Colorants can be added to food systems after a technological extraction or could be part of the colored raw material. However, as some of the natural bioactive compounds that chemically constitute these colorants can be lost due to the matrix storage and processing conditions, some of them can be encapsulated to take advantage of their technological and biological properties (Hidalgo etal.,2018). In addition, encapsulated colorants are easier to handle and often exhibit enhanced physicochemical properties such as better solubility, stability, and flow properties (Labuschagne,2018). Food colorants play a crucial role in food production, masking unpleasant attributes or enhancing the food products' natural properties (Nwoba etal.,2020). Therefore, based on their color, they can also be used for specific purposes. For instance, anthocyaninis highly common watersoluble flavonoids exhibiting pH-dependent colors from red to blue, and recognized by several bioactive properties such as antioxidant, anti-inflammatory, hypoglycemic, and chemo preventive effects (Nwoba etal.,2020). Carotenoids are highly appreciated for their red, orange, and yellow color, primarily fruits and vegetables, contributing to desirable flavors in food and beverages (Rodriguez-Amaya,2019b). Betalains are other type of colorants that have proven to be the most promissory candidates to replace Allura Red AC (Red 40), a synthetic colorant that contains benzidine, a potential human and animal carcinogen (Potera,2010).The aim of the study was to extract, analyze and purify the carotenoid content from various vegetable resources viz. tomato, carrot, broccoli and beetroot and finally

the extracted natural colorant especially from carrot and beetroot was added during processing to develop a popular dairy product (sweetened syrupy cheese ball) rasgulla for enhancing nutraceutical and sensorial qualities. Therefore, the functional aspect of natural colorant will be successfully exploited in milk processing industry to develop the value added dairy products for consumption.

## 2.Material and Methodology

### 2.1. Collection of raw materials and reagents

The vegetables used for the experiment are tomato(*Lycopersicon esculentum*), carrot(*Daucus carota*),broccoli (*Brassica oleracea*) and beetroot (*Beta vulgaricus*) respectively. The fresh vegetables were brought from the localmarket of Kokrajhar, Assam, India adjacent to the institute and stored in the refrigerator.Chemicals or reagents used for the purpose of extraction and analysis are acetone/hexane/ethanol,Nacl,methanol/acetonitrile, starch solution and iodine solution (Ascorbic acid determination), Folin-Ciocalteau reagent, sodiumcarbonate (Total phenolic content) and 2,2-diphenyl-1-picrylhydrazyl/ethanol(Antioxidant activity) respectively.

### 2.2. Extraction of carotenoids

The vegetables used for the study were Tomato (*Lycopersicon esculentum*), Carrot (*Daucus carota*), Broccoli (*Brassica oleracea*), Beetroot (*Beta vulgaris*). The vegetables used were bought fresh from the market and preserved in the refrigerator. A spectrophotometer was used to observe the absorbance at 450 nm. Solvent extraction was done using hexane: acetone (1:1) along withethanol, 10% NaCl solution was also prepared in the laboratory which was used in the extraction (Rebecca *et al.,*2014).

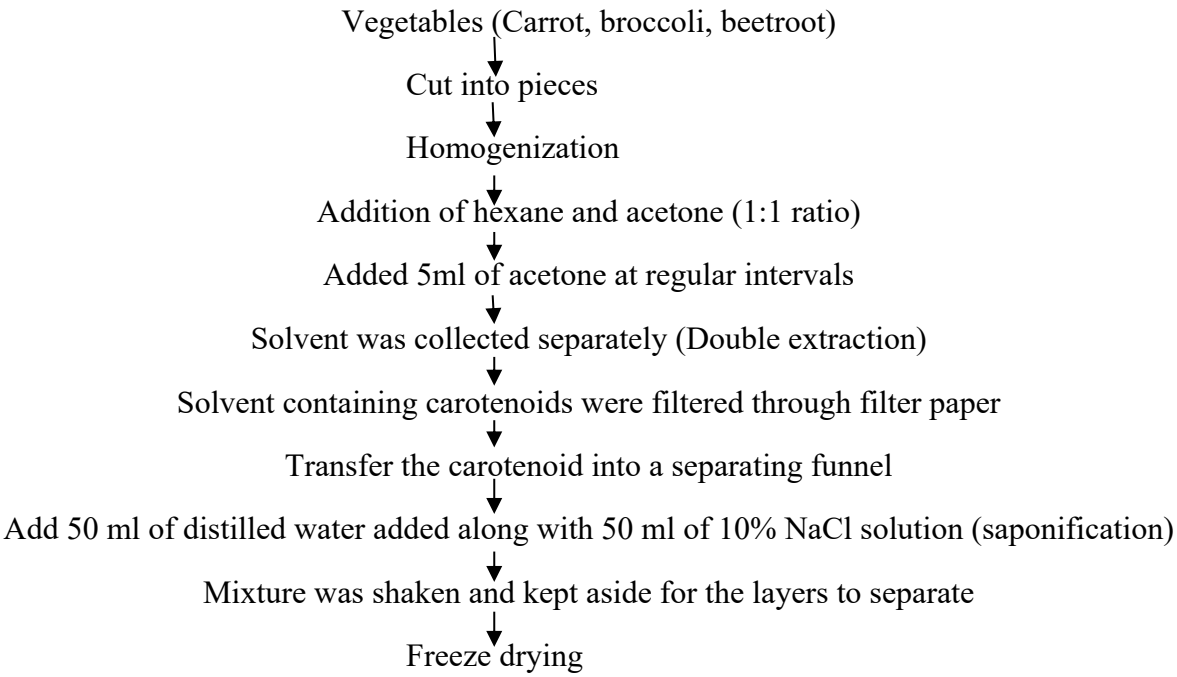


**Figure1.**Extraction of carotenoid from vegetable resources

#### *2.2.1. Extraction of carotenoids using solvent*

Vegetables viz. tomato, carrot, broccoli and beetroot were sliced separately and 100 g of each vegetable was weighed and kept separately. The same extraction procedure was followed for all the vegetables. 100 g of the vegetable was placed in a mortar and crushed with a pestle. A mixture of hexane and acetone in the ratio of 1:1 was added into the mortar and the sample was crushed. About 5ml of acetone was added slowly at regular intervals. The solvents were collected separately and the

process was repeated with the sample again for double extraction. The solvents containing carotenoids were filtered through a filter paper and then transferred into a separating funnel. 50ml of distilled water was added with 50 ml of 10% NaCl solution. The mixture was shaken properly and kept for the layers to separate. The upper layer contained carotenoids and it was collected separately after the removal of the water and NaCl solution. The extract was collected in tubes. The absorbance of the carotenoid was noted at 450 nm. The amount of carotenoid present in 100g of each food sample was calculated (Rebecca *et al.,*2014). The carotenoid extraction process from different vegetable resources will be represented in figure 1

## 2.3. Extraction of Lycopene from Tomato

Fresh tomatoes were first to cut into pieces and seeds were separated. The material was homogenized to form a paste. The paste was extracted with n- hexane (1:1 w/w) for two hours with constant stirring at 40-45 ºC in reaction assembly. The n-hexane layer was separated by using a separating funnel and further saponified for lycopene isolation (Roldan-Gutierrez *et al.,* 2007).

### *2.3.1. Saponification of n-hexane extract*

The oleoresin was mixed with saponification mixture containing 60% propylene glycol, 20% KOH prepared in 45% methanol and 20% water and kept at 65ºC under gentle stirring for 30 minutes followed by n-hexane addition. The mixture was washed with warm water to remove saponified matter and excess propylene glycol and KOH. The lycopene crystals formed were filtered through Whatman filter paper no 1 and dried under vacuum or freeze drier.The lycopene extraction process from tomatoes will be represented in figure 2.

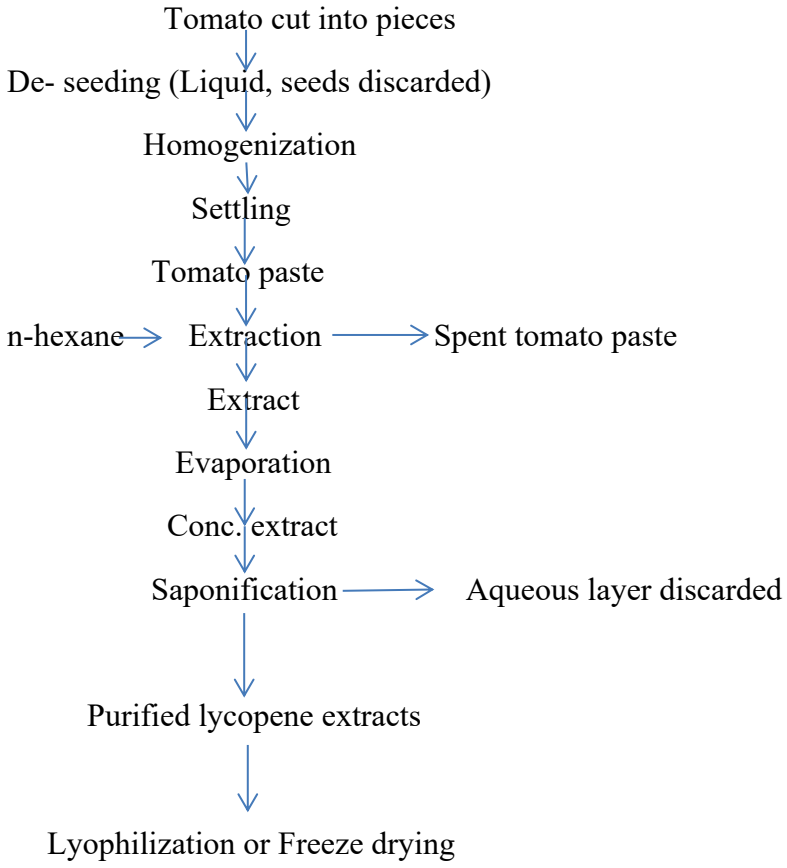


**Figure 2.** Extraction of lycopene (carotenoid) from tomato

### 2.4. Determination of Ascorbic acid

Ascorbic acid was estimated experimentally according to **(**Kaur and Goswami,2018**).** In this technique,20 mL of sample solution was pipetted into a 250 mL conical flask and add about 150 mL of distilled water and 1 mL of starch indicator solution. Titrate the sample with 0. 005 mol L iodine solution. The end point of the titration is identified as the first permanent trace of a dark blue-black colour due to the starch-iodine complex. Repeat the titration with further aliquots of sample solution until obtain the results**.**

#### *2.4.1. Preparation of iodine solution*

Weigh 2 g of potassium iodide into a 100 ml beaker. Weigh 1.3 g of iodine and add it into the same beaker. Add a few mL of distilled water and swirl for a few minutes until iodine is dissolved. Transfer iodine solution to a 1L volumetric flask, making sure to rinse all traces of solution into the volumetric flask using distilled water. Make the solution up to the 1 L mark with distilled water.

#### *2.4.2. Preparation of starch solution*

Starch indicator solution: (0.5%). Weigh 0.25 g of soluble starch and add it to 50 mL of near boiling water in a 100 mL conical flask. Stir to dissolve and cool before using.

### 2.5.Determination of total phenolic content(TPC)

Total phenolic content was determined according to (Annisworthand Gillespie, 2007). The reaction mixture was prepared by mixing 0.2 ml of natural juice separately mixed with .6 ml of distilled water. After addition of .25 ml of Folin- Ciocalteau reagent, 1 ml of saturated sodium carbonate (8% W/V) and 3 ml of distilled water were added. The mixture was then incubated for 30 min at 37 ºC and the absorbance was recorded at 765 nm using an UV-Spectrophotometer. The measurement was compared to calibration curve prepared using the standard gallic acid solution. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per 100 ml of juice.

### 2.6. Determination of antioxidant activity

The antioxidant activity of the extracts was determined using the modified method of(Sharma and Bhat,2009). In this method, 0.001g of DPPH (2,2-diphenyl-1-picrylhydrazyl) in ethanol was prepared and 1ml of this solution was added to a test sample. The reaction mixture was shaken well and incubated for 30 min at 37ºC. The absorbance was read at 517 nm using a UV-Spectrophotometer against the ethanol blank. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of (Yenand Duh, 1994).

$$IP = [(AC\ (0)- AA(t)/\ AC\ (0))] \times 100 \quad (1)$$

Where AC (0) is the absorbance of the control at t = 0 min; and AA(t) is the absorbance of the Anti-oxidants at t = 16 min.

### 2.7. HPLC analysis of carotenoids

HPLC analysis was carried out by the following experimental procedures and the materials are taken such as tomato, carrot, beetroot and broccoli.

#### *2.7.1. Sample preparation*

The solvent mixture used for the HPLC method is generally methanol/Acetonitrile (90:10 v/v). A sample of 5 grams of vegetables (carrot, tomato, beetroot and broccoli) was placed in a vessel, protected from light, and mixed with 100 ml of extraction solvent. The mixture was magnetically stirred during 30 minutes. The extracts were centrifugated to separate the supernatant, and these operations were repeated until the pulp was completely colorless. After that saponification was done with BHT/Methanol (40:60 v/v) (Barba et al.,2006).

#### *2.7.2. Standard carotenoid preparation*

Individual stock standard solutions were freshly prepared every day adding a suitable volume of hexane to the vial containing the carotenoid standard and mixing until complete dissolution; then the solutions were transferred to a volumetric flask and the concentration was determined spectrophotometrically. Individual working standard solutions of around 0.5–9.5

µM were freshly prepared every day from individual stock standard solutions by diluting in hexane.

### *2.7.3. Chromatographic conditions*

Several mobile phases were assayed. Methanol/ ACN (90:10 v/v) and different mixtures of methanol/THF/ water. The mobile phase was filtered through a 0.45 µm membrane, and degassed ultrasonically prior to use. The mobile phase flow rate was 0.9 ml/min. The column temperature was 30 ºC and the absorbance was read at 455 nm and for Lycopene the absorbance was read at 503. The injected volume was 50 µL. The efficiency of the separation was evaluated by the calculation of the number of plates (N), using the width of the peak at half its maximum height. The identification of the peaks was carried out by comparing the retention times with those obtained with a mixed standard solution of all-trans lycopene, b-carotene. The quantification was performed using calibration curves made with different injected amounts of all-trans-lycopene and b-carotene, in a similar proportion as in the samples (Barba et al.,2006).

### *2.7.4. Analysis of ß- carotene*

Determination of ß – carotene was made according to the formula,

$$C\ (\mu g/g) = \frac{A_x \times C_s \times V}{A_s \times P} \quad (2)$$

Where, $A_x$= Carotenoid peak area, $C_s$= Standard concentration, $A_s$ = Standard area, V = Total extract volume, P = Sample weight

## 2.8. Utilization of extracted natural colorant in dairy product preparation viz. Rasgulla

In this study, rasgulla preparation was performed by the addition of extracted and purified colour. Mainly the color obtained from the carrot (beta carotene) and beetroot (beta lain) under the class of carotenoid was considered for the development of the product.

### *2.8.1. Preparation of rasgulla*

The processed dairy product viz. rasgulla preparation will be represented by figure 3, where ingredients are considered as milk, lemon/citric acid, refined sugar, water and colors (from carrot, and beetroot).

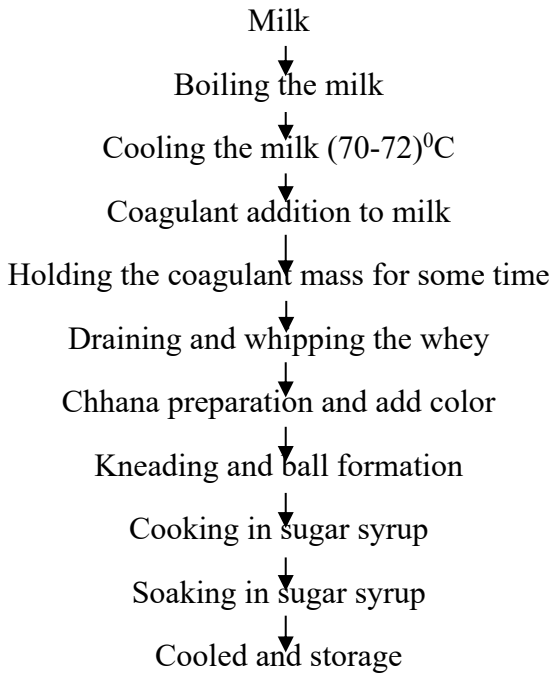


**Figure 3.** Preparation of rasgulla

### 2.9. Sensory evaluation of rasgulla (sweetened syrupy cheese ball)

A total of twenty two panelists including faculty members and students of Central Institute of Technology Kokrajhar, Kokrajhar, Assam, India rated the prepared rasgulla added with extracted natural color for preference of color, appearance, flavor, taste, texture and overall acceptability of the products. Evaluation of organoleptic properties was done by nine point hedonic rating scale from like extremely to dislike extremely according to (Ihekeronye and Ngoddy, 1985).

## 3. Results and Discussions

### 3.1.Extraction of carotenoids as natural colorant

In this study, different vegetables viz. carrot, beetroot, broccoli and tomato were considered for the extraction of natural colors viz. carotenoids e.g. beta carotene, lycopene, betalain etc. by using a mixture of acetone and hexane (1:1) for the first three and hexane only for remaining one respectively. It was suitably represented by table 1. From the experimental investigation, it was found that carrot and broccoli show maximum and minimum extraction efficiency respectively. This is due to the fact of the ability of interaction and dispersibility. The extraction efficiency of either beta carotene or lycopene by using different solvents e.g. hexane, ethanol, acetone etc. depends upon interaction effect and method of extraction (Vieira et al., 2020). Carotenoid extraction from *Bixa orellana L* was shown a lower value by using acetone as solvent (Cruz *et al.,* 2008).Extraction of carotenoids by using a combination of different solvents was shown comparatively better result than that of use of an individual solvent (Amr and Hussein,2013).

**Table 1.** Estimation of extraction of carotenoids from different vegetables and their yield

| Sample | Weight of sample(g) | Extracted sample (ml) | Yield (%) w/v |
|---|---|---|---|
| Tomato | 195.22 | 85 | 43.40±0.42 |
| Carrot | 211.10 | 95 | 45.68±0.34 |
| Beetroot | 200.65 | 88 | 42.36±0.64 |
| Broccoli | 190.55 | 82 | 41.15±0.76 |

### 3.2.Carotenoid content of different vegetables

Carotenoid is a class of natural color contains different carotenes (alpha carotene, beta carotene, Lutein, cryptoxanthin, lycopene etc.). Now different vegetables as indicated in table 2 were considered for carotenoid content (mg/100g). it was observed that the carotenoid content was found to be highest in carrot and conventionally the major fraction is beta-carotene which is the precursor of vitamin A whereas for broccoli it was minimum. Experimental investigation revealed that lycopene content in tomato was varied from 55 to 181 mg/kg (Malviya,2014), 4.31 to 5.97 mg/100 g fw (Shahzad *et al.,*2014).The amounts of major carotenoids of carrots, β-carotene and α-carotene, ranged from 29 to 130 mg/kg and from 9 to 66 mg/kg, respectively (Kocaand Karadeniz,2008; Alasavar*et al.,*2001; Hart and Scott,1995;Kidmose *et al.,*2004; Bushway,1986; Bureau and Bushway,1986; Heinonen *et al.,*1989; Konings and Romans,1997; Niizuand Rodriguez-Amaya,2005).

**Table 2.** Carotenoid content in vegetables

| Vegetables | Carotenoid content(mg/100g) |
|---|---|
| Tomato | 29.34±0.46 |
| Carrot | 37.13±0.32 |
| Beetroot | 25.64±0.74 |
| Broccoli | 23.33±0.86 |

### 3.3. Estimation of Vitamin C

Determination of vitamin C or ascorbic acid is so important in this study and represented by figure 4. The experimental result revealed that tomato and beetroot was shown the maximum i.e. 17.67mg/100g and minimumi.e.7.65mg/100gvitamin C content respectively compared to others. This is due to the inherent characteristics of these two vegetables.

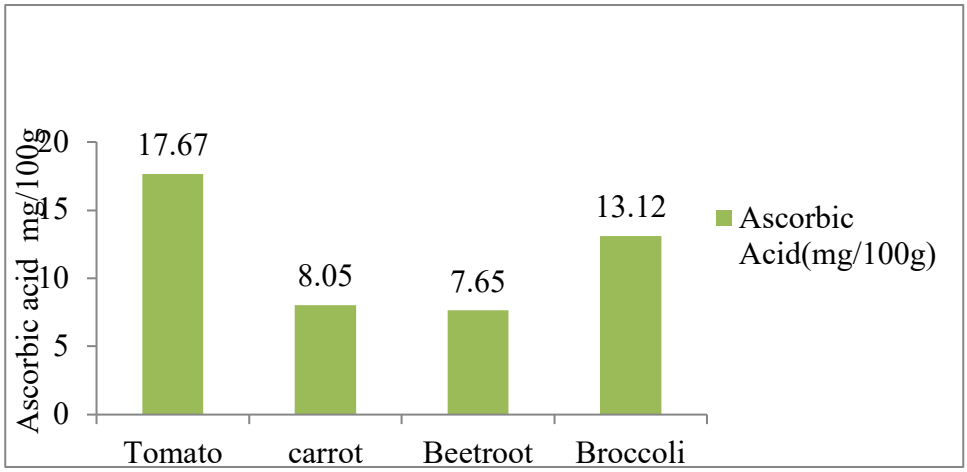


**Figure 4.** Graphical representation of different vegetables vs ascorbic acid

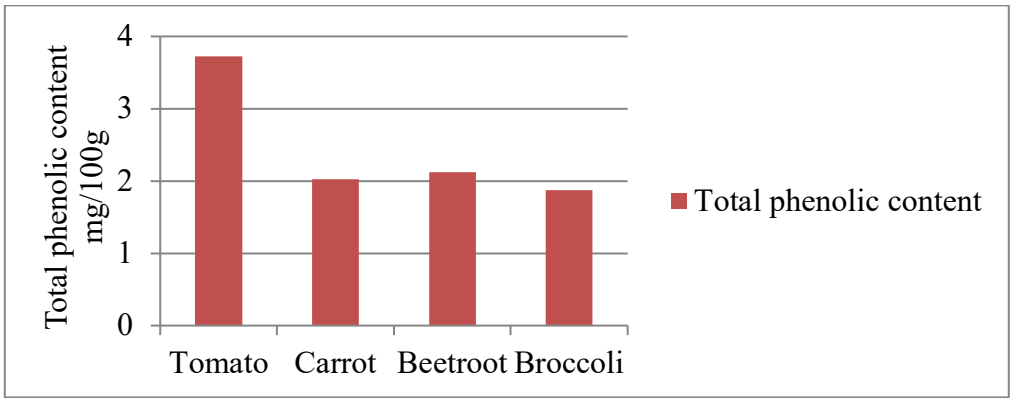


**Figure 5.** Graphical representation of different vegetables vs total phenolic content

### 3.4. Estimation of total phenolic content (TPC)

Determination of total phenolic content (mg GAE/100g) is considered to be a potential marker or indicator for antioxidant content. It acts as an indicator of health benefit. Therefore, it is represented by figure 5. Experimental investigation revealed that tomato and broccoli was shown the maximum i.e. 3.72mg/100g and minimum i.e.1.87mg/100g total phenolic content respectively compared to others. According to (Gebczynski, 2006), the total

polyphenols in fresh carrot was 20.9 mg of chlorogenic acid/100 g. However, (Vinson *et al.,*1998) found 46.4 mg of catechin/100 g FW. On the other hand, the range for total phenolics was also reported by (Kendall, 2006) as 1.1–1.6 mg of gallic acid/g for carrot samples.

### 3.5. Estimation of antioxidant activity by DPPH radical scavenging assay

DPPH reagent is very much useful for free radical scavenging assay of compounds viz. naturally occurring colorants extracted from vegetable resources. The nutraceutical potential of natural colorants is exhibited by the antioxidant activity. The experimental result was shown by figure 6. It was observed that carrot and beetroot were shown maximum(56.46%)and minimum (36.52%) values respectively compared to others. (Ou*et al.,* 2002) reported that the antioxidant activity varied considerably from variety to variety in carrots.(Kendall, 2006) found that antioxidant activities against ABTS cation radicals in carrots varied between 3.1 and 7.2 µmol TE/g DW.

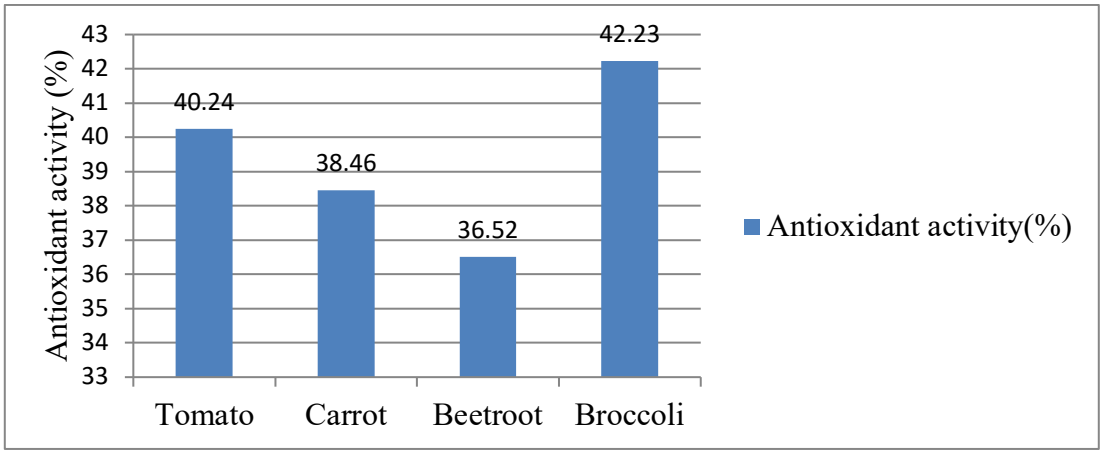


**Figure 6.** Graphical representation of different vegetables vs antioxidant activity (%)

### 3.6. HPLC analysis of extracted carotenoids from carrot

This experiment was considered to be important for the purpose of purification of extracted carotenoid.Enhancement of nutraceutical potential was occurred.Here,experimental investigation was carried out for purification of carotenoid from carrot and represented by figure 7.

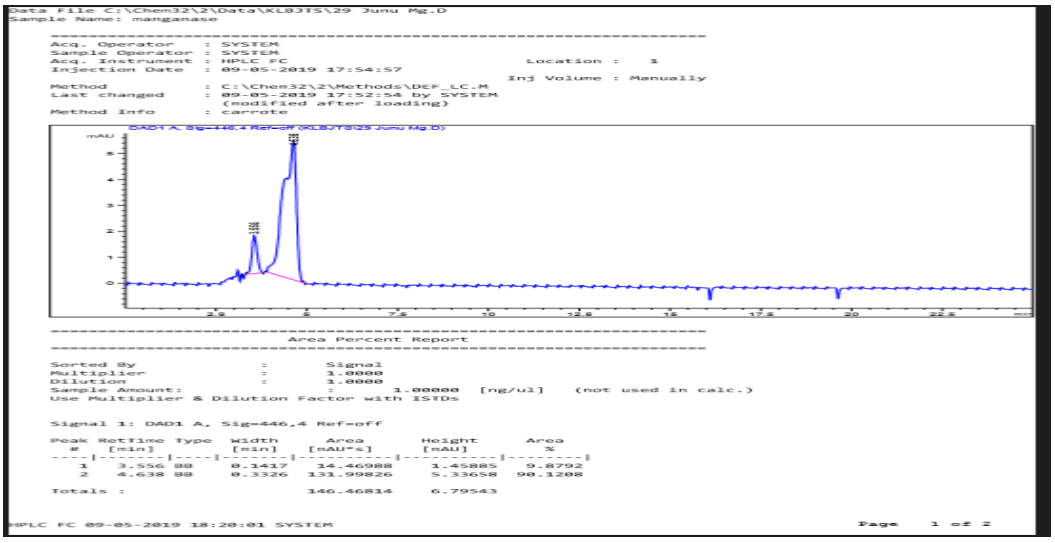


**Figure 7.** HPLC analysis of carotenoid (carrot)

### 3.7. HPLC analysis of lycopene from tomato

This experiment was considered to be important for the purpose of purification of extracted lycopene. Enhancement of nutraceutical potential was occurred. In this study, experimental investigation was carried out for purification of lycopene from tomato and represented by figure 8.

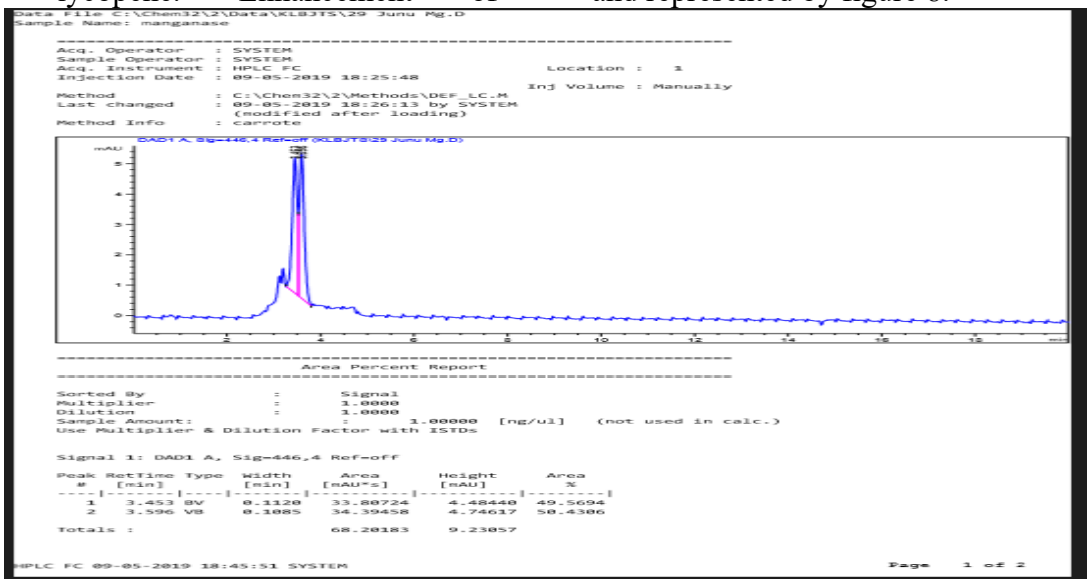


**Figure 8.** HPLC analysis of lycopene ( Tomato)

### 3.8. Evaluation of sensory quality of rasgulla

The sensory analysis of rasgulla was done by 9 point Hedonic Rating Test with the help of twenty two panelists and represented by table 3. During this test natural color extracted from carrot i.e. beta-carotene and natural color extracted from beetroot i.e. betalain were added in rasgulla sample (S1) and rasgulla sample (S2) respectively**.**

**Table 3.** Sensory characteristics of rasgulla sample (S1) and (S2)

| **Parameters** | **Sample (S1)** | **Sample(S2)** |
|---|---|---|
| Color | 7.25±0.43 | 6.5±0.5 |
| Appearance | 6.5±0.5 | 6.75±0.43 |
| Flavor | 6.25±0.82 | 6.25±0.43 |
| Taste | 6.25±0.82 | 5.75±0.82 |
| Texture | 7±0.70 | 7±0.70 |
| Overall acceptability | 6.75±0.43 | 6.25±0.43 |

From table 3. it was evident that there was no such significant variation of overall acceptability of both the two kinds of rasgulla samples i.e. S1 and S2. However, the color ofthe rasgulla sample (S1) i.e.beta carotene added was more attractive according to hedonic rating score in comparison to the color of rasgulla sample (S2) i.e. betalain added.

## 4. Conclusions

The vegetables from where the colors were extracted are the source of ascorbic acid, total phenolic content and possess of significant of antioxidant activity. The antioxidant activity was found to be 40.24%, 56.46%, 36.52%, 42.23%respectively for tomato, carrot, beetroot and broccoli. Similarly, the total phenolic content was 3.72 mg GAE/g for tomato, 2.02 mg GAE/g for carrot, 2.12 mg GAE/g for beetroot and 1.87 mg GAE/g for broccoli respectively. Again, the ascorbic acid content was found to be 20.67 mg/100g, 8.05 mg/100g, 7.65 mg/100g and 15.12 mg/100g respectively for tomato, carrot, beetroot and broccoli. From this study the amount of carotenoid content in fresh tomato, carrot, beetroot and broccoli were

found as 29.34 mg/100g, 37.13 mg/100g, 25.64mg/100g and 23.33mg/100g respectively. Carotenoids extracted from carrot i.e. beta-carotene and betalain from beetroot were utilized on processed foods i.e. rasgulla preparation and sensory analysis data revealed that beta carotene added rasgulla sample (S1)was found to be satisfactory.

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# SYNTHESIS AND CHARACTERIZATION OF GRAFT COPOLYMERS OF NATIVE AND PHTHALATED CASSAVA (*Manihot esculenta*) STARCHES WITH ACRYLONITRILE AS A GRAFTING AGENT

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**ABSTRACT**

Graft copolymerization is a promising method for properties modification of natural polymers. Cassava starch, a natural polymer was extracted using wet extraction route. Part of the obtained starch was pre-gelatinized at above 70ºC and esterified using phthalic anhydride. CaO (catalyst) was prepared by calcining snail shell powder in a muffle furnace at 800ºC for 4 h. The native and pregelatinized/phthalated starch solutions were grafted with acrylonitrile at 120ºC in the presence of CaO catalyst. The prepared CaO catalyst was characterized using SEM, EDS, TGA, XRD, and FTIR for surface assessment, elemental evaluation, thermal degradation, amorphosity/crystallinity determination, and functional group evaluation, respectively. The SEM-EDS of the prepared CaO catalyst revealed spherical shape and the elemental compositions. The FTIR spectra of the grafted native cassava starch copolymer and grafted phthalated cassava starch copolymer revealed the existence of C≡N- peak at 2364 and 2262 $cm^{-1}$, respectively which is an evidence of grafting. The surface of the native cassava starch grafted copolymer granules was eroded with cracks, while that of the phthalated cassava starch grafted copolymer showed coagulated and coerced morphology. The XRD result of the native cassava starch grafted copolymer revealed one major sharp peak at angle $2\theta=25^{o}$ while that of the phthalated cassava starch grafted copolymer revealed numerous peaks. The TGA analysis revealed that the phthalated cassava grafted copolymer framework was completely broken at 500ºC unlike the native cassava starch grafted copolymer whose complete decomposition was about 420ºC. It was observed that the modified samples had improved features over the native sample.

## 1. Introduction

Plastics as packaging materials perform important roles in the food manufacturing sector because of their lightness, durability, and flexibility unlike ceramics and metals (Kutz, 2012), hence, do not biologically decompose. Incineration and recycling methods had been used to mitigate these problems, however, a few percentage of plastic wastes is recyclable and most of them get migrated into the municipal landfills. One solution to control this environmental menace is the use of degradable polymers most importantly in the packaging sector to substitute petroleum-based polymers which are undegradable (Ali *et al*., 2013; Monica *et al.,* 2015). Recently, attention has been drawn to the production of new materials by incorporating natural biopolymers such as cellulose, chitosan, starch, etc. into many stable synthetic polymers (Salmoral *et al.,* 2000; Park

*et al.,* 2001). The widely proposed and considered natural biopolymer for this purpose has been starch: a biodegradable; relatively cheap; non-toxic; renewable; and readily available in several forms (Suvorova *et al.,* 2000; Vroman and Tighzert, 2009). Starch film has some level of permeability making it useful in food packaging system. When it is used as blends with non-biodegradable polymers, it increases the microbial activities at the surface (Koenig and Huang, 1995).

Watanabe *et al.* (2009) reported that, if starch materials are blended with polyolefin matrix, polysaccharide–loving bacteria, enzymes, fungi, etc, will be attracted to them and degradation will be initiated.

Several studied have been successfully conducted on composites of starch and synthetic polymers (Arvanitoyannis *et al.,* 1999; Graaf *et al.,* 2001). Some researcher used starches that were obtained from rice (El-Rahim *et al.,* 2004), potato (Borghei *et al.,* 2010), and sago (Danjaji *et al.* 2001; Danjaji *et al.* 2002; Abdul-Rahman *et al.,* 2006) to produce biodegradable blends.

One major drawback for starch-based composites is that starch and several polymers are immiscible and this generally leads to the poor properties of starch/polymer blends. However, chemical modification of starch is generally embraced to overcome this drawback by the interaction of appropriate moieties with the hydroxyl groups in starch matrix (Bao *et al*., 2003). The obtained derivatives always have some physico-chemical features which deviate significantly from the parent starches, however, their biodegradability is maintained still. Also, Graft copolymerization is another promising technique to modify some physico-chemical properties of naturally available biopolymers (Lv *et al.,* 2008; Kalia and Kaith, 2008).

The present study is aimed at modifying native starch obtained from cassava tubers with acrylonitrile using CaO obtained from snail shells as catalyst, and compare the properties of both the native and modified starches through instrumental analysis.

## 2. Materials and methods

### 2.1. Materials

Cassava tubers, all the reagents such as acrylonitrile, phthalic anhydride, etc. were of analytical grade.

#### *2.2.1. Samples*

Cassava tubers were obtained from a local market in Ado-Ekiti, Nigeria. The starch was isolated by wet extraction method as described by Wang *et al*. (2006). The fresh tubers were thoroughly washed with water, cut into small pieces, blended for homogeneity, the resultant slurry was packed into a muslin cloth and immersed into a bucket filled with distilled water to expel the starch. The starch was allowed to settle for 24 h and the supernatant was decanted. Further washing of the obtained starch was done using distilled water, this process was repeated till the supernatant was clear, and the wet starch was thereafter air-dried.

#### *2.2.1. Preparation of Pregelatinized Starch Phthalate*

Pre-gelatinized Starch Phthalate was prepared using the method of Surini *et al.* (2014). This method involves two major steps: gelatinization and esterification. Gelatinization was carried out as the starch solution was heated at 70°C, the resulting gel was oven-dried, ground, and sieved to obtain uniform particle size. The esterification process was carried out by contacting 10% pre-gelatinized starch in distilled water with 16.7% phthalic anhydride solution in 96% ethanol. NaOH (10 M) was continuously added during the reaction to keep the pH between 8 and 10, anhydrous sodium sulphate was added to absorb excess moisture with constant stirring at 1000 rpm for 30 more min. The resulting mixture was allowed to stay for 24 h, the mixture pH was adjusted to 6.5 − 7.0 using HCl solution. 50% Ethanol was added to the neutralized solution to wash off the un-reacted phthalate, the final precipitate was dried, ground, and sieved to obtain pre-gelatinized cassava starch phthalate (PCSP).

#### *2.2.2. Preparation of Calcined Snail Shell Powder*

A catalyst (CaO) was prepared according to Nurhayati *et al.* (2016). Snail shells were

thoroughly washed with tap water until they were free from dirt, the clean shells were oven-dried at 105ºC, calcined in a muffle furnace at 800ºC for 4 h. The calcined snail shell ash (CSSA) was pulverised to fine powder as CaO, sieved, and stored in a closed container to avoid exposure to air.

***2.2.3. Preparation of Native and Phthalated Starches Reinforced with Acrylonitrile***

Graft copolymerization of acrylonitrile and starch was carried out using the method described by Pourjavadi *et al.* (2007) with slight modification. 20 g of starch was dispersed in 300 mL of distilled water, 1 g of the prepared CaO was added to the mixture and allowed to interact with the starch solution for 15 min as 20 mL of acrylonitrile was later added to the mixture with constant agitation. Graft copolymerization of acrylonitrile onto starch was done at 120ºC with constant agitation for 6 h, the pH of the product was adjusted to 7, the solution was washed to precipitate the polymer, the precipitate was centrifuged for 15 min at 6000 rpm and the supernatant was decanted. The residue was washed again with water to remove any remaining acrylonitrile, the final product was air-dried to obtain phthalated starch grafted with acrylonitrile.

$$\%\, Yield = \frac{weight\ of\ copolymer\ obtained}{weight\ of\ starch + weight\ of\ acrylonitrile} X\ 100 \quad (1)$$

***2.2.4. Statistical analysis***

All the analyses were done in triplicate and the data were subjected to Analysis of Variance (ANOVA) using SPSS (IBM Statistics 21). The results are presented as means of replicates (determined on a dry weight basis) ± standard deviation. The significant difference was at $p < 0.05$.

## 3.Results and discussions

### 3.1. Calcined Shells as Copolymerisation Catalyst

The percentage yield of calcined snail shell (also known as CaO) as obtained at 800ºC using a muffle furnace was 35.50%. The obtained CaO was used as catalyst in the co-polymerisation reaction.

### 3.2. Grafted Copolymers

The grafted copolymer yield of the native and phthalated starches is shown in Table 1.2. The acrylonitrile grafted percentage yield of native cassava starch copolymer (23.75%) was increased after treatment with phthalic anhydride (30.43%). This increase in percentage yield of phthalated starch acrylonitrile grafted copolymer may be that during the course of grafting, phthalated starch was modified and the modification was not done in native starches

**Table 1.** Yield of grafted copolymer

| Sample | Percentage yield (%) |
|---|---|
| NCSS | 23.75 ± 0.02 |
| PCSS | 30.43± 0.01 |

*NCSS - native cassava starch; PCSS - phthalated cassava starch*

### 3.3. FTIR Analysis of Grafted Copolymer

The FTIR spectra of the grafted native and phthalated starches are presented in Figure 1a and Figure 1b. In the spectrum of native cassava starch grafted copolymer (Figure 1a), the peak at 3454 and 2923 $cm^{-1}$ correspond to O-H and C-H stretches, respectively, while the peaks at 2364, 1447, and 1022 $cm^{-1}$ correspond to C≡N stretch, C-H bend, and C-O stretch, respectively. Similar report was submitted by Mano *et al.* (2003). Phthalated cassava starch grafted copolymer (Figure 1b) showed various peaks at 3428, 2976, and 2262 $cm^{-1}$ which are credited to O-H stretch, C-H stretch, and C≡N stretch, respectively, while the peaks at 1659, 1400, and 1181$cm^{-1}$ correspond to C=O, C-H bend, and C-O stretch, respectively. The spectra of the grafted native cassava starch copolymer and grafted phthalated

cassava starch copolymer which showed the presence of C≡N- at 2364 and 2262 $cm^{-1}$ respectively was an indication of grafting with acrylonitrile.

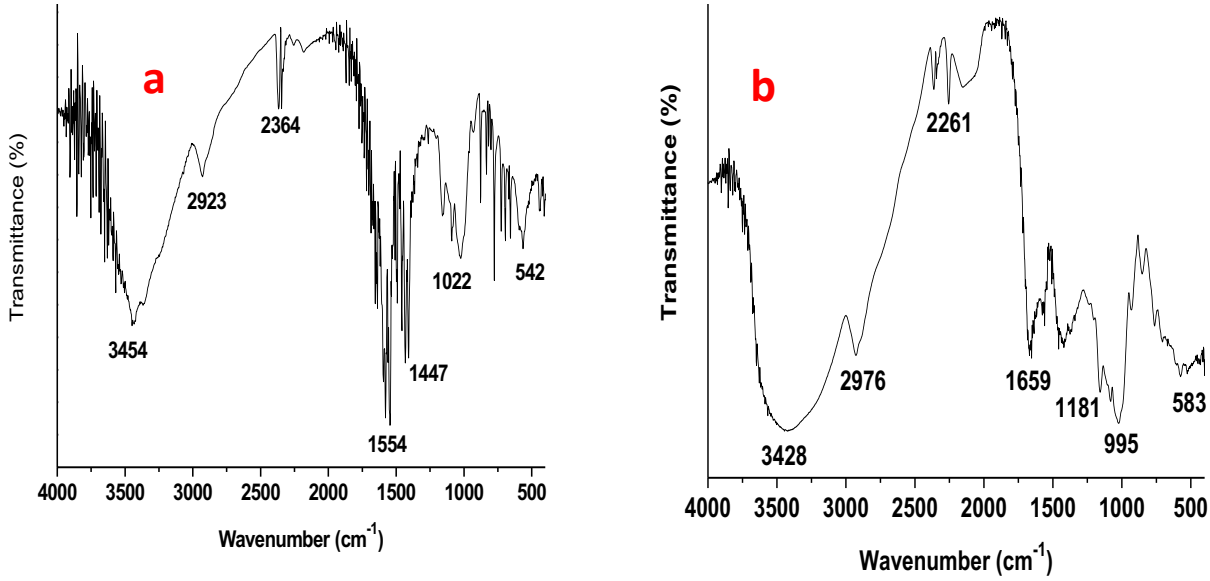


**Figure 1**. FTIR spectra of native cassava starch (a) and phthalated cassava starch grafted with acrylonitrile (b)

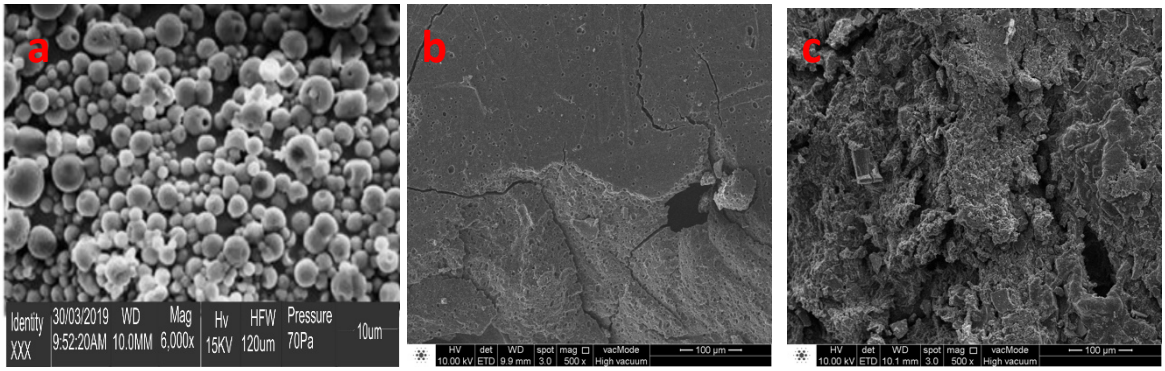


**Figure 2**. SEM of CaO (a), native (b) and phthalated (c) cassava starch grafted copolymers

### 3.4. SEM of CaO (Catalyst) and Grafted Copolymers

SEM analysis was conducted to examine the nature of the surface of the synthesized CaO and the result is presented in Figure 2a. The particles on the surface were spherical in shape, porous, and unevenly distributed probably. The uneven distribution could be due to the high calcination temperature in making the catalyst while liberating $CO_2$ (Ngamcharussrivichai *et al.,* 2010). The shape is similar to that of calcined limestone as reported by Sun *et al.* (2008) whereby the shapes are almost spherical. The surface morphology of native and modified starch grafted copolymers were also verified using Scanning Electron Microscopy (SEM) (Hitachi SU8030 FE-SEM Tokyo, Japan). The micrograph of the native cassava starch grafted copolymer is presented in Figure 2b. The surface was porous and eroded with cracks, similar morphology has been reported by Xu *et al.* (2004). The surface of phthalated cassava starch grafted copolymer (Figure 2c) showed coagulated and aggregated morphology. Exposure to heat during gelatinization process might have caused the granules of the surface of the grafted copolymer to adhere and stick together to form aggregates of coarse particles.

Also, surface roughness observed on the SEM of the grafted copolymer could be linked to the milling process carried out after drying (Casas *et al.,* 2009). However, rough nature of the surface of the grafted copolymer is expected to be favourable for adhesion improvement because increase in the roughness of the surface will lead to enhancement of surface area in bonding as well as mechanical interlocking (Kaith *et al.,* 2009).

### 3.5. X–Ray Diffraction Pattern of Grafted Copolymers

The XRD analysis was performed to know the effects of grafting on the crystallinity or amorphousity levels of native and phthalated starch grafted copolymers. The XRD patterns of the grafted copolymer of both native and phthalated starches are presented in Figure 3. The XRD pattern of native cassava starch (Figure 3a) had one major and broad diffraction peak at 21° (2θ) and another peak at 45° of angle 2θ, other regions of the spectrum were in the amorphous region. This is in line with the work of Kumar *et al.* (2014) which reported that graft reaction leads to amorphousness of structure. The phthalated cassava grafted copolymer (Figure 3b) showed various peaks at 7°, 18°, 23°, 28°, 29°, 32°, 33°and 45° of angle 2θ. The sharp peaks observed are actually a function of crystallinity in the copolymer. The incorporation of acrylonitrile into the framework of the phthalated cassava starch could have improved the crystalline nature of the copolymer.

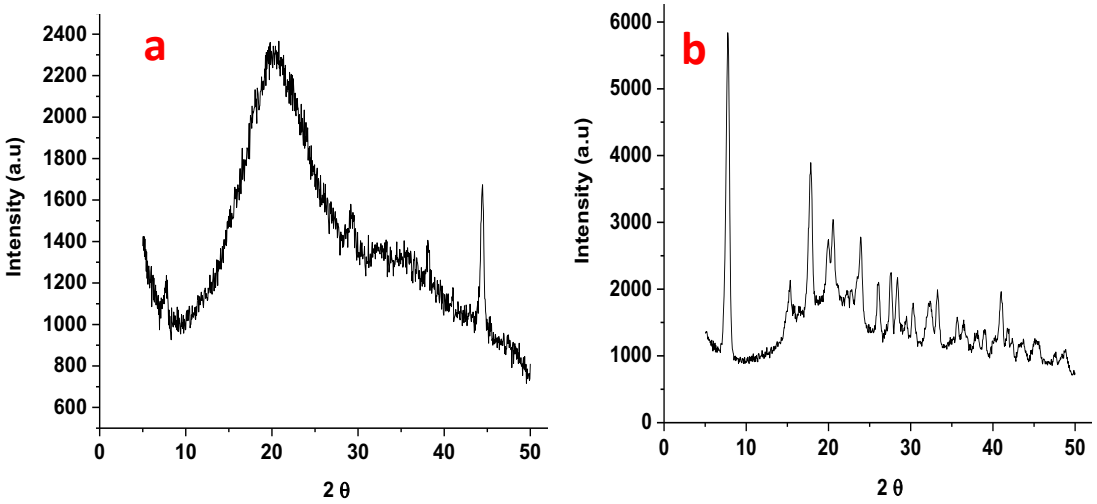


**Figure 3.** XRD of native (a) and phthalated (b) cassava starch grafted copolymers

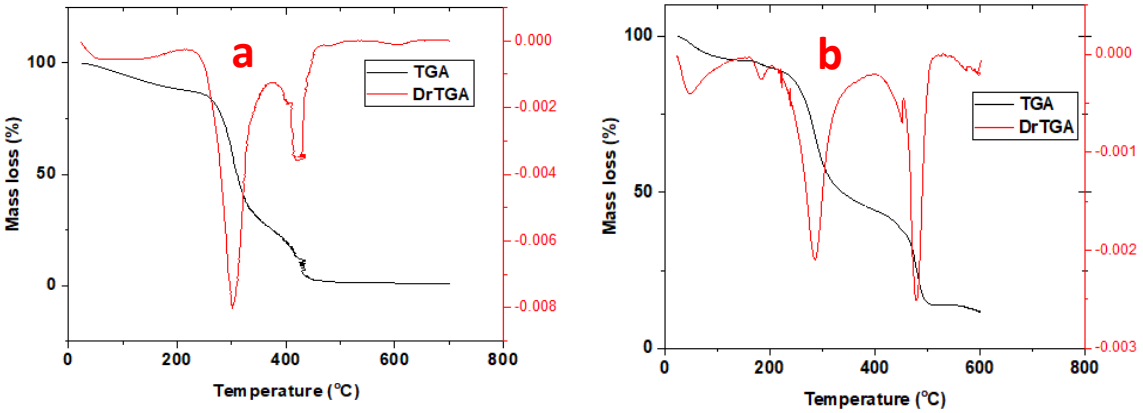


**Figure 4.** TGA of native (a) and phthalated (b) cassava starch grafted copolymers

### 3.6. Thermogravimetric Analysis of Grafted Copolymers

The Thermogravimetry analysis (TGA) curves of the of native and phthalated starch grafted copolymers are presented in Figure 4. The initial weight loss of native cassava starch grafted copolymer (Figure 4a) was observed at a temperature between 50 and 100$^{o}$C indicating the loss of moisture in the copolymer, another weight reduction was observed at 350$^{o}$C, this could be attributed to the decomposition of organic matters in the starch sample, further reduction in the weight of the native cassava starch copolymer was observed at 400$^{o}$C. At 420$^{o}$C, it was observed that the structural matrix has completely collapsed, leaving the residues as ash. For phthalated cassava starch grafted copolymer (Figure 4b), the initial weight loss began at temperature range of 50 to 200$^{o}$C, indicating the removal of water content in the granules. The second weight loss was observed at 300$^{o}$C which was largely due to the decomposition of organic matters that are present in the starch derivative. As the temperature increased, there was further reduction in the weight of the phthalated cassava starch grafted copolymer at 480$^{o}$C. At 600$^{o}$C, the sample has been reduced to ash which is the inorganic part.

## 4. Conclusions

Modification of starches often time is aimed at improving the physical and chemical characteristics of the natural polymers. In this present study, it was observed that the modified sample by Graft copolymerization had its both chemical and physical features improved in comparison to the native sample.

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# TOTAL PHENOLIC AND FLAVONOID CONTENT, AND ANTIOXIDANT ACTIVITY OF WHEAT BREAD ENRICHED WITH PUMPKIN, CHESTNUT AND ROSEHIP FLOUR

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**ABSTRACT**

Pumpkin, chestnut, and rosehip are rich in fibres, vitamins, mineral and antioxidant substances, but their use in bread making is not widespread yet. The objective of this study was to evaluate the effect of pumpkin, chestnut, and rosehip flour enrichment on the antioxidant activity, total phenolic and flavonoids contents of bread. Breads were made by partially replacing of wheat flour with pumpkin, chestnut, and rosehip flour at the level of 5, 10, and 15%. Total phenolic and flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power were examined to determine the content of ethanol extractable bioactive compounds. The results of all studies showed the highest activity and content of phenols and flavonoids in rosehip flour and bread obtained with it, and the lowest in the control sample (without additive). In all three studied supplements, with increasing their concentration, the amount of bioactive substances increases.

## 1.Introduction

In recent years, public attention has increasingly focused on the search for and consumption of healthy foods that reduce the incidence and spread of diseases such as diabetes, cardiovascular disease, obesity. Trends in the food industry are constantly changing to meet consumer demand. There is a tendency to use natural substances in the food as a source of antioxidant and functional ingredients (Meral and Köse, 2019).

Antioxidants eliminate free radicals in the cell and in food and are widely used in dietary formulations (Andrade *et al*., 2015). Polyphenols are natural phytochemicals with antioxidant properties. Flavonoids are phenolic substances isolated from a wide range of plants, where they act as antioxidants. When such substances are added to foods, they prolong their shelf-life. According to Wolfe *et al*. (2003), plant phenols prevent oxidation and maintain food product quality. Bread is one of the most popular foods in the world and is an important part of people's diets. The white bread is defined by a large part of the population as a good source of energy and irreplaceable food (Skrbic and Filipcev, 2008). However, people's increased awareness of the nutritional deficiencies of white bread is growing and this is the reason for looking for substitutes for commercial wheat flour. Besides white bread made from refined wheat flour is a food with a low antioxidant capacity (Meral and Köse, 2019) and low nutritional value (Ibidapo *et al.*, 2020). The health properties of bread can be improved by enriching it with flour rich in bioactive components.

Pumpkin flour (PF) contains phenolic acids, flavonoids, vitamin C, vitamin E, bilirubin, albumin and β-carotene that can act as

antioxidants in the human body (Wahyono *et al*., 2020). Generally, pumpkin flour is used to produce desserts as improved their yellow color and β-carotene content (Pongjanta *et al*., 2006). According to Dabash *et al*. (2017), pumpkin flour has a longer shelf-life and can be used in the manufacture of formulated foods. Pumpkin flour exhibited high levels of carbohydrate (79.57 %), starch (48.30 %), dietary fiber (12.1%) protein (7.81%) and total ash (5.29 %); low contents of lipid (3.60%) and crude fiber (3.65 %) (Saeleaw and Schleining, 2011).

Chestnut flour (CF) is a rich source of bioactive compounds that have high free radical scavenging properties being associated to protective effects against many diseases. The chemical composition of chestnut flour is similar to that of many other cereals (Aponte *et al*., 2014), which is why there is a growing interest in its use in the manufacture of bakery products. The composition of the flour includes: high quality proteins with essential amino acids (4–7%), relatively high amount of sugar (20–32%), starch (50–60%), dietary fiber (4–10%), and low amount of fat (2–4%) (Demirkesen *et al.*, 2010). Other important components of flour are vitamin E, vitamin B group, potassium, phosphorous, and magnesium (Sacchetti *et al*., 2004; Chenlo *et al*., 2007). There is insufficient data in the literature on the use of CF in bread recipes.

Rosehip flour (RF) is characterized by a high content of polyphenols, and has a high antioxidant activity (Ghendov-Mosanu *et al*., 2020). Common food obtained from rose hips are juice, wine, tea, jelly, marmalade, and syrup (Igual *et al*., 2021) and being rich in vitamin C, rosehip powder is successfully used as a health supplement (Larsen *et al*., 2003). There is no information in the literature on the use of RF in the production of bakery products. The only application that is detected is the use of the pomace resulting from juice production.

The objective of this study was to evaluate the antioxidant properties of wheat bread with the addition of pumpkin, chestnut, and rosehip flour (in the amount of 5%, 10% or 15% by the weight of flour).

## 2. Materials and methods

### 2.1. Materials

#### *2.1.1. Raw materials*

For the preparation of the bread samples, the following materials were used:

− commercial wheat flour (type 500) (average chemical composition: fat 0.9g/100 g of which saturated 0.3 g; carbohydrates 70.3 g/100 g, of which sugars 3.4 g, fiber 4.0 g/100 g; protein 10.8 g/100 g);

− pumpkin flour from defatted peeled pumpkin seeds (average chemical composition: fat 10g/100 g of which saturated 1 g; carbohydrates 34 g/100 g, of which sugars 0 g, fiber 16 g/100 g; protein 56 g/100 g);

− chestnut flour (average chemical composition: fat 3.7g/100 g of which saturated 0.7 g; carbohydrates 70.9 g/100 g, of which sugars 29.5 g, fiber 10.8 g/100 g; protein 6.4 g/100 g);

− rosehip flour (average chemical composition: fat 0 g/100 g of which saturated 0 g; carbohydrates 38 g/100 g, of which sugars 3 g, fiber 24 g/100 g; protein 2 g/ 100 g);

− water – according to ISO 6107-1:2004;

− commercial yeast (Lesafmaya);

− salt – according to Codex Standard for Food Grade Salt CX STAN 150-1985.

All chemicals used were of analytical grade and were obtained from Merck KGaA (Darmstadt, Germany) via Fillab (Plovdiv, Bulgaria).

### 2.2. Methods

#### *2.2.1. Preparation of dough and bread samples*

Bread was obtained by a two-phase method. Initially, knead the yeast, flour (control and experimental samples to obtain 100 g) and water of dough in a 1:1 ratio in kneading machine (Labomix 1000, Hungary). The dough thus prepared matured for 60 min at 33 °C and then was mixed to obtain a homogeneous mass by adding the remainder of the flour according to the formulation and salt (1.33 kg/100 kg flour). The bread dough was divided into pieces of a certain weight (440 g) and was formed, matured

for 55 minutes at 36 °C (Tecnopast CRN 45–12, Novacel ROVIMPEX Novaledo, Italy). After the final fermentation, the pieces of dough were put into an electric oven (Salva E-25, Spain) pre-heated to 220–230 °C. The baking time was 24 min. After baking, the bread was allowed to cool down for 3 h at room temperature.

The details of bread formulations are given in Table 1.

***2.2.2. Extraction of bioactive compounds***

Before the extraction, breads were sliced (about 1.5 cm thick), dried (40 °C, 24 h), ground in a mill, and sieved (0.5 mm sieve). After that the extraction of bioactive compounds was carried out with 70% ethanol (solid to liquid ratio 1:20) in an ultrasonic bath (VWR, Malaysia; 45 kHz, 30 W) at 45 °C for 15 min according to Vasileva *et al.* (2018). Solid remnants of bread were removed by centrifugation at 1800xg for 15 min (MPW-251, Med. Instruments, Poland) and obtained supernatants were used for further studies.

***2.2.3. Analytical methods***

Folin-Ciocalteu's reagent was used to determine of total polyphenols (Ainsworth and Gillespie, 2007) and the results were expressed as mg equivalents gallic acid (GAE) per gram dry weight (DW). Total flavonoids were determined using $Al(NO_3)_3$ reagent (Kivrak *et al.,* 2019) and the results were expressed as mg equivalents quercetin (QE) per gram DW.

***2.2.4. In vitro determination of antioxidant activity***

DPPH radical scavenging activity was estimated according to Dimov *et al.* (2018) with some modification – in a test tube were mixed 0.15 ml of ethanolic extract with 2.85 ml 0.06 mM DPPH (dissolved in 96% ethanol; freshly prepared). After 30 minutes in the dark at room temperature, the absorbance at 517 nm was read by spectrophotometer and the results were compared with the blank containing 70% ethanol (without sample addition). The results of DPPH analysis were expressed as mM Trolox equivalents (TE) per gram DW.

FRAP method – Ferric Reducing Antioxidant Power was measured according to the method of Dimov *et al.* (2018) with modifications – three ml FRAP reagent (freshly prepared) were mixed with 0.1 ml of the extracted sample. After incubation for 10 min at 37 °C (in the darkness), the absorbance was measured at 593 nm against blank prepared with 70% ethanol (without sample addition). A standard curve was built with $FeSO_4.7H_2O$. The results of FRAP analysis were expressed as μmol $Fe^{2+}$ equivalents per gram DW.

**Table 1.** The formulations of breads (% on the flour basis)

| Ingredients | Bread samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | with PF (%) | | | with CF (%) | | | with RF (%) | | |
| | | 5 | 10 | 15 | 5 | 10 | 15 | 5 | 10 | 15 |
| WF, g | 450 | 427.5 | 405.0 | 382.5 | 427.5 | 405.0 | 382.5 | 427.5 | 405.0 | 382.5 |
| Water, ml | 248 | 248 | 248 | 248 | 248 | 248 | 248 | 248 | 248 | 248 |
| Yeast, g | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 |
| Salt, g | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 |
| PF, g | - | 22.5 | 45.0 | 67.5 | - | - | - | - | - | - |
| CF, g | - | - | - | - | 22.5 | 45.0 | 67.5 | - | - | - |
| RF, g | - | - | - | - | - | - | - | 22.5 | 45.0 | 67.5 |

WF – wheat flour, PF – pumpkin flour, CF – chestnut flour, RF – rosehip flour.

### *2.2.5. Data analysis*

The tests were conducted with three replications. Data were analyzed by one-way analysis of variance (ANOVA) using Statgraphics Centurion statistical program (version XVI, 2009) (Stat Point Technologies, Ins., Warrenton, VA, USA). To compare the means, Fisher's least significant difference test was used for paired comparison with a significance level $\alpha = 0.05$.

## 3.Results and discussions

### 3.1. Determination of bioactive substances in different flours

The results of all analyzes of the flours are summarized in Table 2. Total flavonoid content ranged from 0.15 to 1.77 mg QE/g DW, as the highest value was registered at RF, while the lowest value, as expected, showed the control commercial wheat flour. From the studied 3 types of substitute flours the lowest content of total flavonoids showed PF (0.27 mg QE/g DW). These results show that it is likely that in the course of defatting of pumpkin seeds, treatment with organic solvents also affected the content of bioactive substances. The results obtained by us for the RF were similar to the data from the literature on the high content of common flavonoids. Igual *et al*. (2021) found 0.43 g/100 g DW total flavonoid content in rosehip pomace.

**Table 2.** Total flavonoids and phenols, and antioxidant activity of ethanolic extracts from flours

| Flour sample | Total flavonoids, mg QE/g DW | Total phenols, mg GAE/g DW | DPPH, mM TE/g DW | FRAP, µmol $Fe^{2+}$/g DW |
|---|---|---|---|---|
| WF | 0.15±0.00$^{d}$ | 0.98±0.03$^{d}$ | 0.35±0.03$^{d}$ | 2.38±0.05$^{d}$ |
| CF | 1.04±0.00$^{b}$ | 1.99±0.06$^{b}$ | 7.25±0.03$^{b}$ | 25.63±0.07$^{b}$ |
| PF | 0.27±0.01$^{c}$ | 1.25±0.05$^{c}$ | 2.41±0.23$^{c}$ | 10.43±0.20$^{c}$ |
| RF | 1.77±0.00$^{a}$ | 9.56±0.04$^{a}$ | 8.37±0.01$^{a}$ | 57.76±0.00$^{a}$ |

*$^{a-d}$: Means in a column without a common letter differ significantly ($p < 0.05$).*
*Results were calculated on a dry matter basis (DW): 87.49% ± 0.04 for WF, 92.12% ± 0.17 for CF, 92.95% ± 0.02 for PF, and 88.59% ± 0.06 for RF.*

The polyphenol content of the flour follows the same order as the results for flavonoids, in increasing order, as follows: WF, PF, CF, RF (0.98-9.56 mg GAE/g DW). The results obtained by us for wheat flour were lower than those of Zhang *et al*. (2021) but probably the duration of storage of the flour and the extraction conditions are the reason for these differences. The values of total phenols in PF were again lower than those reported by another team of authors, according to which total polyphenols in pumpkin seed flour are in the range 2.44–3.82 mg GAE/g (Peng *et al*., 2021). The most significant source of phenols from the three substitute flours studied is RF (9.56 mg GAE/g DW) in which the amount of phenols was almost 10 times higher than the control sample of flour (0.98 mg GAE/g DW).

The data from DPPH and FRAP methods follow the same trend as those for total phenols and flavonoids. Again, the highest values of the studied 3 additives were measured for RF as in comparison with the control the increase was by 24 times in both methods – from 0.35 to 8.37 mM TE/g DW and from 2.38 to 57.76 µmol $Fe^{2+}$/g DW, respectively. Huang *et al*. (2015) also reported that the antioxidant activity is directly proportional to the total phenol. As a result, the higher was the phenolic content in a compound the higher was its antioxidant activity.

### 3.2. Determination of bioactive substances in breads

As can be seen from Table 3, an increase ($p < 0.05$) in total phenols and flavonoids, and DPPH and FRAP values was observed with the addition of substitute flours to the formulations. In our research, bread containing CF, PF and RF showed in most of the cases higher values in

comparison with the control bread. However, in general, the amount of bioactive substances was significantly lower than the reported values in flour (Table 2). A similar decrease in *in vitro* antioxidant activity in the production of bread with the addition of herbs has been reported by another authors (Dimov *et al.*, 2018). Probably, the heat treatment during the baking of the bread damages or destroys the antioxidant compounds presented in different flours. According to Chlopicka *et al*. (2012), during thermal processes, the antioxidants are subject to modifications.

**Table 3.** Total flavonoid and phenol contents of ethanolic extract from bread samples

| Bread sample | Total flavonoids, mg QE/g DW | Total phenols, mg GAE/g DW |
|---|---|---|
| Control | $0.12\pm0.00^{g}$ | $0.55\pm0.03^{h}$ |
| CF5 | $0.14\pm0.00^{f,B}$ | $0.96\pm0.03^{f,C}$ |
| CF10 | $0.16\pm0.00^{cd,A}$ | $1.06\pm0.02^{e,B}$ |
| CF15 | $0.16\pm0.01^{bc,A}$ | $1.28\pm0.04d^{,A}$ |
| PF5 | $0.15\pm0.00^{de,B}$ | $0.32\pm0.01^{i,C}$ |
| PF10 | $0.16\pm0.00^{bc,A}$ | $0.80\pm0.06^{g,B}$ |
| PF15 | $0.16\pm0.00^{b,A}$ | $1.09\pm0.02^{e,A}$ |
| RF5 | $0.15\pm0.00^{e,C}$ | $1.49\pm0.03^{c,C}$ |
| RF10 | $0.16\pm0.00^{bc,B}$ | $1.95\pm0.02^{b,B}$ |
| RF15 | $0.17\pm0.00^{a,A}$ | $2.86\pm0.03^{a,A}$ |

*$^{a-i}$: Means in a column without a common letter differ significantly ($p < 0.05$).*

*$^{A-C}$: Means of the three samples (in a column) with various concentrations (5, 10 and 15 %) for a certain type of bread without a common letter differ significantly ($p < 0.05$).*

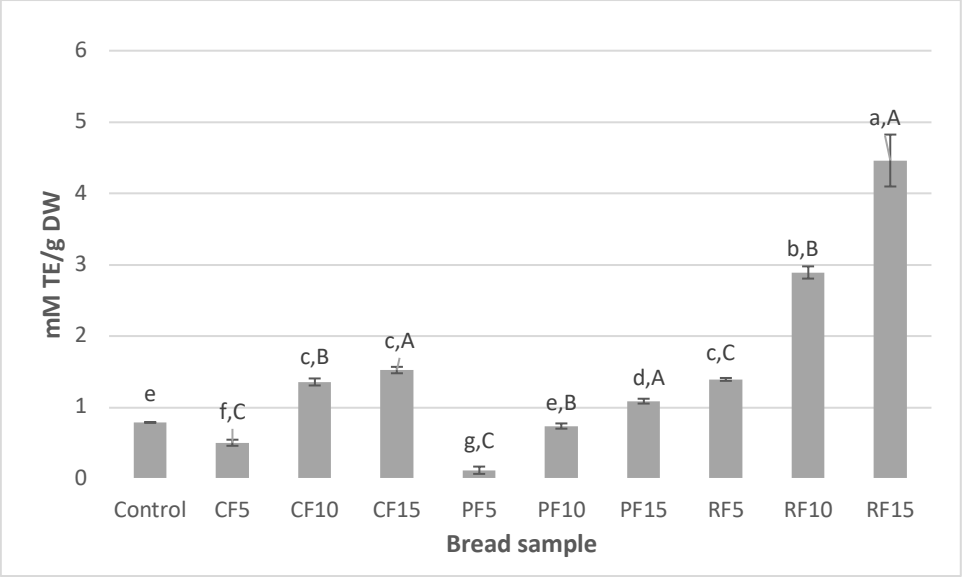


**Figure 1.** DPPH radical scavenging activity of ethanolic extracts from bread samples

*$^{a-e}$: Means without a common letter differ significantly ($p < 0.05$).*

*$^{A-C}$: Means of the three samples with various concentrations (5, 10 and 15 %) for a certain type of bread without a common letter differ significantly ($p < 0.05$).*

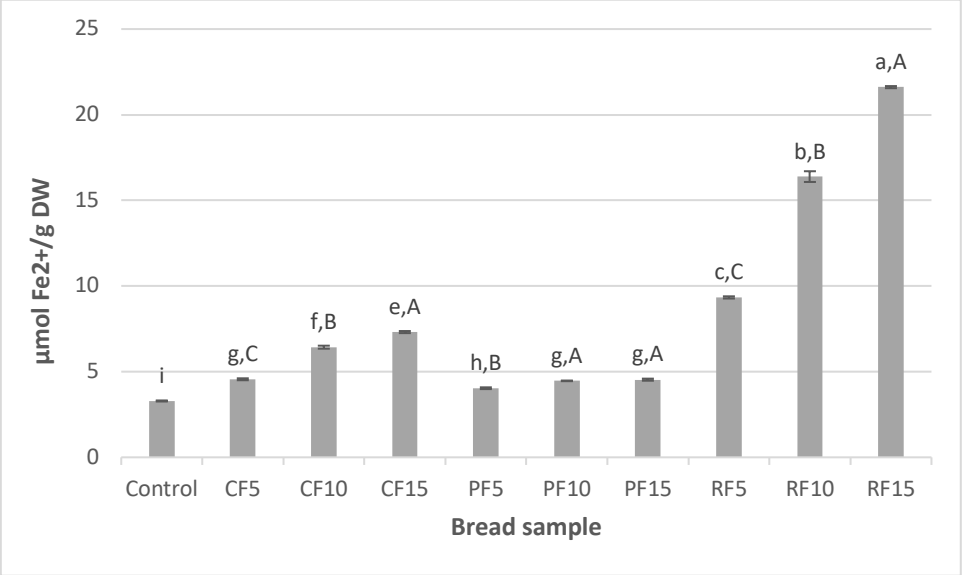


**Figure 2.** Ferric reducing antioxidant power of ethanolic extracts from bread samples
*[a-i]: Means without a common letter differ significantly ($p < 0.05$).*
*[A-C]: Means of the three samples with various concentrations (5, 10 and 15 %) for a certain type of bread without a common letter differ significantly ($p < 0.05$).*

*Results in Table 3, and Figures 1 and 2 were calculated on a dry matter basis (DW): Control – 95.14% ± 0.57, CF5 – 95.63% ± 0.22, CF10 – 96.10% ± 0.13, CF15 – 96.54% ± 0.46, PF5 – 96.06% ± 0.25, PF10 – 95.63% ± 0.08, PF15 – 95.95% ± 0.25, RF5 – 95.77% ± 0.13, RF10 – 95.94% ± 0.14, RF15 – 96.20% ± 0.04.*

The amount of total flavonoids in the breads was in the range 0.12-0.17 mg QE/g DW and that of total phenols was 0.55-2.86 mg GAE/g DW. The results demonstrated that these contents also raised with the increase in the level of substitute flours in breads. The highest total polyphenol content was found in a bread sample with the addition of 15% of RF, while the lowest one was seen in bread without additive. The percentage increase in bioactive substances in breads was not as noticeable as in flour. For example, the lowest results were observed for flavonoids, where the greatest increase compared to the control was less than once. In studies by other authors, flavonoids in bread were not even registered. For example, Petkova *et al*. (2018) found 45.6 mg QE/100 g total flavonoids in flour mixture, but any flavonoids were not detected in bread samples prepared from the mixture. This due to the greater sensitivity of flavonoids at high baking temperatures, because most bioactive compounds become unstable when exposed to heat. The extractability of the bioactive components after baking the bread at high temperature decreased, which was associated with the formation of complexes between polyphenols (e.g. flavonoids) with proteins and starch. According to Zhang *et al*. (2011), the starch interacts with quercetin, which reduces the digestibility of starch, as these interactions affect the bioactivity of antioxidants. Antioxidant activity of bread enriched with CF, PF and RF are shown in Figures 1 and 2. In Fig. 1, antioxidant activity of bread measured by the ability of the test sample to scavenge DPPH radical. From the three studied concentrations of added flours, the highest values were observed at 15% concentration. Rosehip flour addition significantly affected ($p < 0.05$) antioxidant activity (DPPH) of enriched bread (4.46 mM TE/g DW and 21.61 µmol $Fe^{2+}$/g DW). From the data in Fig. 1 interesting impression was made by the use of 5% supplement of CF and PF – DPPH values were lower compared to the control bread. Probably, that is due to the

insufficient amount of imported substitute flour since wheat flour was dominant in the mixture. Wahyono *et al*. (2020) also established that increased pumpkin powder concurrently increased antioxidant activity of resulting bread. The highest values were measured when using the highest percentage of added flour (20%) and the control has the lowest antioxidant activity.

Of interest is the not so big difference in the increase of the value in the FRAP method, when 5, 10 and 15% PF were used as an additive. When adding this flour in a concentration of 5%, the value was 4.03 μmol $Fe^{2+}$/g DW, at 10% - 4.46 μmol $Fe^{2+}$/g DW and at 15% - 4.53 μmol $Fe^{2+}$/g DW, as between 10 and 15% there was no statistically significant difference in the results obtained by the FRAP method. When using CF, the increase in FRAP units with an increase in the concentration of the additive compared to the control was 1.39, 1.95 and 2.21 times, respectively. However, when RF is added in the same concentrations, the increasement was with 2.84, 4.81, and 6.57 times, respectively. Irakli *et al*. (2015) replaced wheat flour with rice bran in the bread recipe resulted in breads with a significantly increased total antioxidant activity. The authors found that with the increase of the level of rice bran from 10 to 30%, total antioxidant activity increased 2 to 5 times compared to the control. Increase in antioxidant activity within the same limits reported and Selimović *et al*. (2014) when replacing wheat flour with buckwheat flour. The inclusion of 15-40% buckwheat flour led to an increase in antioxidant activity from 2 to 5 times. According to our findings, chestnut flour, pumpkin flour, and especially rosehip flour are good sources of phenols and possessed good antioxidant activity.

## 4. Conclusions

The addition of CF, PF and RF increased phenolic compounds, flavonoids and antioxidant activity when compared to the control bread. Their quantity increased with the increasing of the level of the substitute flour. The highest content of total phenols and flavonoids, and the highest antioxidant activity, measured by DPPH and FRAP methods, showed RF and the breads obtained from it, while the lowest were the results for PF and the breads obtained from its use. As the results were higher than those of the control, these flours have proven to be an effective ingredient to enrich bread, which can improve its taste, aroma and shelf-life.

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# COMPARATIVE ANALYSES OF PROXIMATE COMPOSITION, BIOACTIVE COMPOUND AND ANTIOXIDANT ACTIVITY IN DIFFERENT PARTS OF GREEN AND RIPE PASSION FRUIT

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**ABSTRACT**

The current research was undertaken to compare the proximate composition and antioxidant activity of different parts of green and ripe passion fruit. The proximate composition, bioactive compounds and antioxidant activity of different parts of passion fruit were analyzed by standard methods. According to the results, the ash content of the peel and seed of ripe passion fruit was higher than that of the green one, but only significant ($P<0.05$) for the seed. Besides, the crude fiber, carbohydrates, and protein content of the peel, pulp, and seed of ripe passion fruit were relatively higher ($P<0.05$) than those of the green one. At $P<0.05$, the fat content of green peel and pulp was higher than that of ripe, but the seed content of ripe was higher. The peel and pulp of green passion fruit contain the highest amounts of total polyphenol content (TPC) and total flavonoid content (TFC) than ripe passion fruit ($P<0.05$). The highest amount of TPC is contained in the seed of green passion fruit than in ripe fruit ($P>0.05$), but the seed of ripe fruit contains TFC almost twice as much as green fruit ($P<0.05$). It was also found that the peel and pulp of ripe passion fruit contain a higher percentage of total antioxidant activity (TAA) and vitamin C ($P<0.05$) than the pulp of a green one. The seed of the green one contains a slightly higher percentage of TAA than the seed of the ripe fruit ($P<0.05$), but vitamin C was opposite ($P<0.05$). It is concluded that the pulp, peel, and seed of green and ripe passion fruit have great potential as a health-promoting source that is normally underutilized.

## 1. Introduction

Micronutrients may be found in abundance in fruits and vegetables. Furthermore, these foods are high in phytochemical compounds (mostly polyphenols), which have a variety of wellbeing properties (Singh *et al.,* 2016a). These phytochemicals reduce oxidative stress and have synergistic actions, resulting in anti-inflammatory, antibacterial, anti-mutagenic, anti-tumor, and cognitive activities (Kang *et al.,*

2011; Zielinski *et al.,* 2014; Sing *et al.,* 2016b). Polyphenols have been found in a broad variety of fruits and vegetables. Furthermore, they provide enough amounts of dietary fiber, which is crucial for maintaining intestinal health as well as preventing cardiovascular illnesses, cancer, obesity, and diabetes (Elleuch *et al.,* 2011). As a result, in the human diet, frequent eating of fruits and vegetables is suggested. Bangladesh is endowed with a wide variety of fruits and was ranked sixth in the world for tropical fresh fruit output in 2017 (FAOSTAT, 2019). A large number of tropical fruits, sometimes referred to as indigenous or small fruits, are underutilized. These fruits are not commonly available in national or international marketplaces since they are not commercially grown. These fruits are typically found in backyards, unused highlands, hill tracts, and along roadsides. These fruits require no special care or agricultural management, which is why they are referred to as "underutilized small indigenous fruits" in Bangladesh, where they are being decimated year after year. These small fruits, on the other hand, can be an excellent source of vitamins and antioxidants (Molla *et al.,* 2021).

Passion fruit, or *Passiflora edulis*, is a tropical and subtropical fruit that is important due to its balanced nutrition and health advantages (He *et al.,* 2020). The genus Passiflora is the biggest in the Passifloraceae family, with over 500 species. Passiflora edulis, in particular, stands out due to its economic and therapeutic significance. (Dhawan *et al.,* 2004). It is commonly planted in tropical and subtropical locations across the world, particularly in South America, Florida, the Caribbean, South Africa, and Asia (Zhang *et al.,* 2013; Yuan *et al.,* 2017; Hu *et al.,* 2018). In Bangladesh, passion fruit, locally named Tang Phal, which is high in vitamins A and C, has been grown in Chittagong's hill areas for a few years, but it is not available in other areas. However, the Bangladesh Agricultural Research Institute developed a local variety called BARI Passion Fruit-1 in 2003, which has been growing in some areas of Panchagarh district (Daily Star, 2022).

Apart from their enormous economic and nutritional potential (Santana & Naves, 2003), there are very few manufactured goods on the market. However, there is very little information on the chemical characteristics and potential of these fruits, especially when it comes to their green to ripe, pulps and edible seeds, and the material that is accessible is dispersed or does not meet scientific requirements. Furthermore, in Bangladesh, research on the bioactive components and antioxidant activity of passionfruit is pretty scarce. As a result, the current study was conducted to determine the proximate composition, bioactive compounds, and antioxidant activity of different parts of green and ripe passion fruit accessible in Chittagong, Bangladesh, in order to enhance awareness of these fruits and to expand the possibilities for their use in new high-value products.

## 2. Materials and methods

### 2.1. Research plan and study location

Investigative research based on proximate composition, bioactive compound and antioxidant activity of different parts of green and ripe passion fruit. Samples of green and ripe passion fruit were purchased from the local market in Cox's Bazar, Chittagong, Bangladesh. The study was conducted in the research laboratory of the Food Technology and Nutritional Science (FTNS) department at Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh.

### 2.2. Analysis of proximate composition

#### *2.2.1. Analysis of moisture content*

The percentage of moisture was examined by the procedure given in AOAC (2005) method. At first, the empty crucible was dried in the oven at 105℃ for 3-4 h and transferred to the desiccators to cool. Then the empty crucible was weighed through a digital electronic balance, and 3 g of sample was placed in the crucible. Again, the weight of the crucible with the sample was collected. Then the sample in the

crucible was reserved in the oven at 105℃ for 3-4 h. After heating it was cooled in desiccators. Again, the weight of the crucible with the sample was determined until the weight became stable. The following equation was used for the calculation of moisture percentage.

$$\text{Moisture content (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Sample weight}} \times 100. \quad (1)$$

### *2.2.2. Analysis of ash content*

Ash content was measured using the AOAC (2005) method's instructions. A sample of about 3 g was taken, heated in a muffle furnace for 3–4 h at 105°C, and then heated for 3–4 h at 600°C (JSMF-45T). The sample was then cooled in desiccators, and the percentage of ash content was determined using the equation below.

$$\text{Ash content (\%)} = \frac{\text{Weight of the ash}}{\text{Sample weight}} \times 100. \quad (2)$$

### *2.2.3. Analysis of crude fat*

According to the AOAC (2005) method, the crude fat content was assessed. Overnight, a beaker containing the extracted oil was left in the incubator at 105°C. To filter and wrap, weigh approximately 5 g of the sample. The sample was then transferred into an extraction thimble, placed in a Soxhlet, which was then filled with petroleum ether in an amount equal to about 250 mL, and placed on a heating mantle. The heating mantle was turned on after the Soxhlet apparatus was connected and the water was turned on to cool them. The sample was heated at a rate of 150 drops/m for about 3–4 h. Then the beaker that had previously been weighed was filled with the solvent-extracted oil. The beaker was then kept in the oven and heated to between 80 and 90 degrees Celsius until the solvent had completely evaporated and the beaker was dried. The beaker was then moved to the desiccators to cool after drying, and its dried contents and the sample's fat content were calculated using the equation below.

$$\text{Fat content (\%)} = \frac{\text{Weight of the extracted oil}}{\text{Sample weight}} \times 100 \quad (3)$$

### *2.2.4. Analysis of crude protein*

According to the AOAC (2005) method, the crude protein content was assessed. By estimating the nitrogen content of a food product and multiplying the nitrogen value by 6.25, one can determine the protein content of that food product. Since the nonprotein content (NPN) of the material is taken into account, this is referred to as the crude protein content. By taking non protein nitrogen out, one can calculate the true protein nitrogen (which is estimated by pirating the protein in the sample with trichloro acetic acid, copper hydroxide and determining the residual nitrogen in the protein-free filtrate). The Kjeldahl method was used to estimate the nitrogen content because it is based on the fact that organic nitrogen is converted to ammonium sulfate when it is digested with sulfuric acid in the presence of copper, a catalyst. The protein content of the sample was determined using the following equation by distilling the ammonium released during the alkalinization of the solution into a known volume of a sulfuric acid.

$$\text{Protein content (\%)} = \frac{(c-b)\times 14 \times d \times 6.25}{a \times 1000} \times 100. \quad (4)$$

Here, a = sample weight in gm, b =volume of NaOH to neutralize 25mL of 0.1N $H_2SO_4$, c =volume of NaOH to neutralize 0.1N $H_2SO_4$ in control or back titration, d =strength of NaOH (normality of NaOH), 6.25 =gravimetric factor of protein in food.

#### *2.2.4.1. Digestion*

A precise measurement of 0.54 g of the sample's weight was made on weighing paper. This sample was poured into a 500 mL clean, oven-dried Kjeldal flask, and then 25 mL of pure $H_2SO_4$ and 5 g of digestion mixture were added. The flask was filled with a glass rod to prevent foaming and clumping. With the exception of the sample materials for comparison, black was transported with all reagents. The flask was subsequently heated in a Kjeldahl digestion chamber, first at a low temperature (40°C) until the mixture was no longer forth, then the temperature was raised to 60°C, and heating was continued until the solution was colorless. The

flask was cooled after the digestion period and then diluted with 100 mL of distilled water. A sample piece of litmus paper was placed in the solution and the reaction was found to be acidic.

***2.2.4.2. Distillation***

Before beginning the distillation process, the Kjeldahl apparatus distillation set was thoroughly cleaned with distilled water. The receiving 250 mL conical flask received 0.25 mL of 0.1N $H_2SO_4$. 75 mL of 40% NaOH was poured down the side of the Kjeldahl flask from a measuring cylinder. The solution turned alkaline, as indicated by the litmus paper turning blue. The flask's mouth was sealed with a stopper that had a connecting tube that led to a flask holding 0.1N H2SO4 for receiving ammonia. Water and ammonia distilled over at a steady, moderate rate as the mixture was heated to a boil. In order to prevent the $H_2SO_4$ solution from being drawn into the Kjeldahl flask, the heating was neither too slow nor too fast. So that the distilling ammonia did not escape the $H_2SO_4$ without absorption.

***2.2.4.3. Titration***

Utilizing three drops of methyl red as an indicator, the ammonia absorbed in the receiving flask containing 0.1N $H_2SO_4$ was titrated with 0.1N NaOH. A blank reagent was also distilled and titrated similarly.

$$\text{Protein (\%)} = \frac{(\text{mL of sample} - \text{mL of blank}) \times 0.1 \times 1.4007}{\text{sample weight}} \times \text{Protein factor.} \quad (5)$$

***2.2.5. Determination of crude fiber content***

According to the AOAC (2005) method, the crude fiber content was assessed. 200 mL of boiling 0.225 N (1.25% w/v) sulfuric acid was added to the sample after it had been weighed to be about 5 g (W) of moisture and fat free sample. After 30 m of boiling, water was added to the mixer to keep the volume constant. After boiling, the mixture was filtered, and the remaining material was repeatedly washed in hot water to remove any remaining acids. Then, to the acid-free residue, 200 mL of 0.313 N (1.25%) NaOH were added. After 30 m of boiling, filtering, and hot water washing, the residue was made alkali-free. After cleaning the mixture with ether and alcohol, respectively, the residue was prepared for estimating crude fiber. Following that, the porcelain crucible was weighed, the leftovers were kept inside, and it was heated for 3 h at 105°C in the oven. After that, the sample was cooled in desiccators. The sample's weight is determined and considered (A). The sample is then heated to 600ºC for 3–4 h in a muffle furnace. The crucible containing the sample was then cooled in desiccators. Next, the sample is weighed. This weight is considered as weight (B). The crude fiber content of the sample is determined by subtracting the weight (B) from the weight (A). Crude fiber content was calculated using the following equation:

$$\text{Crude fiber (\%)} = \frac{A-B}{W} \times 100. \quad (6)$$

where, W = weight of moisture and the fat-free sample taken, A = weight of the crucible with contents after ashing, and B= weight of the crucible with contents before ashing.

***2.2.6. Determination of carbohydrate content***

The anthrone method was used to calculate the carbohydrate content. In order to convert carbohydrates into simple sugars, concentrated sulfuric acid is used. Glucose is dehydrated to hydroxymethyl furfural in a hot acidic medium. The product of this compound and anthrone has a green color and a maximum absorption wavelength of 620 nm. In a different test tube, 0.5 mL of standard glucose solution, 4 mL of anthrone reagent, and a previously prepared sample are combined for this purpose. Using a UV spectrophotometer, measure the absorbance at 620 nm after mixing and boiling the ingredients. The following equation was used to determine the amount of carbohydrates.

Conc. of unknown = (Absorbance of unknown/Absorbance of standard) × Conc. of standard. (7)

**2.3. Analysis of bioactive compounds**

***2.3.1. Determination of total phenolic content (TPC)***

Using the Folin-Ciocalteu method, the TPC of all the herbs and spices was ascertained. By creating the dilutions of (0.1, 0.5, 1.0, 2.5, and 5 mg/mL) in methanol, a standard gallic acid curve was created. Each of these dilutions was

diluted each time in 100 µL of water, which was then combined with 100 µL of Folin-Ciocalteu reagent and left to stand for 6 m. 500 µL of distilled water and 1 mL of sodium carbonate at 7% were then added to the reaction mixture. At 760 nm, the absorbance was measured spectrophotometrically after 90 m. All the samples underwent the exact same process. The amount of gallic acid equivalents (mgGAE/g) used to measure the TPC of the herbs and spices

***2.3.2. Determination of total flavonoid content (TFC)***

The TFC was determined by an aluminum chloride complex-forming assay. A calibration curve for quercetin was constructed by preparing the dilution of (0.1, 0.5, 1.0, 2.5 and 5mg/mL) in methanol. 100 µL of each of the quercetin dilutions was mixed with 500 µL of distilled water and then with 100 µL of 5% Sodium nitrate and allowed to stand for 6 minutes. Then 150 µL of 10% aluminum chloride solution was added and allowed to stand for 5 m after which 200 µL solution of 1M Sodium hydroxide was added sequentially. On a UV spectrophotometer, the absorbance of this reaction mixture was measured at 510 nm. All the samples underwent the exact same process. And (mgQE/g) was calculated as the TFC.

**Table 1.** Concentration and absorbance of gallic acid

| Concentration (mg/mL) | Absorbance at 760 nm |
|---|---|
| 0.1 | 0.71 |
| 0.5 | 1.024 |
| 1 | 1.23 |
| 2.5 | 2.007 |
| 5 | 3.088 |

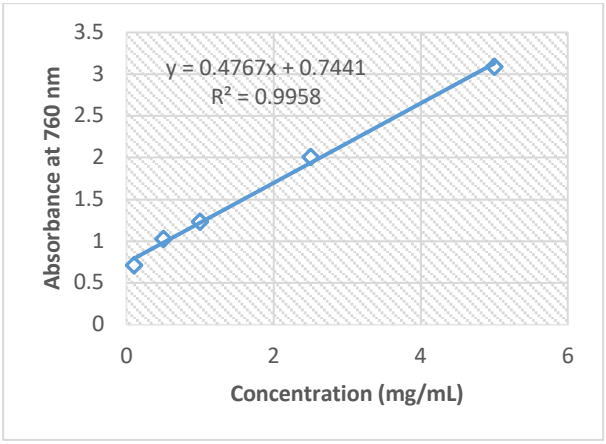


**Figure 1:** Standard curve of gallic acid

**Table 2:** Concentration and absorbance of quercetin

| Concentration (mg/mL) | Absorbance at 510 nm |
|---|---|
| 0.1 | 0.089 |
| 0.5 | 0.168 |
| 1 | 0.244 |
| 2.5 | 0.436 |
| 5 | 0.733 |

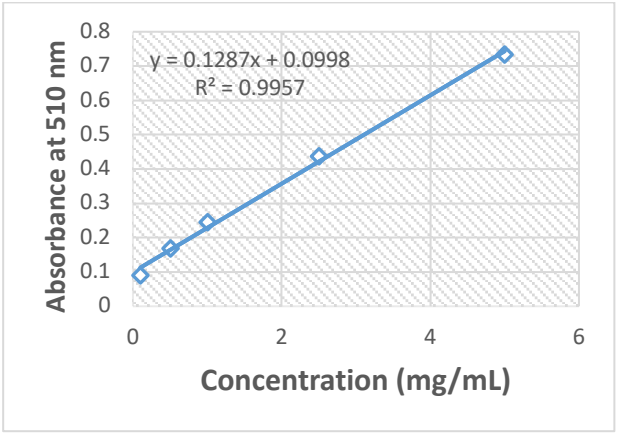


**Figure 2:** Standard Curve of quercetin

## 2.4. Analysis of antioxidant activity

***2.4.1. Determination of total antioxidant activity***

Using the free radical scavenging technique 2,2-Diphenyl-1-picrylhydrazyl (DPPH), the antioxidant properties of the extracts were assessed (Brand-Williams et al., 1995). In methanol, oxidized DPPH produces a deep violet hue. An antioxidant compound reduces

DPPH by giving it an electron, changing its color from deep violet to yellow as a result. A spectrophotometer is used to measure the absorption at 515 nm, which yields the sample's percent inhibition (Shirazi, 2014). Getting the sample extract ready Weighing 2g of the sample, 10 mL of methanol was added to the beaker. then extracted for 4 h while using a magnetic stirrer. After extracts were obtained, what-man filter paper No. 42 was used to filter them. The absorbance of a freshly made 0.002% solution of DPPH in methanol was measured at 515 nm. A 3 ml solution of DPPH was added to 50 µl of the pure extract, which was then left to stand in the dark for 15 m. At 515 nm, the absorbance was once more measured. The formula below was used to determine the extract's percentage inhibition of DPPH:

Percent of Inhibition: (A-B/A) × 100. (8)

Where, A is the absorbance of pure DPPH in oxidized form and B is the absorbance of the sample taken after 15 m of reaction with DPPH.

#### *2.4.2. Determination of vitamin C content*

According to the AOAC method (2000), vitamin C content was measured. This was accomplished by titrating 10 mL of a standard vitamin C solution with the dye solution in a conical flask. A sample of 4-6 g was taken, thoroughly homogenized with 3% metaphosphoric acid, and then filtered through two layers of muslin cloth. After centrifuging the filtrate at 3,000 rpm for 10 m, the supernatant was titrated with a solution of 2, 6-dichlorophenol indophenols. By contrasting the titration results with a standard vitamin C solution, the amount of vitamin C in the extract was calculated. The following formula was used to determine the vitamin C content:

Percentage (%) of vitamin C content (mg/100g) = (mg of vitamin C obtained weight of sample (g)) × 100. (9)

### 2.5. Statistical analysis

Descriptive statistics were calculated for all variables using the SPSS software package (version 25.0; IBM Corp., Armonk, New York, NY, USA), and all of the values are expressed as the mean ± SD. The significance of the differences between the means of the two samples was determined by comparing the means of paired samples t-test. Differences were considered to be significant at $p<0.05$ (*).

## 3. Results and discussions

### 3.1. Proximate composition in different parts of passion fruit

#### *3.1.1. Moisture content in different parts of passion fruit*

The current study has found that the moisture content of the peel, pulp, and seed of green passion fruit was 85.98±1.69, 92.11±1.70, and 27.30±0.79 g/100g respectively, and the moisture content of the peel, pulp, and seed of ripe passion fruit was 76.24±0.93, 85.02±0.47 and 28.95±0.97 g/100g respectively. It is clear that the moisture content in peel and pulp of green passion fruit was higher and statistically significant ($P<0.05$) than ripe one but in seed, ripe passion fruit has higher moisture content but not statistically significant ($P<0.05$). However, among peel, pulp and seed of green and ripe passion fruit, pulp contain highest amount of water. The moisture content of pulp of green passion fruit is almost similar to previous study (Adeyeye and Aremu, 2017), who has found the moisture content of pulp was 87.5±0.028 g/100g. On the other hand, the moisture content of pulp of ripe passion fruit is slightly higher than previous study (Kulkarni and Vijayanand, 2010) where they found the moisture content of pulp of ripe passion fruit was 81.5±0.2 g/100g, and also higher in case of seed of ripe passion fruit than previous study done by Ramaiya, Bujang and Zakaria (2018) who have found that the moisture content of seed of ripe passion fruit was 11.09±0.40 g/100g.

#### *3.1.2. Ash content in different parts of passion fruit*

The present study found that the ash content of the peel, pulp, and seed of green passion fruit was 1.32±0.03, 0.94±0.04 and 0.29±0.08 g/100g respectively. On the other hand, the current research found that the ash content of the peel,

pulp, and seed of ripe passion fruit was 1.38±0.16, 0.42±0.29 and 0.76±0.20g/100g respectively. So, it is clear that the ash content of the peels of both green and ripe passion fruit was almost the same, whereas the pulp of green passion fruit contained more than double that of the ripe one but was statistically non-significant (P<0.05) and the ash content of the seed of green and ripe passion fruit was statistically significant (P<0.05). The previous study was done by Adeyeye and Aremu (2017) and found that the ash content of the peel, pulp, and seed of passion fruit was 0.898±0.612, 0.34±0.014, and 2.26±0.014 g/100g respectively.

### *3.1.3. Crude fat content in different parts of passion fruit*

The present study has found the fat content of peel, pulp, and seed of green passion fruit of 1.70±0.02, 1.39±0.02, 16.32±0.56g/100g, and 0.38±0.03, 0.86±0.06, 23.79±0.38g/100g respectively in the case of ripe passion fruit. It is stated that the seed of both green and ripe passion fruits contains highest amount of fat than peel and pulp of both types of passion fruit. However, peel and pulp of green passion fruit contains slightly higher fat than ripe one but seed of ripe passion fruit contain relatively higher fat than seed of green passion fruit but all the samples of green and ripe passion fruit showed statistically significant at p<0.05. One study (Adeyeye and Aremu, 2017) found that the fat in peel was 0.805±0.693g/100g and (Ramaiya, Bujang and Zakaria, 2018) found fat in seed was 29.65±0.41g/100g and Kawakami *et al.* (2022) found between 12.31±0.68 to 32.65±0.45g/100g in seed in different varieties. However in the case of the pulp and peel one study (Adeyeye and Aremu, 2017) found a lower amount of fat than the present study.

### *3.1.4. Crude protein content in different parts of passion fruit*

The present study found that the protein content of peel, pulp and seed of green passion fruit was 1.76±0.08, 3.37±0.11 and 8.11±0.23g/100g and 7.49±0.18, 3.95±0.10 and 16.21±0.32g/100g respectively for ripe passion fruit. From our study we can see the protein content of pulp of ripe passion fruit is slightly higher than green fruit but protein content of peel and seed of ripe fruit is too much higher than of green passion fruit and seed contain the higher amount of protein but all the samples of green and ripe passion fruit showed statistically significant at p<0.05. Previous study done by Kawakami *et al.* (2022**)** found that the protein content of seed of ripe passion fruit between 13.07±0.12to 17.57±0.31g/100g whereas, Ramaiya, Bujang and Zakaria (2018**)** found 12.71±0.10g/100g of protein in seed of ripe passion fruit and Silva *et al.* (2015**)** found 11.80±0.20g/100g of protein in seed of passion fruit. According to Fonseca *et al.* (2022**)** peel, pulp and seed of passion fruit contain 6.47-7.5, 2.2-3.0 and 12.2-13.2g/100g of protein, which is almost similar to the present study.

### *3.1.5. Crude fiber content in different parts of passion fruit*

The crude fiber content of the peel, pulp, and seed of green passion fruit was 26.43±0.09, 1.35±0.06 and 41.45±0.53 g/100g respectively, whereas ripe passion fruit was 32.20±0.34, 1.64±0.05 and 47.68±0.54 g/100g respectively. It is clear that the crude fiber content increases with the maturity of passion fruit as the crude fiber content of ripe passion fruit is higher than that of green passion fruit in peel, pulp, and seed, which is statistically significant (P<0.05). Among the three samples, seed contains the highest amounts of crude fiber, pulp contains the lowest amounts, and peel is in the middle. The fiber content of peel and seed in the present study of both green and ripe was almost the same as in the previous study (26.98±0.48) and (48.18±0.64) g/100g respectively), but in the case of pulp it was negligible, which is not related to our present study (Ramaiya, Bujang and Zakaria, 2018; Kawakami *et al.*, 2022).

### *3.1.6 Carbohydrate content in different parts of passion fruit*

The present study found that the carbohydrate content of peel, pulp, and seed of green passion fruit was 41.36±0.42, 10.25±0.28 and 38.73±0.32 g/100g respectively, whereas in ripe passion fruit it was 50.51±0.28, 12.71±0.14 and 54.85±0.31 g/100g respectively in case of peel, pulp, and seed. So, it is suggested that all

parts of ripe passion fruit have contains more carbohydrates than all parts of green passion fruit, and it is statistically significant at $p<0.05$. However, previous studies showed that seed of passion fruit has contain more carbohydrates than peel and pulp. Kawakami *et al.* (2022) found carbohydrates between 49.44±1.16 to 71.07±0.00 g/100g in seed, and Adeyeye and Aremu (2017) found 77.6±0.049 g/100g in seed, whereas 11.9±0.028 g/100g in pulp and 66.2±2.83 g/100g in peel.

**Table 3.** Proximate composition of passion fruit

| Parameters (g/100g) | Peel | Mean±SD | P-value | Pulp | Mean±SD | p-value | Seed | Mean±SD | p-value |
|---|---|---|---|---|---|---|---|---|---|
| Moisture | Green | 85.98±1.69 | 0.010 | Green | 92.11±1.70 | 0.030 | Green | 27.30±0.79 | 0.071 |
| | Ripe | 76.24±0.93 | | Ripe | 85.02±0.47 | | Ripe | 28.95±0.97 | |
| Ash | Green | 1.32±0.03 | 0.628 | Green | 0.94±0.04 | 0.068 | Green | 0.29±0.08 | 0.025 |
| | Ripe | 1.38±0.16 | | Ripe | 0.42±0.29 | | Ripe | 0.76±0.20 | |
| Crude Fiber | Green | 26.43±0.09 | 0.002 | Green | 1.35±0.06 | 0.034 | Green | 41.45±0.53 | 0.009 |
| | Ripe | 32.20±0.34 | | Ripe | 1.64±0.05 | | Ripe | 47.68±0.54 | |
| CHO | Green | 41.36±0.42 | 0.002 | Green | 10.25±0.28 | 0.003 | Green | 38.73±0.32 | 0.000 |
| | Ripe | 50.51±0.28 | | Ripe | 12.71±0.14 | | Ripe | 54.85±0.31 | |
| Fat | Green | 1.70±0.02 | 0.000 | Green | 1.39±0.02 | 0.003 | Green | 16.32±0.56 | 0.005 |
| | Ripe | 0.38±0.03 | | Ripe | 0.86±0.06 | | Ripe | 23.79±0.38 | |
| Protein | Green | 1.76±0.08 | 0.001 | Green | 3.37±0.11 | 0.041 | Green | 8.11±0.23 | 0.001 |
| | Ripe | 7.49±0.18 | | Ripe | 3.95±0.10 | | Ripe | 16.21±0.32 | |

*Data are expressed as mean±standard deviation (SD). *P<0.05 considered as statistically significant when compared different parameters of green and ripe passion fruit. CHO: carbohydrates.

## 3.2. Bioactive compounds of passion fruit

### *3.2.1. TPC in different parts of passion fruit*

The TPC of peel, pulp and seed of green passion fruit were 57.04±2.35, 67.06±3.23 and 156.35±10.15mgGAE/g respectively. On the other hand, in ripe passion fruit it was 34.96±2.35, 42.25±3.02 and 116.48±16.35mgGAE/g of peel, pulp, and seed respectively. It is noted that TPC in all the parts like peel, pulp, and seed of green passion fruit were relatively higher than the ripe one. It is also found that seed contain the highest amount of TPC where, peel contain the lowest amounts. The difference between peel and pulp of green and ripe passion fruit was statistically significant at $p<0.05$ but in the case of seed it showed non-significance at $p<0.05$. The previous study (Gonzalez *et al.*, 2019) found that the total phenolic content of peel of the ripe fruit between 37.7±0.13 to 46.8±0.18mgGAE/g which is moderately different from the present findings and also found TPC between 105.6±0.35 to 153.4±0.78mgGAE/g in seed of ripe passion fruit using different extraction method. Janzantti *et al.* (2012) found that 41.566±0.00mgGAE/g TPC in pulp of ripe passion fruit, which is also close to the present findings.

### *3.2.2. TFC in different parts of passion fruit*

The TFC found by the current study in peel, pulp, and seed of green passion fruit were 54.82±1.71, 72.71±1.68, and 12.99±1.47mgQE/g respectively and 47.21±2.44, 56.27±1.82 and 26.66±1.18mgQE/g of TFC respectively. It is noted that the highest TFC is in the pulp of green passion fruit, and lowest in seed of green passion fruit. Overall, peel, and pulp of green fruit contain higher TFC, but seed of green fruit contain lower amount of TFC than ripe fruit but all the samples of green and ripe passion fruit were statistically significant at $p<0.05$. A previous study done by Gonzalez *et al.* (2019) found that the TFC of peel of ripe passion fruit was 55.6±0.11 mgQE/g which is almost similar to the present study, but TFC of seed in ripe passion fruit was 53.2±0.09 mgQE/g which is quite higher than current findings.

**Table 4.** Bioactive compounds content of different parts of green and ripe passion fruit

| Parameters | Peel | Mean±SD | p-value | Pulp | Mean±SD | p-value | Seed | Mean±SD | p-value |
|---|---|---|---|---|---|---|---|---|---|
| TPC (mgGAE/g) | Green | 57.04±2.35 | 0.000 | Green | 67.06±3.23 | 0.004 | Green | 156.35±10.12 | 0.113 |
| | Ripe | 34.96±2.35 | | Ripe | 42.25±3.02 | | Ripe | 116.48±16.35 | |
| TFC (mgQE/g) | Green | 54.82±1.71 | 0.005 | Green | 72.71±1.68 | 0.001 | Green | 12.99±1.47 | 0.00 |
| | Ripe | 47.21±2.44 | | Ripe | 56.27±1.82 | | Ripe | 26.66±1.18 | |

* Data are expressed as mean±standard deviation (SD). *P<0.05 considered as statistically significant when compared different parameters of green and ripe passion fruit.

### 3.3. Antioxidant activity of passion fruit

#### *3.3.1. Total antioxidant activity in different parts of passion fruit*

In the current study, the percent of inhibition was determined in order to determine the antioxidant activity. Percent inhibition in peel, pulp, and seed was 54.09±0.14, 51.23±0.22 and 60.44±0.53 respectively of green passion fruit. On the other hand, we found the percent of inhibition of peel, pulp and seed of ripe passion fruit to 63.16±0.18, 58.65±0.16 and 57.86±0.24 respectively. These data show that the percent inhibition of the peel and pulp of ripe fruit is higher than that of the peel and pulp of green passion fruit, but the seed of ripe fruit has a lower percent inhibition than the seed of green fruit. However, all the samples of green and ripe passion fruit showed statistical significance at p<0.05.

#### *3.3.2. Vitamin C content in different parts of passion fruit*

The vitamin C content of the peel, pulp, and seed of green passion fruit were 1.37±0.11, 7.06±0.10 and 0.80±0.04mg/g respectively, whereas the vitamin C content of the peel, pulp, and seed of ripe passion fruit were 6.76±0.25, 3.82±0.13 and 2.59±0.12mg/10g respectively. It is clear that the seed of the passion fruit contains the lowest amount of vitamin C, and peel and seed of ripe passion fruit contain a higher amount of vitamin C, but pulp of ripe passion fruit contains a lower amount of vitamin C than that of green passion fruit, but all the samples of green and ripe passion fruit showed statistical significance at p<0.05. Previous studies done by Fonseca *et al.* (2022) found that the pulp of ripe passion fruit contained 3.0±0.00mg/10g whereas, Genovese *et al.* (2008) found 4.30±0.06 mg/10g. These findings are almost same for the pulp of the ripe passion fruit.

**Table 5.** Overview of antioxidant activity of different parts of green and ripe passion fruits.

| Parameters | Peel | Mean±SD | p-value | Pulp | Mean±SD | p-value | Seed | Mean±SD | p-value |
|---|---|---|---|---|---|---|---|---|---|
| TAA (%) | Green | 54.09±0.14 | 0.000 | Green | 51.23±0.22 | 0.000 | Green | 60.44±0.53 | 0.009 |
| | Ripe | 63.16±0.18 | | Ripe | 58.65±0.16 | | Ripe | 57.86±0.24 | |
| Vit-C (mg/10g) | Green | 1.37±0.11 | 0.000 | Green | 7.06±0.10 | 0.000 | Green | 0.80±0.04 | 0.002 |
| | Ripe | 6.76±0.25 | | Ripe | 3.82±0.13 | | Ripe | 2.59±0.12 | |

*Data are expressed as mean±standard deviation (SD). *$P$<0.05 considered as statistically significant when compared different parameters of green and ripe passion fruit. TAA: total antioxidant activity.

## 4. Conclusions

In summary, the present study concluded that passion fruit has great potential for human health. Currently, the pulp of ripe passion fruit is widely consumed as raw or processed fruit, however, the peel and seed of green and ripe passion fruit are still underutilized. But the current study showed that besides ripe passion fruit, all the parameters of green passion fruit also have great nutritional properties. It is also demonstrated that underutilized peel and seed are good sources of dietary fiber, protein, and fat and are also rich sources of TPC, TFC, percentage of total antioxidants, and vitamin C contents. This study will be useful to consumers to plan rich fiber, protein, bioactive compounds, and antioxidant diets and to estimate the daily intakes and their impact on health. Therefore,

there is a high potential for the use of the peel, pulp, and seed of green and ripe passion fruit as a health promoting and disease-preventing source that is normally underutilized.

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# DIMETHYL CARDAMONIN FROM *CLEISTOCALYX OPERCULATUS* LEAVES: OPTIMISED EXTRACTION CONDITIONS AND INHIBITIVE ACTIVITY AGAINST FOOD-POISONING BACTERIA

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**ABSTRACT**

In this study, the predominant phenolic compound of *Cleistocalyx operculatus* leaves harvested in Vietnam was identified as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (also called dimethyl cardamonin) by using UHPLC-DAD-HRMS. The extraction of this compound from leaves was optimised using response surface methodology. A second-order polynomial model with three important variables (ethanol concentration, temperature and extraction time) was used. A rotatable central composite design consisting of 17 experimental runs with three replicates at the center point was applied to describe the experimental data as the dimethyl cardamonin apparent content of leaves. The experimental results fit well to the model with $R^2$ equal to 0.9618 and without a lack of fit. The optimised conditions were as follows: 80% ethanol, 85 °C and extraction time of 22 min. These conditions were applied to produce the extract from *C. operculatus* leaves. The obtained freeze-dried extract powder exhibited inhibitive activity against five food poisoning bacterial strains belonging to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* species, with the inhibitive zone ranging from 1.83 to 9.17 mm. The antibacterial activity of the extract was dose dependent. The results indicated the potential application of *C. operculatus* leaves as a source of natural antimicrobial agents for food preservation.

## 1. Introduction

*Cleistocalyx operculatus* (synonyms: *Cleistocalyx nervosum*, *Syzygium nervosum* and *Eugenia operculata*; common name "Voi" in Vietnamese) belongs to the Myrtaceae plant family (Do, 2004) and is widely distributed in tropical areas of Southeast Asian countries (reviewed by Pham *et al*., 2020). The decoction of *C. operculatus* leaves and flower buds is a popular beverage for Vietnamese people. In addition, parts of this plant have been used in traditional Vietnamese medicine for a long time. The leaves and flower buds promoted digestion. The leaves have antibiotic activity and have been used to treat boils, sores and scabies (Do, 2004).

*C. operculatus* has important pharmacological activities that have been demonstrated in several studies. Extracts from the flower buds of *C. operculatus* showed cytotoxic activity against cancer cell lines, including HeLa, HL-60 and A549 (Min *et al*., 2010), SMMC-7721, 8898, HeLa, SPC-A-1, 95-D and GBC-SD (Ye *et al*., 2004), PANC-1 and MIA PACA2 (Huynh *et al*., 2019). Ethanol extract of *C. operculatus* flower buds showed

anti-inflammatory effects in an experimental lipopolysaccharide-induced sepsis mouse model (Tran *et al*., 2019). Truong *et al*. (2009) reported that the aqueous extract of *C. operculatus* flower buds showed a strong antioxidant effect and antihyperlipidemic effect through the suppression of pancreatic lipase activity in diabetic rats. Oral administration of an aqueous extract from *C. operculatus* buds to diabetic rats for 8 weeks resulted in a significant reduction in the levels of glucose, total cholesterol and triglycerides in plasma. According to Nguyen *et al*. (2017), a methanolic extract of *C. operculatus* leaves inhibited bacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus* mutans GS-5) and three multiresistant bacteria (*Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535 and *Staphylococcus aureus* North German epidemic strain) with inhibition zone diameters ranging from 7 to 16 mm. Eighty-six phytochemical compounds have been identified in *C. operculatus* belonging to three main groups, including terpenoids, flavonoids and phloroglucinols (Pham *et al*., 2020). Among them, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone or dimethyl cardamonin, a phenolic compound, is considered the major constituent and pharmacologically responsible of this medicinal plant (Ye *et al*., 2004; Huynh *et al*., 2019; Tran *et al*., 2019; Pham *et al*., 2020).

Food poisoning or foodborne disease is one of the main problems in public health worldwide. According to the WHO, an estimated 600 million people in the world, almost 1 in 10 people, fall ill after eating contaminated food, and 420,000 die every year. Children under 5 years of age carry 40% of the foodborne disease burden, with 125,000 deaths every year. One hundred and ten billion American dollars are lost each year in productivity and medical expenses resulting from unsafe food in low- and middle-income countries (WHO, 2020). Approximately 70% of foodborne diseases result from food contaminated with a microorganism (Hernández-Cortez *et al*., 2017). Among the microorganisms causing foodborne diseases, bacteria have the highest significance with respect to public health (Park *et al*., 2001). Bacteria frequently documented as foodborne disease agents include *Bacillus cereus*, *Escherichia coli*, *Clostridium botulinum*, *Staphylococcus aureus*, *Salmonella* and *Listeria monocytogenes* (Park *et al*., 2001; Kirk *et al*., 2015).

The objectives of this study were to identify the major phenolic compounds in *C. operculatus* leaves harvested in Vietnam and to optimise the extraction of this compound from leaves using response surface methodology (RSM). The antibacterial activity against some food poisoning microorganisms of the freeze-dried leaf extract was then evaluated.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ethanol, formic acid, and myricetin standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile was purchased from Merck (Germany). Dimethyl sulfoxide (DMSO) was produced in Japan. Methanol, agar, yeast extract, meat extract, peptone and tryptone were obtained from China. Antibiotics were bought in pharmacies in Hanoi.

Five food poisoning bacterial strains belonging to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* species were taken from the microorganism collection of Gene Key Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology.

### 2.2. Sample collection

*C. operculatus* leaves were harvested in Hai Duong Province (Vietnam) in July 2019. All leaves were first washed with tap water and rinsed in distilled water. They were then freeze-dried at -58 °C for 3 days and ground to a fine powder with a particle size of less than 0.3 mm using a Tecator Cyclotec 1093 Sample Mill (Foss Tecator AB, Sweden). The leaf powder was stored in an airtight bag and kept at 4 °C until further use.

### 2.3. Identification of dimethyl cardamonin in *C. operculatus* leaves harvested in Vietnam by UHPLC-DAD-HRMS

#### *2.3.1. Extraction of phenolic compounds from C. operculatus leaves*

Due to the lack of dimethyl cardamonin standards on the market, a primary extract of *C. operculatus* leaves harvested in Vietnam was prepared and sent to the Luxembourg Institute of Science and Technology to identify the peak of dimethyl cardamonin. Briefly, 0.5 g of freeze-dried *C. operculatus* leaf powder was mixed with 10 mL of 70% ethanol and shaken for 60 min at 40 °C. The mixture was then centrifuged at 3,642 × g for 10 min at 4 °C. The supernatant was collected, and the solvent in the extract was evaporated in a R210 rotary evaporator (Buchi, Switzerland) at 40 °C/58 mbar for 30 minutes. The concentrated extract was freeze-dried, and the extract powder was sent to Luxembourg Institute of Science and Technology.

#### *2.3.2. Identification of dimethyl cardamonin in the extract powder*

Extract powder was redissolved in 70% methanol and analysed with a Waters Acquity UPLC I-class system (Milford, MA) equipped with a diode-array detector (UPLC PDA eLambda) and hyphenated to a high-resolution time-of-flight mass spectrometer (TripleTOF 6600, AB Sciex, Concord, Ontario, Canada). The separation of the 5-µL aliquot was performed in two technical replicates on a reverse-phase Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size, Waters). In both ionisation modes, the eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was as follows: 0 min, 1% B; 4 min, 1% B; 16 min, 5% B; 35 min, 40% B; 45 min, 100% B; 50 min, 100% B; 53 min, 1% B; 60 min, 1% B. The flow rate was 0.5 mL/min, and the column temperature was 50 °C. The total wavelength chromatogram was first acquired between 190 and 800 nm. Analytes were then ionised with an electrospray ionisation (ESI) source. For MS1 and MS2, full HR-MS spectra between 100 and 1300 mass-to-charge ratios (m/z) and between 25 and 1300 m/z were recorded. The structure of the predominant peak was putatively identified by comparison of its UV–VIS and mass spectral data with previous literature and reference data from the PubChem Compound database (http://pubchem.ncbi.nlm.nih.gov). The molecular formula was considered valid when the mass error was below 4 ppm.

### 2.4. Modelisation and optimisation of dimethyl cardamonin extraction from *C. operculatus* leaves

#### *2.4.1. Effect of the solid-to-liquid ratio on the extraction of dimethyl cardamonin*

Freeze-dried *C. operculatus* leaf powder was mixed with 5 mL of 60% ethanol to have a solid-to-liquid ratio ranging from 1/5 to 1/25 and shaken for 60 min at 40 °C. The mixture was then centrifuged at 3,642 × g for 10 min at 4 °C. The supernatant was collected, and the dimethyl cardamonin content was analysed by HPLC. Extraction was performed in triplicate.

#### *2.4.2. Modelization and optimisation of dimethyl cardamonin extraction*

Response surface methodology was used to optimise the extraction of dimethyl cardamonin from freeze-dried *C. operculatus* leaf powder. A three-factor and rotatable central composite design consisting of 17 experimental runs with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68 from the design center) and three replicates at the center point (Table 1) was employed. The design variables were ethanol concentration ($X_1$), extraction temperature ($X_2$) and extraction time ($X_3$). The optimised solid-to-liquid ratio was determined in the previous experiment and kept at a constant value. The response was the apparent dimethyl cardamonin content of *C. operculatus* leaves. Three replicates at the central points of the design were used to allow for estimation of a pure error sum of squares.

For all runs, extractions were performed in 15 mL Falcon tubes. The extraction was terminated by centrifugation of the mixture at 3,500 × g for 10 min at 4 °C. The supernatant was collected, filtered and analysed for dimethyl cardamonin content by using an HPLC equipped

with a multiple wavelength detector (HPLC-MWD). The experimental data were fitted to the following second-order polynomial model.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} x_i x_j$$

where Y is the response, βo, βi, βii, βij are regression coefficients for intercept, linear, quadratic and interactions terms, respectively, and xi, xj are the coded values of the independent variables. The formula to convert coded values to real values and vice versa was as follows: $x_i = (X_i - X_0)/\Delta X_i$, where xi and Xi are the coded and real values of the independent variable i (i = 1, 2, and 3), respectively, $X_0$ is the real value of the independent variable i at the central point, and $\Delta X_i$ is the step change of $X_i$ corresponding to a unit variation of the coded value.

The optimum conditions for dimethyl cardamonin extraction were determined by maximising the desirability with JMP 10 software. Four separate experimental extractions under optimised conditions were performed. The experimental and predicted values were then compared to validate the model.

### 2.5. Quantification of dimethyl cardamonin in *C. operculatus* leaves by HPLC

Quantification of dimethyl cardamonin was performed by HPLC using an Aligent system 1260 (Santa Clara, CA) equipped with G1311B-Quat pumps, G1329B auto sampler, G1330B thermostat, and G1365 MWD VL lamp. A 20 µL aliquot of the extract was injected onto a Kinetex EVO C18 column (150x4.6 mm i.d:5 µm particle size) equipped with a guard column of the same type (Phenomenex, Netherlands). The mobile phases were A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The flow rate was 1 mL/min, and the column temperature was set at 30 °C. The mobile phase gradient was as follows: 0 min, 0% B; 1 min, 25% B; 5 min, 50% B; 6 min, 70% B; 12 min, 100% B; 15 min, 100% B; 18 min, 0% B; 20 min, 0% B. Monitoring was set at 360 nm. The identification of the dimethyl cardamonin peak was previously performed by using UHPLC-DAD-HRMS at Luxembourg Institute of Science and Technology as described above. Myricetin was used as a standard, and dimethyl cardamonin content was calculated by using a five-point calibration curve y = 28.346*x - 140.98 with $R^2 = 0.9999$ (y: peak area and x: myricetin content (µg/mL)).

### 2.6. Evaluation of the inhibitive activity against food-poisoning bacteria of *C. operculatus* leaf extract powder

#### *2.6.1. Preparation of C. operculatus leaf extract powder*

Freeze-dried *C. operculatus* leaf powder was extracted under the optimal conditions obtained for the modelisation and optimisation experiment. The extraction was terminated by centrifugation of the mixture at 3,500 × *g* for 10 min at 4 °C. The supernatant was collected, filtered and concentrated in an R210 rotary evaporator. The concentrated extract was then freeze-dried.

#### *2.6.2. In vitro antimicrobial activity test*

The antimicrobial activity of *C. operculatus* leaf extract powder was measured by using the agar well diffusion method described in the work of Dang *et al*. (2015). First, bacteria were transferred to the corresponding liquid media and then incubated at 37 °C in a shaking incubator (200 rpm) for 12 hours. Meat-peptone-agar medium (peptone 5 g/L, NaCl 5 g/L, meat extract 5 g/L) was used for *Salmonella, E. coli* and *Vibrio parahaemolyticus,* while Luria Bertami medium (NaCl 10 g/L; tryptone 10 g/L; yeast extract 5 g/L) was used for *Staphylococcus aureus* and *Listeria monocytogenes*.

One hundred microliters of bacterial culture adjusted to a microorganism concentration of $10^{10}$ colony forming units per mL (CFU/mL) was spread on a Petri dish containing 25 mL of their specific media. *C. operculatus* leaf extract powder was dissolved in DMSO to make concentrations of 10, 20, 30, 40 and 50 mg/mL for the antibacterial activity test. One hundred microliters of extract solution at different concentrations was added to test wells (dimension of 8 mm), while the negative control well consisted of 100 µL DMSO and the positive control wells consisted of 300 µg/mL

antibiotics. Petri dishes were kept at 4 °C for 2 hours and then incubated at 37 °C for 20 hours. After incubation, the zone of inhibition was measured as the difference between the dimension of the inhibition zone surrounding the well and the dimension of the well (8 mm). Experiments were performed in triplicate.

### 2.7. Statistical analysis

Data were analysed using the statistical software SAS 9.4 (SAS Institute, Cary, NC). In the experiment determining the effect of the solid-to-liquid ratio on extraction, the dimethyl cardamonin apparent content was expressed as the mean ± standard deviation of three extraction replications. One-way analysis of variance (ANOVA) and Duncan's test were used to determine the differences among the means. $p$ values < 0.05 were considered to be significantly different. Analysis of variance was carried out using a generalised linear model (GLM) procedure to determine the effect of the bacteria, extract concentration and their interactions on the zone of inhibition. The model configuration was $Y = a + b_1*X_1 + b_2*X_2 + b_{12}*X_1*X_2$ (Y: zone of inhibition; $X_1$: bacteria and $X_2$: extract concentration). In the RSM experiment, multiple linear regression analysis was performed by JMP 10 software (SAS Institute, Cary, NC).

## 3. Results and discussions

### 3.1. Identification of the major compound in *C. operculatus* leaf extract

The predominant peak in the HPLC chromatogram (Figure 1) had a retention time of 38.37 and exhibited molecular ions $[M-H]^+$ at $m/z$ 299.1289 and ion $[M-H]^-$ at $m/z$ 297.1141, with mass errors of 3.7 and -0.4 ppm, respectively, which were below our cut-off of 4 ppm.

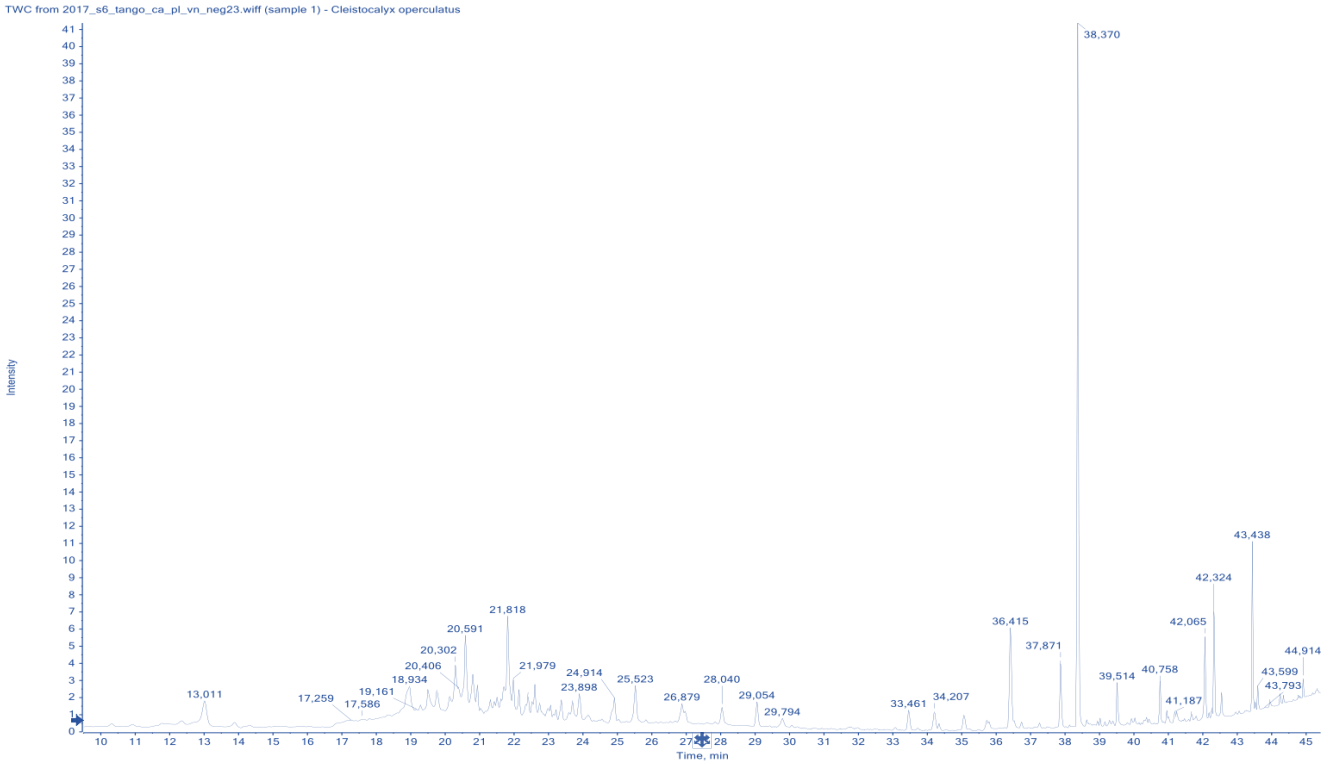



**Figure 1.** Total wavelength chromatogram of *C. operculatus* leaf extract**.**

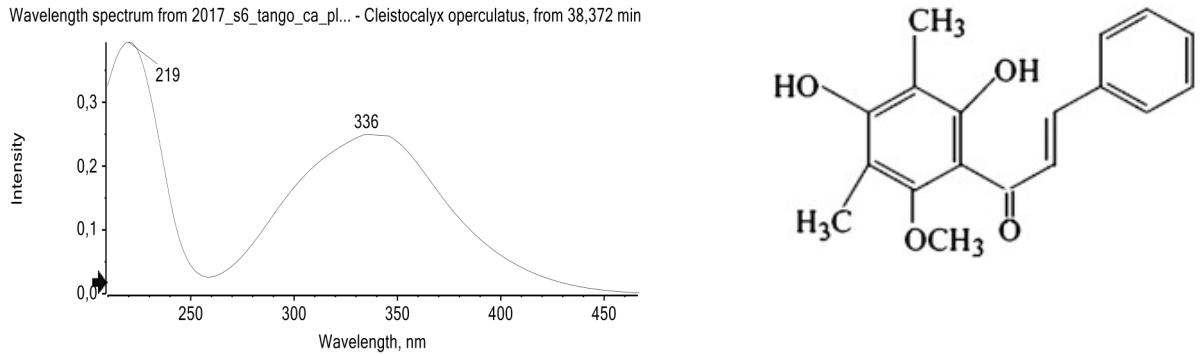


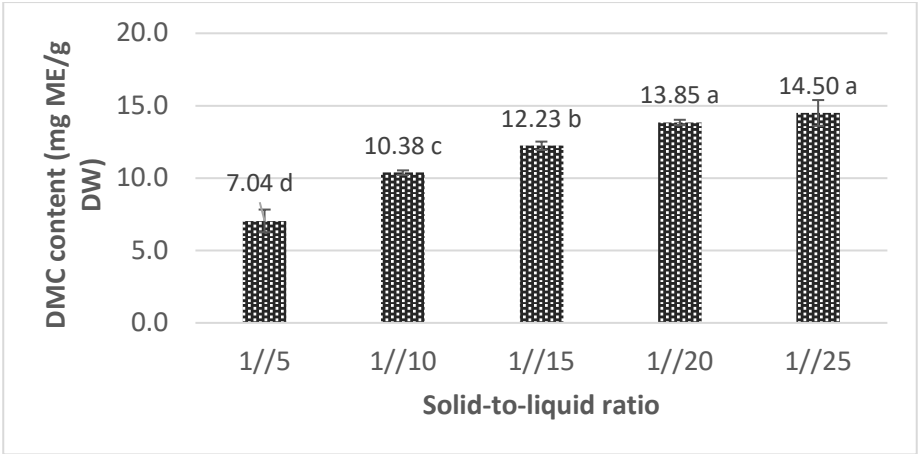

**Figure 2.** Absorbance spectrum and structure of dihydroxy-methoxy-dimethyl-chalcone (predominating peak) in *C. operculatus* leaf extract**.**

The molecular formula of the compound was hence inferred to be $C_{18}H_{18}O_4$. Fragmentation in positive mode (*m/z* 195.0726 (90) and 180.0442 (80)) was similar to the fragmentation pattern of protonated chalcones reported in the work of Tai *et al*. (2006) and Ye *et al*. (2004). This fragmentation has also been previously reported for 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone by Malterud *et al.* (1977). In addition, the absorbance spectrum of this peak with $\lambda_{max}$ (336 nm) was very close to that described for 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone in the report of Malterud et al. (1977). All these data suggested that the predominant peak was a dihydroxy-methoxy-dimethyl-chalcone (Figure 2). 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone, also called dimethyl cardamonin, has been reported to be present in the buds and leaves of *C. operculatus* (Pham *et al*., 2020) and showed anti-inflammatory (Yu *et al*., 2015; Tran *et al.*, 2019) and antitumor activity against six established human cancer cell lines, including SMMC-7721, 8898, HeLa, SPC-A-1, 95-D and GBC-SD cells (Ye *et al*., 2004), as well as antiviral properties against H1N1, H9N2, and novel H1N1, through strong enzymatic inhibition of various virus neuraminidases (Ha *et al*., 2016).

### 3.2. Modelization and optimisation of dimethyl cardamonin extraction from *C. operculatus* leaves

#### *3.2.1. Effect of the solid-to-liquid ratio*

The impact of the solid-to-liquid ratio on the extraction of dimethyl cardamonin from *C. operculatus* leaves is presented in Figure 3.




** Mean ± SD (n = 3 experimental replications). Means followed by the same letter do not differ statistically using Duncan test with 5% probability.*

**Figure 3.** Effect of solid-to-liquid ratio on dimethyl cardamonin apparent content of *C. operculatus* leaf.

The results of the one-way analysis of variance showed that the solid-to-liquid ratio had a significant effect on the dimethyl cardamonin extraction ($p < 0.0001$). The quantity of extracted dimethyl cardamonin initially increased when the ratio varied from 1/5 to 1/20. This is consistent with mass transfer principles. The driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a higher solvent to solid ratio is used (Elboughdiri, 2018). However, the dimethyl cardamonin content remained fairly constant when the ratio varied from 1/20 to 1/25. A similar effect of the solid-to-liquid ratio on extraction yield was reported for the extraction of phenolic compounds from *Inula helenium* (Wang *et al.*, 2013), *Inga edulis* leaves (Silva *et al.*, 2007), olive leaves (Elboughdiri, 2018), green coffee bean (Lai *et al*., 2018) and brewers' spent grain (Andres *et al*., 2020). A ratio of 1/20 gave a high dimethyl cardamonin content and hence was chosen for the dimethyl cardamonin extraction.

### *3.2.2. Modelization and optimisation of dimethyl cardamonin extraction*

The experimental design of the five-level, three-variable central composite rotatable design (CCRD) and the experimental results of extraction are shown in Table 1. By applying a multiple regression analysis, the relation between the tested independent variables and the response was explained in Equation 1, in which $x_i$ were standardized or coded variables.

$$Y = 16.56 + 5.29*x_1 + 4.01*x_2 + 0.28*x_3 + 0.33x_1* x_2 – 0.44x_1*x_3 – 0.35x_2*x_3 – 1.77x_1^2 + 1.33x_2^2 – 0.51x_3^2 \quad (1)$$

**Tables 1.** Rotatable central composite design setting in the coded form ($x_1$, $x_2$, $x_3$) and real values of the independent variables.

| Run | Standard variables | | | Real variables | | | Dimethyl cardamonin apparent content (mg/g DW) |
|---|---|---|---|---|---|---|---|
| | $x_1$ | $x_2$ | $x_3$ | Ethanol concentration (%) | Temperature (°C) | Time of extraction (min) | |
| 1 | 1 | 1 | 1 | 80 | 85 | 45 | 23.04 |
| 2 | -1 | 1 | 1 | 40 | 85 | 45 | 12.53 |
| 3 | 1 | -1 | 1 | 80 | 45 | 45 | 15.66 |
| 4 | -1 | -1 | 1 | 40 | 45 | 45 | 7.04 |
| 5 | 1 | 1 | -1 | 80 | 85 | 15 | 24.47 |
| 6 | -1 | 1 | -1 | 40 | 85 | 15 | 12.74 |
| 7 | 1 | -1 | -1 | 80 | 45 | 15 | 16.26 |
| 8 | -1 | -1 | -1 | 40 | 45 | 15 | 5.31 |
| 9 | 1.68 | 0 | 0 | 93.6 | 65 | 30 | 21.99 |
| 10 | -1.68 | 0 | 0 | 26.4 | 65 | 30 | 3.88 |
| 11 | 0 | 1.68 | 0 | 60 | 98.6 | 30 | 29.56 |
| 12 | 0 | -1.68 | 0 | 60 | 31.4 | 30 | 13.87 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 13 | 0 | 0 | 1.68 | 60 | 65 | 55.2 | 17.81 |
| 14 | 0 | 0 | -1.68 | 60 | 65 | 4.8 | 15.19 |
| 15A | 0 | 0 | 0 | 60 | 65 | 30 | 17.39 |
| 15B | 0 | 0 | 0 | 60 | 65 | 30 | 16.01 |
| 15C | 0 | 0 | 0 | 60 | 65 | 30 | 15.81 |

To fit the response function and experimental data, the linear and quadratic effects of the independent variables, as well as their interactions on the response, were evaluated by analysis of variance (ANOVA), and regression coefficients were determined (Tables 2 and 3).

**Table 2.** Analysis of variance for the response surface quadratic model of dimethyl cardamonin apparent content of *C. operculatus* leaf.

| Source | Degree of freedom | Sum of square | Mean square | F |
|---|---|---|---|---|
| Model | 9 | 687.76 | 76.42 | 19.56 |
| Error | 7 | 27.34 | 3.91 | $p = 0.0004$ |
| Lack of fit | 5 | 25.86 | 5.17 | 6.98 |
| Pure error | 2 | 1.48 | 0.74 | $p = 0.1299$ |
| Total | 16 | 715.10 | | |

**Table 3.** Parameter estimates of the predicted second-order model for the response (dimethyl cardamonin apparent content of *C. operculatus* leaf).

| Term | Estimate | Standard error | t ratio | *p* |
|---|---|---|---|---|
| Intercept | 16.5617 | 1.1389 | 14.54 | <.0001 |
| Ethanol concentration | 5.2916 | 0.5348 | 9.89 | <.0001 |
| Temperature | 4.0198 | 0.5348 | 7.52 | 0.0001 |
| Time | 0.2852 | 0.5348 | 0.53 | 0.6102 |
| Ethanol concentration*Temperature | 0.3338 | 0.6988 | 0.48 | 0.6475 |
| Ethanol concentration*Time | -0.4438 | 0.6988 | -0.64 | 0.5456 |
| Temperature*Time | -0.3463 | 0.6988 | -0.50 | 0.6354 |
| Ethanol concentration* Ethanol concentration | -1.7719 | 0.5886 | -3.01 | 0.0197 |
| Temperature* Temperature | 1.3323 | 0.5886 | 2.26 | 0.0580 |
| Time*Time | -0.5115 | 0.5886 | -0.87 | 0.4137 |

The ANOVA of the regression model showed that the model was highly significant due to a low probability value ($p < 0.0004$) (**Table 2**). The fitness of the model was judged by the coefficient of determination ($R^2$). In this study, the $R^2$ value for the regression model of the dimethyl cardamonin apparent content of *C. operculatus* leaves was 0.9618, which was close to 1, suggesting that the predicted second-order polynomial model well defined the dimethyl cardamonin extraction process from *C. operculatus* leaves and that 96.18% of the variation in the dimethyl cardamonin apparent content was attributed to the three studied

factors (Bharathi *et al.*, 2011). In addition, the lack of fit test is used to verify the adequacy of the model. The sum of squared errors (SSE) is split into two components called pure error (variation between observed and average values at X) and lack of fit (variation between average and predicted values at X). In our study, the absence of lack of fit ($p = 0.1299$) meant that the total error of the model was due to the pure error. This strengthened the reliability of the model (Table 2).

The effects of ethanol concentration, temperature and time of extraction on the apparent dimethyl cardamonin content of *C. operculatus* leaves are presented in Table 3 and Figure 4. As illustrated in Table 3, the ethanol concentration and temperature showed significant linear effects on the dimethyl cardamonin apparent content ($p < 0.0001$ and $p = 0.0001$, respectively), while the time of extraction had no effect ($p = 0.6102$). Among them, ethanol concentration appeared to be the most affecting factor of the dimethyl cardamonin extraction process from *C. operculatus* leaves because its coefficient had the highest value (5.2916). As shown in Figure 4, the quantity of extracted dimethyl cardamonin increased as the ethanol concentration increased. Similarly, higher temperatures of extraction resulted in higher dimethyl cardamonin apparent contents. Moreover, a high quantity of dimethyl cardamonin (29.56 mg/g DW) was obtained at 98.6 °C (run 11), indicating that this compound was a thermoresistant compound.

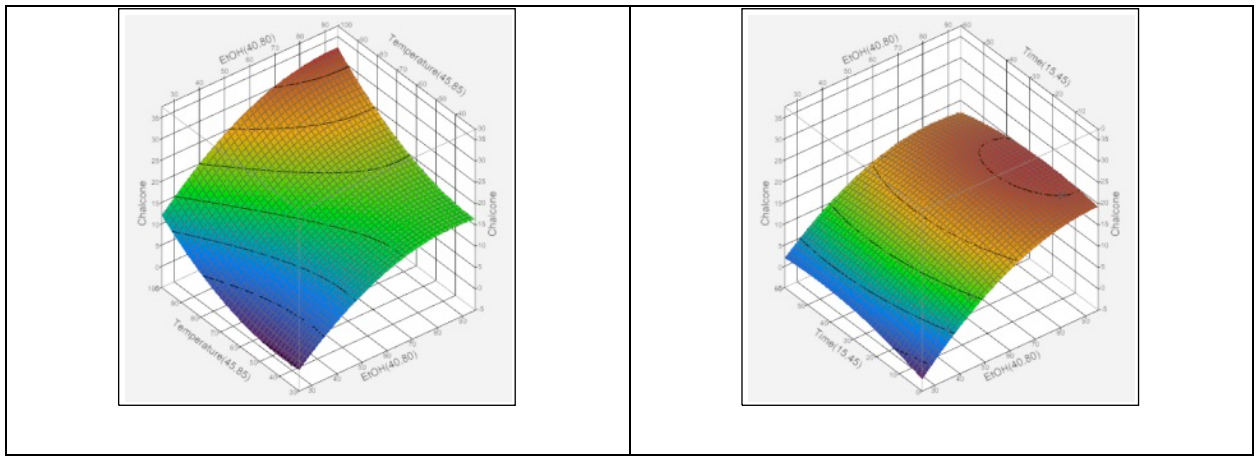



**Figure 4.** Response surface for dimethyl cardamonin apparent content in function of ethanol concentration, temperature and time of extraction

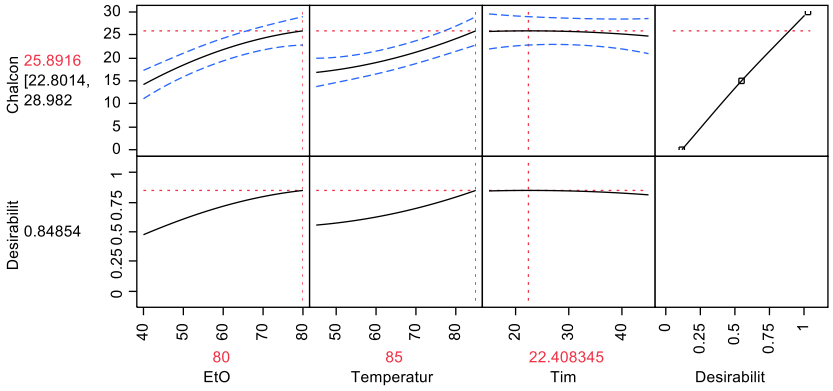



**Figure 5.** Ethanol concentration, temperature and time of extraction as well as predicted response at point with maximal desirability.

Concerning time extraction, this factor had a nonsignificant effect on the dimethyl cardamonin apparent content of *C. operculatus* leaves. In runs 1 and 14 (Table 1), a small increase in dimethyl cardamonin quantity (17.81 - 15.19 = 2.62 mg/g DW) was observed when the time of extraction increased from 4.8 to 55.2 minutes. Similarly, when the time of extraction increased from 4.8 (run 14) to 30 min (run 15), the dimethyl cardamonin content slightly increased (from 15.19 to 16.4 mg/g DW). This indicated that an important quantity of dimethyl cardamonin could be extracted during the first minutes of extraction. Accordingly, the maximal rates of extraction of phenolic compounds from agrimony, sage and savoury leaves and green coffee bean were found to take place during the first minutes of extraction (Kossah *et al.*, 2010; Lai *et al*., 2018).

The optimum conditions of dimethyl cardamonin extraction from *C. operculatus* leaves were acquired by using JMP 10 software. The software was set to determine the optimum desirability of the response, which was the maximum dimethyl cardamonin apparent content of the leaves. The optimum conditions were found as follows: ethanol concentration, 80%; temperature, 85 °C; and time of extraction, 22 min, as shown in Figure 5. To examine the validity of the model, extraction was performed with four replicates under these optimised conditions. The measured values (23.52, 25.50, 23.81 and 24.84 mg/g DW) lay within a 95% mean confidence interval of the predicted value (22.80 - 28.98 mg/g DW). These results confirmed the predictability of the model. The second-order polynomial model (Eq. 1) can thus be effectively applied to predict the amount of dimethyl cardamonin extracted from *C. operculatus* leaves.

### 3.3. *In vitro* antimicrobial activity against food-poisoning bacteria of *C. operculatus* leaf extract powder

Table 4 shows the antibacterial activities against five food poisoning bacteria of *C. operculatus* leaf extract powder measured by the agar well diffusion method. The *C. operculatus* leaf extract exhibited inhibitory activity against both gram-positive and gram-negative bacteria. The inhibition zone for the five food-poisoning bacteria ranged from 1.83 to 9.17 mm and significantly depended on the bacterial strain ($p < 0.0001$). Among the five tested bacteria, *Staphylococcus aureus* and *Listeria monocytogenes* were the most sensitive strains to *C. operculatus* leaf extract, followed by *Salmonella* and *Escherichia coli,* while *Vibrio parahaemolyticus* was the least sensitive. The extract concentration significantly affected the antimicrobial activity ($p < 0.0001$). In general, higher concentrations of leaf extract resulted in higher inhibition zones. However, the interaction between bacterial strain and extract concentration had no significant effect on the inhibitory zone ($p = 0.4567$).

**Table 4.** Inhibition zones (mm) of *C. operculatus* leaf extract powder against five food poisoning bacteria*.

| Bacteria | Extract concentration | | | | | Antibiotic (300 µg/mL) |
|---|---|---|---|---|---|---|
| | 10 mg/mL | 20 mg/mL | 30 mg/mL | 40 mg/mL | 50 mg/mL | |
| *S. aureus* | 4.75±0.25$^{aD}$ | 6.92±0.14$^{aC}$ | 8.08±0.38$^{aB}$ | 8.33±0.76$^{abAB}$ | 9.17±0.88$^{aA}$ | 10.58±0.52 (Norfloxacin)<br>13.58±0.29 (Gentamicin)<br>1.83±0.14 (Ceftriaxone) |
| *V. parahaemolyticus* | 1.83±1.04$^{bC}$ | 4.58±0.76$^{bB}$ | 6.00±0.43$^{bA}$ | 6.42±0.63$^{cA}$ | 7.17±0.14$^{bA}$ | 10.83±0.14 (Norfloxacin)<br>1.83±0.29 (Ceftriaxone)<br>13.75±0.25 (Chloramphenicol) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *E. coli* | 4.42±0.80$^{aC}$ | 5.92±0.29$^{abBC}$ | 6.58±0.88$^{abAB}$ | 7.00±0.87$^{ABbc}$ | 7.67±1.15$^{bA}$ | 0 (Aztreonam)<br>14.22±0.38 (Norfloxacin)<br>0.92±0.29 (Amoxycillin) |
| *Salmonella* | 5.33±0.58$^{aB}$ | 6.08±1.42$^{abAB}$ | 6.92±1.01$^{abAB}$ | 7.58±0.95$^{abcA}$ | 7.67±0.38$^{bA}$ | 0 (Aztreonam)<br>12.75±0.25 (Norfloxacin)<br>15.75±0.25 (Piperacillin) |
| *L. monocytogenes* | 5.33±0.58$^{aC}$ | 6.50±1.32$^{aBC}$ | 6.76±1.38$^{abAB}$ | 8.67±0.80$^{aA}$ | 9.00±0.43$^{aA}$ | 13.33±0.14 (Spectinomycin)<br>4.58±0.38 (Gentamicin)<br>1.33±0.52 (Amoxycillin) |

** Mean ± SD (n = 3 experimental replications). Means followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically using Duncan test with 5% probability.*

Antibacterial activity of *C. operculatus* leaf extract has not been extensively studied. In the work of Nguyen *et al.* (2017), a methanolic extract of *C. operculatus* leaves inhibited bacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus mutans* GS-5) and three multiresistant bacteria (*Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535 and *Staphylococcus aureus* North German epidemic strain) with inhibition zone diameters ranging from 7 to 16 mm. Phytochemical screening of the extract using thin layer chromatography indicated the presence of flavonoids, which could contribute to the antibacterial activity of the extract. In our study, *C. operculatus* leaf extract inhibited the growth of five food poisoning bacteria, indicating the potential application of *C. operculatus* leaves as a source of antimicrobial agents for food preservation.

## 4. Conclusions

In this study, by using UHPLC-DAD-HRMS, the predominant compound in *C. operculatus* leaves harvested in Vietnam was identified as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone, also called dimethyl cardamonin. The extract of this compound from leaves was optimised by using response surface methodology. The optimised extraction conditions were as follows: ethanol concentration of 80% and extract temperature of 85 °C for 22 min. The obtained extract powder exhibited inhibitive activity against five food poisoning bacterial strains belonging to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* species. The results indicated the potential application of *C. operculatus* leaf extract powder as a natural antibacterial preservative

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# ASSESSMENT OF PURPLE SWEET POTATO FLOUR ANTIN 2 AND ANTIN 3 VARIETIES NUTRITIONAL QUALITY USING VARIOUS DRYING TEMPERATURE

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**ABSTRACT**

To produce purple sweet potato flour (*Ipomoea batatas* L.) through natural drying under sunlight is weather dependent and has fluctuating quality. Drying with a cabinet dryer has the potential to ensure consistency and time efficiency. This study aims to assess differences in purple sweet potato varieties and drying temperature on antioxidant activity, anthocyanin levels, and chemical characteristics of flour in Antin 2 and Antin 3 varieties. The study was designed using a randomized complete design with two factors, including factor I (Antin 2 and Antin 3 varieties) and factor II of drying cabinet drying temperature (40°C, 50°C, and 60°C), while fresh tuber as a control. Parameters observed included antioxidant activity, anthocyanin content, water content, ash content, protein content, fat content, carbohydrate content, vitamin A, vitamin C, and purple sweet potato flour. Data were analyzed using the ANOVA test. The Antin 3 variety has higher levels of anthocyanins and antioxidants than the Antin2 variety. Antin 3 has a significantly higher fat and Vitamin A content than Antin 2 variety. Antin 2 has significantly higher protein and vitamin C content than Antin 3 variety. The temperature has a significant effect on water and carbohydrate content.

## 1.Introduction

Sweet potato [*Ipomoea batatas* L. (Lam.)] is one of the world's most important and versatile food crops (Alam et al., 2010; Escobar-Puentes et al., 2022). Indonesia is one of the five sweet potato-producing countries with total exports of more than 2.3 million tons (FAO 2013). Sweet potato contains a variety of nutrients and xenobiotic phytochemicals with antioxidant, anticoagulant, antibacterial, anti-nyctalopia/xerophthalmia, hepatoprotective/ spasmolytic, and antidiabetic potential (Escobar-Puentes et al., 2022). There are many types of purple sweet potato in Indonesia, both local and introduced. Antin 2 and 3 are new varieties resulting from the breeding of the Research Institute for Nuts and Tubers. Both varieties have a high level of productivity and anthocyanin content. The Antin 3 variety contains 7 times higher anthocyanin than the local purple sweet potato of Gunung Kawi (Kurniasari et al., 2021). The results of other studies also reported that the Antin 3 variety had anthocyanin content of 150.7 mg/100 g while Antin 2 was 130.2 mg/100g (Anonim, 2020).

Anthocyanins function as powerful natural antioxidants to reduce and neutralize free radical compounds in the body. The chemical components of purple sweet potato are influenced by the type, variety, maturity level, processed form, and environmental conditions such as temperature, pH, and light.

Anthocyanins are natural antioxidants that are highly reactive, easily oxidized, and reduced and their glycoside bonds are easily hydrolyzed, causing damage and decreased antioxidant activity due to contact with air, changes in temperature, pH, and processing. Anthocyanin color stability and anthocyanin antioxidant activity can be influenced by external and internal factors. External factors include pH, temperature, humidity, light, salinity, and storage conditions. Meanwhile, internal factors other than ascorbic acid and sugar are the presence of enzymes, proteins, metal ions, and other polyphenols as well as intramolecular complexation. Furthermore, it is known that the activity and stability of anthocyanins can be maintained at pH below 4.6, and in storage of food processing products anthocyanins are recommended to be maintained at pH 3 (Chen et al., 2019). This is confirmed by the results of another study which reported the antioxidant activity of two anthocyanin fractions and co-pigment (primary phenolic acid) of purple sweet potato cultivar Eshu No.8 which was simulated in vitro and found to be stable in gastric digestion under acidic conditions (Yang et al., 2019). conducted in China showed that there were differences in nutritional content, dietary fiber, total phenolic, antioxidant activity, and anthocyanin content in four sweet potato cultivars.

Purple sweet potato processed in different ways will affect the nutritional content and antioxidant activity of anthocyanins. Problems that often occur in the use of tubers in fresh conditions, among others, require a large storage space, are less practical and easy to rot. Processing tubers into flour has advantages over fresh form because it can be stored longer, is more practical and efficient. Processing in the form of flour can also increase its economic value and can provide raw materials for an industry so that it has the potential to become alternative flour as a raw material for cakes and biscuits. On the other hand, purple sweet potato processing can affect antioxidant activity and anthocyanin stability. Processing of purple sweet potato reduces the level of raw antioxidants as indicated by a very strong IC50 value of 5.0 mg/L which decreases to 47.82 mg/L after steaming and becomes 82.22 mg/L after boiling. The decrease in antioxidant levels was directly proportional to the decrease in total phenolic and anthocyanin levels of purple sweet potato extract. Besides being able to reduce antioxidant activity, sweet potato processing can also reduce nutritional content such as water content, ash content, fat content, protein content, fiber content, and total carbohydrates (D'Amelia et al., 2022; Putri, 2019). Processing of fresh purple sweet potato into powder form requires appropriate techniques and methods to maintain color, nutritional content, antioxidant activity, and anthocyanin levels.

The data generated from previous research on the manufacture of purple sweet potato flour only focused on certain components for drying techniques and methods, use of tools and analysis of chemical components, antioxidant activity, and anthocyanin levels. The drying method by drying in direct sunlight takes quite a long time (2 - 3 days). Due to the erratic weather, this drying method is less effective and produces fluctuating products, which can even fail. Therefore, it is necessary to research drying temperature treatments of 40ºC, 50ºC, and 60ºC using a cabinet dryer in the manufacture of purple sweet potato flour varieties Antin 2 and Antin 3 to maintain nutrient content, antioxidant activity, and anthocyanin levels to produce quality sweet potato flour suitable for industrial scale.

## 2. Materials and methods

The purple sweet potato varieties Antin 2 and Antin 3 were collected from Blitar and Malang Regency. The chemicals used in the analysis are distilled water, diethyl ether or petroleum ether, hexene, H2SO4, HCl, K2SO4,

NaOH, 40% NaOH, 4% boric acid, 96% ethanol, DPPH solution (1,1-diphenyl-2-picrylhydrazyl), SDS (Sodium dodecyl sulfate, Brand), and other analytical chemicals.

### 2.1. Sweet Potato Flour Preparation

The production of purple sweet potato flour of Antin 2 and Antin 3 varieties was done by modifying the method from another study (Moloto et al., 2021), through the stages of sorting, cleaning, washing and peeling fresh sweet potatoes with good quality (purple sweet potato flesh and skin). The sweet potato tubers were peeled into thinly sliced using a saw blade to produce of aproximately 3 mm chips. The raw chips were dried using a cabinet dryer with a temperature treatment (40°C, 50°C, 60°C) for 2 x 48 hours (2 days) at a constant temperature. After drying process, the chips were floured using a chopper and filtered using an 80-mesh sieve to produce fine flour.

### 2.2. Antioxidant Activity Test

The antioxidant activity of sweet potato has been modified using 2-2 diphenyl 1-picrylhydrazyl (DPPH) as a free radical following previous studies(Yang et al., 2019; (Damat et al., 2020). The sample solution was made by mixing of 5 grams of sweet potato, 50 ml volumetric flask and water/ethanol to the mark. Then the 4 ml sample solution, 2 ml DPPH (0.001 g DPPH and methanol were mixed in a 50 ml volumetric flask) was put in a vial, shaken to homogenize, and incubated for 30 minutes in a dark room. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 515 nm. The formula used to calculate antioxidant activity is as follows

$$\text{Antioxidant activity (\%)} = \frac{(\text{Absorbent Blank} - \text{Absorbent Sample})}{(\text{Absorbent Blank})} x\ 100\% \qquad \textbf{(1)}$$

where :

Absorbent Blank = Absorbance of DPPH before being reacted with the sample

Absorbent Sample = Absorbance of DPPH after being reacted with the sample

### 2.3. The total anthocyanin

The total anthocyanin content of purple sweet potato using UV-VIS spectrophotometric differential pH method adapted from [7, 13, 14]. 50 mg of purple sweet potato extract was acidified with 1% HCl until it reached pH 1.0 then diluted with 10 ml of 96% ethanol. Furthermore, 1 ml of the extract solution was put into 2 small tubes, each added with 5 ml of KCl buffer pH 1.0 and 5 ml of Na-Acetate buffer pH 4.5, shaken until dissolved for approximately 30 minutes. Absorbance measurements were repeated twice at wavelengths 505 and 700 nm. The total anthocyanin content was calculated using the following formula:

$$\text{Total anthocyanin content} = \frac{\text{A} \times \text{MW} \times \text{DF} \times \text{V} \times 100}{\varepsilon \times \text{I} \times \text{W}} \qquad \textbf{(2)}$$

Where :

| | |
|---|---|
| A | = Sample absorbance value |
| MW | = Molecular weight cyanidin-3-glucoside (449.2 g/mol) |
| DF | = Dilution factor |
| V | = Primary liquor volume |
| W | = Extract weight (g) |
| 100 | = Conversion factor calculation in mg/100g sample |
| ε | = Molar absorptivity Sianidin-3-glukosida = 26,900 L/(mol.cm) |
| I | = Cuvette Width at 1 cm |

### 2.4. Color Test

Color determination was carried out using a color reader based on the method referred to a previous study (Ruttarattanamongkol et al., 2016). This analysis uses the L*, a*, and b* hunter system, where colors are divided into 3 color dimensions; namely the symbol L (Lightness) is the level of brightness, and the symbol a* for the dimensions of redness and greenness, the symbol b* for the dimensions and bluish.

### 2.5. Proximate Analysis

Testing the nutritional content of purple sweet potato using proximate analysis that has been done following the other study (Ramdath

et al., 2020). It consists of water content (gravimetric), ash content (AOAC 923.03), dietary fiber (AOAC 991.43), protein content (AOAC 992.15), fat content (AOAC 922.06), carbohydrate content based on differences (CHO) % = [100% moisture% – protein% – fat% – ash%]). Vitamin A was measured by Spectrophotometric Method (AOAC), whilte Vitamin C was measured by Iodine Titration Method (Ramdath et al., 2020).

### 2.6. Data Analysis

The study adopted a randomized complete design with two factors, including factor I (Antin 2 and Antin 3 varieties) and factor II of drying cabinet drying temperature (40ºC, 50ºC, and 60ºC), while fresh tuber as a control/comparison. Parameters observed included antioxidant activity, anthocyanin content, water content, ash content, protein content, fat content, carbohydrate content, vitamin A, vitamin C, and purple sweet potato flour. Each treatment was repeated 4 times (except for anthocyanin content) so that there were 32 experimental trials. The anthocyanin content was repeated two times. Data on measure variables were analyzed using the two-way ANOVA eccept data of Vitamin A. Data of Vitamin A did not meet the requirements of the normality test. So the data of Vitamin A was analyzed non-parametrically using the Kruskal-Wallis test.

## 3.Results and discussions

### 3.1. Total Anthocyanin Content

The total anthocyanin contents of the fresh tuber of Antin 2 variety was 1349.46 mg/kg, while that in Antin 3 variety was 2407.16 mg/kg.

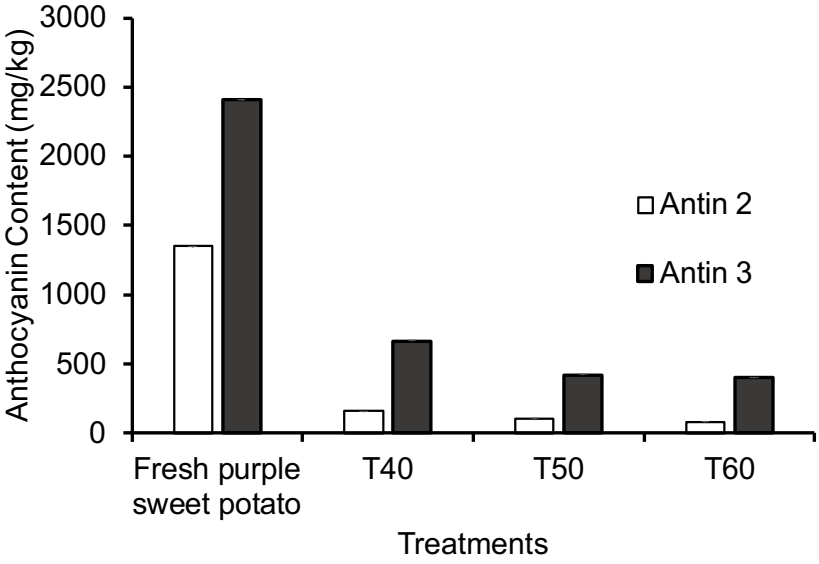


**Figure 1.** The average total anthocyanin content of purple sweet potato varieties Antin 2 and Antin 3 treated with drying temperatures of 40ºC (T40), 50ºC (T50) and 60ºC (T60) (N = 2)

After the purple sweet potato was processed into flours with the difference in drying temperatures, the total anthocyanin content of the purple sweet potato flours in the Antin 2 variety ranged from 82.02 mg/kg to 159 mg/kg, while those in Antin 3 variety ranged from 399.5 mg/kg to 661.97 mg/kg. overall, the total anthocyanin contents in Antin 3 variety were higher than those in Antin 2 variety. Anthocyanin levels in both varieties decreased with increasing temperature (Figure 1).

The difference in the anthocyanin content of the two varieties can be physically observed in the different colors of the tuber flesh. The red and purple colors are determined by the type of

anthocyanin, and the red color is mostly determined by cyanidin and peonidin. The purple sweet potato of the Antin 3 variety has blackish purple flesh, while the purple sweet potato of the Antin 2 variety is reddish purple.

This study indicates that the Antin 3 variety has higher levels of anthocyanins and antioxidants than the Antin2 variety. The heating process during drying was able to reduce the anthocyanin content of purple sweet potato which was indicated by the change in the color of the flour produced. The stability, quality, and color of anthocyanins are determined by changes in temperature, pH, and the ratio of solid-liquid concentrations, where the pH change factor is more dominant than temperature changes. (Rodriguez-Amaya, 2018; Tang et al., 2019). Furthermore, it is shown by the other study that longer exposure to heat will result in lower anthocyanin levels. Anthocyanins are heat-sensitive pigments that cause changes in the anthocyanin structure, namely the opening of the aglycone ring of the flavylium cation and the formation of colorless carbinol and chalcone compounds. The decrease in color intensity affects the color absorption performance in the total antioxidant test (Ticoalu & Maligan, 2016).

The blue to purple color is influenced by delphinidin, petunidin, and malvidin, while pelargodin is responsible for the orange color (D'Amelia et al., 2022). Each type of purple sweet potato in addition to having a different color also has different chemical characteristics and antioxidant activity depending on the type of anthocyanin and phenolic compounds contained in it (Ji et al., 2015).

The heating process during drying was able to reduce the anthocyanin content of purple sweet potato which was indicated by the change in the color of the flour produced. Furthermore, longer exposure to heat lead to lower anthocyanin levels. Anthocyanins are heat-sensitive pigments that cause changes in the anthocyanin structure, namely the opening of the aglycone ring of the flavylium cation and the formation of colorless carbinol and chalcone compounds. The decrease in color intensity affects the color absorption performance in the total antioxidant test (Ticoalu & Maligan, 2016). The heating process during drying was able to reduce the anthocyanin content of purple sweet potato which was indicated by the change in the color of the flour produced.

### 3.2. Average of Antioxidant Activity, Protein, Fat, Carbohydrate and Vitamin C content

The average antioxidant activity of fresh tuber of Antin 3 variety was 82.07% ± 0.12, while that in Antin 2 variety was 49.83% ± 2.85. After the purple sweet potato was processed into flours with the difference in drying temperatures, the total anthocyanin content of purple sweet potato flours in the Antin 3 variety ranged from 59.81% – 77.68%, while those in Antin 2 variety ranged from 23.19% – 38.80% (Table 1). The water content of fresh tubers of Antin 2 variety was 60.52±0.29, while that in Antin 3 was 59.68±0.23. The highest water content was found in the 40$^{o}$C treatment, while the lowest was in 60$^{o}$C. The water content decreases with increasing temperature (Table 1). The average ash content of potato flours in the Antin 3 variety ranged from 0.97% – 1.06%, while those in Antin 2 variety ranged from 0.97% – 0.98% (Table 1).

The protein content of purple sweet potato flour in the Antin 2 variety was higher than the Antin 3 variety. The average protein content of fresh tuber of Antin 2 variety was 6.99% ± 0.63, while that in Antin 3 variety was 4.57% ± 0.56. After flour processed, protein content of purple sweet potato flours in the Antin 2, it ranged from 5.33% to 6.30%, while the Antin 3 variety produced an average protein content of 2.21% - 2.71%. The highest protein content was found in fresh Antin 2, while the lowest was found in fresh Antin 2 treatment in 60$^{o}$C (Table 1). The high and low levels of protein in food have influenced the variety of purple sweet potatoes, temperature, maturity and processing time. Protein is a source of amino acids that contain elements C, H, O, and N.

The average fat content of the Antin 3 variety was higher than that of the Antin 2 variety. The fat content of the Antin 3 variety ranged from

1.27% to 2.58%, while the Antin 2 variety ranged from 1.08% - 1.41%. The lowest fat content was found in the Antin 2 treatment in 60ºC, while the highest was found in fresh Antin (Table 1). The average carbohydrate content of the Antin 3 variety was higher than that of the Antin 2 variety. The carbohydrate content of the Antin 3 variety ranged from 31.47% to 85.74%, while the Antin 2 variety ranged from 30.91% to 83.72%. The highest carbohydrate content was found at Antin 3 treatment in temperature of 60ºC, while the lowest was found in fresh Antin 2. The average vitamin A content of the Antin 3 variety was higher than that of the Antin 2 variety. The Vitamnin A content of the Antin 3 variety ranged from 42.05 to 51 mg/100g, while those in the Antin 2 variety ranged from 3.76 to 21.13 mg/100g (Table 1). The average vitamin C content of the Antin 3 variety was higher than that of the Antin 2 variety. The vitamin C content of the Antin 2 variety ranged from 1.06 to 1.52 mg/100g, while the Antin 3 variety ranged from 0.76 to 1.23 mg/100g (Table 1).

**Table 1.** Average of antioxidant, water, ash, protein, fat, carbohydrate and Vitamin C content in purple sweet potato of Antin 2 and Antin 3 varieties before and after drying treatment

| Treatment | Antioxydant (%) | Water (%) | Ash (%) | Protein (%) | Fat (%) | Carbohydrate (%) | Vitamin A (mg/100g) | Vitamin C (mg/100g) |
|---|---|---|---|---|---|---|---|---|
| **Antin 2 fresh** | 49.83±2.85 | 60.75±0.30 | 0.92±0.03 | 6.99±0.63 | 1.32±0.14 | 30.91±0.73 | 21.13±2.04 | 1.52±0.12 |
| **Antin 2 T40** | 38.80±0.67 | 11.98±0.11 | 0.98±0.00 | 6.30±0.96 | 1.41±0.09 | 80.88±0.91 | 10.55±1.72 | 1.24±0.27 |
| **Antin 2 T50** | 37.05±0.98 | 11.79±0.13 | 0.97±0.00 | 5.41±0.54 | 1.15±0.04 | 81.55±1.23 | 3.76±0.00 | 1.17±0.29 |
| **Antin 2 T60** | 23.19±0.32 | 9.54±0.25 | 0.98±0.00 | 5.33±0.57 | 1.08±0.01 | 83.72±1.11 | 3.90±0.40 | 1.06±0.37 |
| **Antin 3 fresh** | 82.07±0.12 | 59.99±0.36 | 0.96±0.02 | 4.57±0.56 | 2.58±0.29 | 31.47±0.80 | 51.00±2.81 | 0. 94±0.12 |
| **Antin 3 T40** | 73.60±1.15 | 11.16±0.13 | 0.97±0.00 | 2.51±0.37 | 2.31±0.07 | 82.42±0.89 | 48.81±3.77 | 0.82±0.15 |
| **Antin 3 T50** | 77.68±4.77 | 9.13±0.05 | 1.06±0.03 | 2.71±0.37 | 2.22±0.07 | 83.66±1.28 | 43.40±1.01 | 0.76±0.12 |
| **Antin 3 T60** | 59.81±0.64 | 8.71±0.12 | 0.97±0.00 | 2.21±0.24 | 1.27±0.45 | 85.74±1.06 | 42.05±8.81 | 0.76±0.12 |

The effect of sweet potato variety was significant on Antioxydant (3), Protein (2), Fat (3) and Vitamin C content (2), while the effect of temperature was significant on Water, Ash, Carbohydrate (Table 2). The effect of sweet potato variety was significant ($\chi = 17.280$, $P < 0.001$) on Vitamin A content (3), while that of drying temperature was not significant ($\chi = 2.727$, $P > 0.05$).

**Table 2.** Statistical F value and significance from the results of parametric analysis on the chemical composite of purple sweet potato average variable

| Treatment | Antioxydant | Water | Ash | Protein | Fat | Carbohydrate | Vitamin C |
|---|---|---|---|---|---|---|---|
| **Effect of variety** | | | | | | | |
| **F-value** | 105.654 | 0.026 | 3.176 | 41.577 | 19.726 | 0.036 | 9.101 |
| **Sig.** | 0.000 | 0.874 | 0.085 | 0.000 | 0.000 | 0.851 | 0.006 |
| **Effect of drying temperature** | | | | | | | |
| **F-value** | 2.117 | 6,328 | 5.178 | 1.684 | 2.082 | 1,232 | 0.701 |
| **Sig.** | 0.121 | 0.000 | 0.006 | 0.193 | 0.126 | 0.000 | 0.562 |

Anthocyanin level has a significant positive correlation with water and vitamin C content. It has a significant negative correlation with carbohydrates. Antioxidant levels were significantly positively correlated with fat and vitamin A levels. Water content was significantly positively correlated with vitamin C. Water content was significantly negatively correlated with carbohydrates. Protein content has a significant negative correlation with Vitamin A. Fat content has a significant positive correlation with Vitamin A. Carbohydrate content has a significant negative correlation with Vitamin C (Table 3).

**Table 3.** Correlation between variables

| | Ac | Ao | Wa | Ash | Pro | Fat | Car | VA | VC |
|---|---|---|---|---|---|---|---|---|---|
| **Ac** | 1.000 | 0.606 | 0.898 | -0.422 | 0.095 | 0.619 | -0.897 | 0.559 | 0.865 |
| **Ao** | | | 0.290 | 0.263 | -0.623 | 0.901 | -0.289 | 0.959 | 0.391 |
| **Wa** | | | | -0.619 | 0.461 | 0.286 | -1.000 | 0.218 | 0.939 |
| **Ash** | | | | | -0.557 | 0.279 | 0.609 | 0.202 | -0.536 |
| **Pro** | | | | | | -0.466 | -0.468 | -0.728 | 0.448 |
| **Fat** | | | | | | | -0.291 | 0.829 | 0.441 |
| **Car** | | | | | | | | -0.213 | -0.942 |
| **VA** | | | | | | | | | 0.267 |

Note Ac = Antocyanin, Ao = Antioxydant Wa = Water Pro = Protein Car = Carbohydrate VA = Vitamin A, VC = Vitamin C. cell with grey color means that correlation between variable is significant ($P < 0.05$).

The high antioxidant activity of purple sweet potato in the Antin 3 variety is due to the high content of anthocyanins and beta carotene (precursor of vitamin A) which is indicated by a darker purple color compared to the Antin 2 variety which has a reddish-purple color. The difference in color between the two varieties of purple sweet potato is caused by the addition or subtraction of hydroxyl groups, the position of hydroxyl groups, methylation of hydroxyl groups, sugars bound to molecules, number and location of sugars bound to molecules, and aliphatic acids or aromatic acids attached to the molecule. Sugar. The color produced in plants will be stronger with the increasing number and length of conjugated double bonds in the anthocyanin structure (Alappat & Alappat, 2020; Amoanimaa-Dede et al., 2019; Hellmann et al., 2021). Antioxidants are compounds capable of eliminating, purifying, counteracting, or combining the effects of reactive oxygen. The main function of antioxidants is to try to minimize the oxidation of fats and oils, minimize the occurrence of spoilage processes in food products, prolong the shelf life in the food industry, increase the stability of fats contained in food, and prevent the degradation sensory and nutritional qualities (Minah et al., 2015).

The treatment of different drying temperatures for the manufacture of purple sweet potato flour on the Antin 2 and Antin 3 varieties decreased the antioxidant activity. Generally, the higher the drying temperature, the lower the antioxidant activity. A drying temperature of 60OC was able to reduce the antioxidant activity of fresh purple sweet potato Antin 3 varieties by 82.07% to 59.35%, while fresh Antin 2 varieties with antioxidant activity 50.07% to 23.28%. This shows that one of the factors that affect antioxidant activity can be heating when dried. Several factors that affect antioxidant activity include changes in pH, the presence of oxygen, processing, and light (Jiang et al., 2019; Safari et al., 2019). The processing of purple sweet potato reduces antioxidant levels, where the decrease in antioxidant levels is directly proportional to the decrease in total phenolic and anthocyanin levels from purple sweet potato extract (Salim et al., 2017).

Antin 3 has a significantly higher fat and Vitamin A content than Antin 2 variety. Antin 2 has significantly higher protein and vitamin C content than Antin 3 variety. Vitamin C is a water-soluble compound that is unstable and easily damaged by heating. The heating process can result in oxidation processes and non-enzymatic browning reactions that can reduce the vitamin C content in food. which are rich in vitamin C as in fruits. This is following the results of research by (Ameliya & Handito, 2018) that the higher the temperature and the duration of heating (boiling) the degradation of vitamin C in cherry syrup is also greater. Oxidation of vitamin C (ascorbic acid) will convert ascorbic acid into L-dehydroascorbic

acid which is chemically very labile and can undergo further changes to L-ketogulonic acid which has no vitamin C activity (Ioannou, 2013).

The increase in the brighter color of purple sweet potato flour was due to the inhibition of polyphenol oxidase (PPO) activity (Ruttarattanamongkol et al., 2016). Generally, the processing of flour causes a loss of anthocyanins. Frying causes a total loss of anthocyanins about 46% of Blue Star varieties. Although there is a difference with cooking using a microwave which causes an increase in the total content of raw materials (D'Amelia et al., 2022)

The temperature has a significant effect on water and carbohydrate content. Water content decreases, while carbohydrate content increases with increasing temperature. The higher the temperature of the dryer, the more water that comes out of the material causing the lower the moisture content. Low water content can extend the shelf life to prevent damage caused by microorganisms or chemical damage. This shows that the water activity in the material is an important factor in the shelf life of dry food (D'Amelia et al., 2022; Ruttarattanamongkol et al., 2016).

Several factors that determine the high and low ash content of a material, among others, are caused by the different mineral content in the source of the raw material, the type of material, the method of ashing, the time, and the temperature used during drying. Ash content is inorganic substances or minerals contained in a material, the determination of ash content and composition depends on the type of material and the method of ashing. Determination of total ash content is useful as a parameter of the value of a food product because, with increasing ash content, the minerals contained in food products also increase(Setyowati & Nisa, 2014).

The protein content of food varies both in amount and type. Proteins from different sources have certain functional properties that affect the characteristics of the food. Proteins from different sources have certain functional properties that affect the characteristics of the food. The presence of the element N in determining the amount of protein can be determined by determining the amount of nitrogen (N) present in food. Nitrogen is the main element of protein because it is present in all proteins, which account for 16% of the total protein (Sundari et al., 2015). Protein in fresh food (before processing) has a higher protein content than after processing. The processing process with high-temperature heating and for a long time can damage the protein structure so that the protein content of the processed food decreases (Rijal et al., 2019). Proteins from different sources have certain functional properties that affect the characteristics of the food. The presence of the element N in determining the amount of protein can be determined by determining the amount of nitrogen (N) present in food. Nitrogen is the main element of protein because it is present in all proteins, which account for 16% of the total protein (Sundari et al., 2015). Protein in fresh food (before processing) has a higher protein content than after processing. The processing process with high-temperature heating and for a long time can damage the protein structure so that the protein content of the processed food decreases (Rijal et al., 2019).

Differences in protein content results can be caused during the processing/preservation of protein foods that are not controlled properly and can reduce protein levels. A processing process mainly using heat, such as sterilization, boiling, and drying can affect the protein content of foodstuffs. Excessive heat or other types of processing can damage the protein from a nutritional point of view, and it is also affected by the presence of other nutritional compounds contained in the ingredient. Processing causes changes in protein structure due to temperature increases, although not all of these changes are undesirable (Ariani et al., 2017). In addition to the type/variety of sweet potato, the protein content of sweet potato flour is also influenced by the peeling process during production. The excessive peeling process can cause the protein-rich part of the sweet potato meat to be wasted (Ariani et al., 2017).

Fat is an organic material that is soluble in organic solvents and is non-polar. The fat content of foodstuffs can affect the shelf life, the higher the fat content the lower the shelf life. This is evident in the storage of research raw materials, for the Antin 2 variety has a longer shelf life than the Antin 3 variety. Low-fat content can extend the shelf life of foodstuffs in the dry form (Damat et al., 2020). The results of the study using a cabinet dryer produced a higher fat content compared to the results of the other study on the purple sweet potato flour of the Ayamurasaki variety which was dried using an oven with a temperature of 40ºC which produced a fat content of 0.40% (Kassegn, 2018).

The carbohydrates contained in purple sweet potato flour are mostly in the form of starch, dietary fiber, and several types of sugar. soluble substances such as maltose, sucrose, fructose, and glucose (Ranonto & Razak, 2015). β-carotene tends to be damaged with higher heating. The decrease in beta-carotene content will be greater with increasing temperature and heating time as a result of damage to β -carotene by high temperatures. During drying, changes occur due to the isomerization reaction of cis-trans oxidation with the formation of epoxy carotenoids and apocarotenes (Ioannou, 2013). This causes a decrease in the content of beta-carotene in the material with increasing temperature. Factors that reduce and destroy β -carotene are oxygen, light, and heat. -carotene is easily oxidized in the air, this is due to the presence of a double bond structure in the β -carotene molecule. Oxidation will take place more rapidly in the presence of light, high-temperature heating, and metal catalysts. Decreased levels of -carotene can also occur with a longer heating process. Another factor that causes a decrease in beta-carotene levels in the production of flour from sweet potatoes is the milling process. During the milling process, there is contact with oxygen and there is constant friction between the sweet potato flour particles and the grinder wall, which generates a lot of heat (Kassegn, 2018).

### 3.3. Color Observation

Color analysis is carried out using a color reader with three value dimensions, namely, the first dimension is the L value which indicates the brightness level with a scale of 0-100, a value of 0 indicates a tendency for black or dark colors, while 100 indicates a tendency for white or light colors. The second parameter dimension a (+) value indicates the achromatic color of the red mixture with a scale of 0 – 80, and the value 0 – (-80) indicates a green color tendency. The third parameter, the b (+) value, shows a mixed achromatic color of yellow with a +b value of 0- (+70) and blue with a –b value of 0- (-70).

**Table 3.** Average Value of L, a+ and b+ Purple Sweet Potato Flour Antin 2 and Antin 3 varieties with different drying temperatures

| Treatment | Color | | |
|---|---|---|---|
| | L | a (+) | b (+) |
| **Antin 2 T40** | 60,00 | 10,95 | 5,75 |
| **Antin 2 T50** | 61,30 | 9,65 | 8,35 |
| **Antin 2 T60** | 62,80 | 8,40 | 9,70 |
| **Antin 3 T40** | 54,45 | 11,00 | 1,70 |
| **Antin 3 T50** | 54,10 | 10,85 | 3,15 |
| **Antin 3 T60** | 55,25 | 10,20 | 3,75 |

Based on the table data, it is known that the Antin 2 purple sweet potato flour has a higher brightness level (L) value than the Antin 3 variety. An increase in the drying temperature of the purple sweet potato flour in both varieties shows a brighter color. Purple sweet potato flour in the dimensions of the a (+) value for the greenish-red color intensity is known to be the higher the drying temperature, the smaller the a

(+) value produced with a range of values ranging from 8.40 - 11.00. A decrease in the value of a* indicates color damage during processing using heat caused by a significant decrease in anthocyanin content.

The results of the study indicate that the intensity of the yellowish color (b + value) was inversely proportional to the reddish color intensity (a+) value, and an increase in the drying temperature increased the intensity of the yellowish color (b+) value. This shows that the heating process will produce flour with a color that leads to a yellowish and brighter larger L value. flour that acquires a greater heat intensity during the drying process. The increase in dark yellow color during the drying process is thought to be caused by a non-enzymatic reaction (Maillard reaction) that occurs between reducing sugars and free amine groups of amino acids and/or proteins.

## 4. Conclusions

This study indicates that the Antin 3 variety has significantly higher levels of anthocyanins, antioxidants, fat, and Vitamin A than the Antin2 variety. Antin 2 has significantly higher levels of protein and Vitamin C content than Antin 3 variety. The temperature has a significant effect on water and carbohydrate content. Water content decreases, on the contrary, carbohydrate content increases with increasing temperature.

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# PHYSICO-CHEMICAL, TECHNOLOGICAL AND SENSORIAL QUALITIES OF MEAT AND SAUSAGE FROM BROILER CHICKENS FED DIETARY WHITE AND CAYENNE PEPPER POWDERS

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**ABSTRACT**

Repeated purchase of edible products by customers can be attributed to quality and taste – a target for producers and processors. The technological, physico-chemical and sensorial qualities of meat and sausage of broiler chickens fed dietary white pepper (WP) and cayenne pepper (CP) powders was investigated. Fifty-six broiler chickens with average weight ranging between 2.0 – 2.2 kg were selected. Prior to the experiment, broiler birds were selected from seven experimental groups supplied seven experimental diets. Post - evisceration, meat technological and physico-chemical indices were taken while sensory scores were awarded by semi-trained panelists. Analysis was carried out using One-Way ANOVA and Linear Mixed Model of SPSS with significant means ($p<0.05$) separated using Duncan Multiple Range Test. Results reveal overall minimization of loss and improved instrumental colour values of meat from B+200CP group was better but B+200WP group had better technological indices. Sausage from meat of birds fed B+200CP and B+125WP+125CP diets had preferred ($p<0.05$) loss and instrumental colour values. Panelists adjudged that meat from birds fed B+125WP+125CP diet had higher ($p<0.05$) overall flavour and acceptability sensorial scores though similar ($p<0.05$) as B+100WP+100CP and B+250CP groups; but identical in juiciness score as sole WP groups. Sausage from groups fed the Basal and B+125WP+125CP diets had better overall sensory scores, as panelists choice for or against pungency was the distinguishing index. B+200WP, B+200CP and B+125WP+125CP diets can be fed to broiler chickens to enhance meat technological, physico-chemical and sensorial characteristics respectively but meat from the latter and Basal groups are suggested for sausage.

## 1.Introduction

There are evidences suggesting a relationship exists among product quality, product cost and product safety (Cruz, 2015). Consumers are the target of every production chain and every production line is flawed without careful pre-consideration of the needs of consumer, knowing fully well that satisfying consumer expectations affect shopping behaviour. Satisfaction derived from the quality of a product strongly influence the intention to repeatedly purchase a product while quality cues or attributes of meat affect choice of purchase. Font-I-Furnols and Guerrero (2014) explained that the intrinsic (colour, amount of fat and layering) and extrinsic (price, origin, quality labels) properties of a product and their interactions impact the eventual sensations experienced by consumers. Consumer perception of meat and preference for certain meat qualities encourage consumer to purchase meat products. Sensations experienced from

meat are based on attributes such as tenderness, juiciness and taste which correlate highly with the intent to buy at the point of purchase (Banović *et al*., 2009; Garmyn, 2020).

Poultry meat contain essential desirable nutritional profile such as low lipid content and relatively high proportion of unsaturated fatty acids essential for healthy growth and development. Nutriment profile of poultry meat reveals chicken meat is comparable to other meats in cholesterol profile but offers superior nutrients when the protein, total fat and calorie content is juxtaposed with meat from other sources (Babic *et al*. 2014; Marangoni *et al.*, 2015). Nowadays, investigations of meat qualities of broiler chickens subjected to nutritional modification raises curiosity among consumers, and notable studies have been embarked on to connect causal factors affecting consumer attitudes with meat attributes. Since product quality is retained as best as possible under cold storage, the physical and nutritional qualities of perishable products is judged when stored meat is bought; largely affecting the quality of product and appeal to purchasers. Also, the technological properties of meat such as its water holding and absorptive properties influence its interaction with other ingredients (flour) and food additives or marinades for the production of chicken meat sausage (Lees *et al*., 2019; Mudgil and Barak, 2019; Szendrő *et al*., 2020). Hence, producers and processors see it expedient to modify meat properties to satisfy consumer expectations.

Additives have been incorporated into broiler diets to improve the sensory characteristics of food. (Kralik *et al*., 2017; Sanwo *et al*., 2019). Exploration of these substances influence decision to purchase since properties of meat and meat products are critical to purchase. Hot red pepper and cayenne pepper contain capsaicin, vitamins, potassium, manganese and flavonoids that contribute to performance and quality (Younis and Abdel-Latif, 2017; Adegoke *et al*., 2018). On the other hand, Akinoso *et al*. (2013) reported that *Piper nigrum* contribute piquant taste and flavour to cuisines as a result of its piperine (the principal functional compound) content alongside minerals such as calcium, manganese, iron, zinc, potassium, magnesium, carotene, zeaxanthin, lycopene (excellent sources of vitamin B complex groups such as pyridoxine, riboflavin, thiamine and niacin) and rotundone – an aromatic compound (Drew *et al*., 2016).

Studies on performance among birds fed dietary pepper powders suggests sensorial and eating qualities of meat are likely sacrificed at higher levels of inclusion. Notably, a study conducted by Sanwo *et al*. (2019) showed that cayenne pepper inclusion at minimal dosage exhibited improved meat quality profile. Since consumer satisfaction and product quality are intrinsically connected to repeated purchase and limited information currently exists on the impact dietary pepper powders exert on quality of meat and sausage from meat-type chickens; an experiment was therefore conducted to investigate the physico-chemical, technological and sensorial qualities of meat and sausage from broiler chickens fed dietary white and cayenne pepper powders.

## 2.Materials and methods

Meat of broiler birds fed diets containing pepper powders were sourced. Diet layout birds were subjected to comprise:

Treatment (T) 1 - Basal diet (B) (No additive added)

T2 - Basal diet + 200 g White Pepper (WP) (B+200WP)

T3 – Basal diet + 250 g White Pepper (B+250WP)

T4 - Basal diet + 200 g Cayenne Pepper (CP) (B+200CP)

T5 – Basal diet + 250 g Cayenne Pepper (B+250CP)

T6 - Basal diet + 200 g WP and CP (B+100WP+100CP)

T7 - Basal diet + 250 g WP and CP (B+125WP+125CP)

Proximate composition of the Basal diet was determined following AOAC (2005) method with 22.90% crude protein, 12.05% metabolizable energy, 3.80% crude fibre, 3.98% ether extract, 1.36% calcium; 0.58% Phosphorus, 1.61% lysine and 0.54% methionine present.

### 2.1.1. Experimental layout

Twenty-eight broiler chickens with weight range between 2.2 and 2.5 kg were selected for meat quality analysis, while another twenty-eight birds with weight range between 1.8 and 2.2 were sacrificed for sausage. One bird was selected from each of the seven (7) treatment groups that comprise of four (4) replicates. The same procedure was followed for sausage, totaling 56 birds for both meat and sausage quality evaluations. Selected birds were conventionally dressed following ethical guidelines detailed by the Animal Welfare Board of the College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta (FUNAAB, 2016).

### 2.1.2. Procedure for sausage preparation

Chicken meat sausage was formulated up to 500 grams. Composition of chicken sausage is presented in Table 1. Fresh meat from the breast muscles were obtained, weighed and run through a 5 mm plate in a grinder unit of Kenwood (Hampshire U. K) food processor and placed in cellulose casing at 10 - 15 mm length distance. Seven (7) batches of sausage, each corresponding to a treatment was constituted. Each batch was further subdivided into rolls as replicates, comprising four (4) rolls for sensory and technological properties respectively.

**Table 1**. Ingredient composition of chicken sausage

| **Ingredients** | **Composition (%)** |
|---|---|
| Breast Muscles | 48 |
| Wheat Flour | 25 |
| Vegetable Oil | 10 |
| Additives | 2 |
| Ice/Cold Water | 13 |
| Salt | 2 |
| Total | 100 |

* Additives – White pepper 0.3 g, Cayenne pepper 0.3 g, Ginger 0.25 g, Turmeric 0.90 g, Coriander 0.23g, Seasoning 0.02 g

## 2.2. Data Collection

### *2.2.1. Physico-chemical indices of chicken meat and sausage*

*2.2.1.1. pH and temperature evaluation*

Meat pH was taken by placing the electrode of the pH meter (model pH – 108A) deeply into the breast region after standardization (Kim *et al*., 2009), while a simple insertion was performed for raw sausage. Thorough rinsing was done after each dipping.

*2.2.1.2. Colour evaluation*

Two wings from each dressed bird per replicate was neatly separated and meat colour was examined using a colorimeter (CHROMA METER CR – 410, JAPAN). Lightness (L*), redness (a*) and yellowness (b*) colorimetric coordinates were evaluated using details published by Kralik *et al*. (2017) while colour intensity/saturation index (chroma value and hue angle) were calculated according to Melahat Özbek *et al*. (2020) from the a* and b* values for chicken meat and sausage.

*2.2.1.3. Cooking loss determination*

Left thigh of each carcass was apportioned for loss evaluation. Selected samples were weighed, wrapped in separate air-tight

polythene bags and cooked in a water bath at 70 °C for 30 minutes (Sanwo *et al*., 2019). Sausages were labelled and placed in microwave oven (Samsung GE109MST) at 600 Watt power level for 15 min. Subsequently, the internal temperature of each sausage was measured using a digital thermometer probe.
Cooking loss for both meat and sausage were obtained using the formula below.

$$\text{Cooking loss (g)} = \text{Weight before cooking} - \text{Weight after cooking} \quad \textbf{(1)}$$

$$\text{Cooking loss (\%)} = \frac{weight\ before\ cooking - weight\ after\ cooking}{weight\ before\ cooking} X\ 100 \quad \textbf{(2)}$$

*2.2.1.3. Determination of meat and sausage refrigeration loss*

Left drumstick from each carcass was selected per replicate. The samples were weighed and labelled prior to refrigeration, re-weighed after 24 hours of refrigeration for refrigeration loss analysis as follows:

$$\text{Refrigeration loss (g))} = \text{weight before refrigeration} - \text{weight after refrigeration} \quad \textbf{(3)}$$

$$\text{Refrigeration loss (\%)} = \frac{weight\ before\ refrigeration - weight\ after\ refrigeration}{weight\ before\ refrigeration} X\ 100 \quad \textbf{(4)}$$

*2.2.1.4. Assessment of meat water holding capacity*

Fifteen grams of meat from the breast region of each carcass was cut out to determine meat water holding capacity. Samples were blended using conventional blender prior to centrifugation using 22.5 ml of 0.6 M saline solution. Afterwards, contents were stirred with a glass rod for 1 minute as described by Hamm (1975). Next, samples were placed in the refrigerator for 15 min as slurry formed was stirred for 1 min before placement into centrifuge **(**MERLIN 503, Spectra scientific Ltd, Great Britain**)** for 15 mins. Supernatant layer gotten was decanted and the solution obtained was documented. Liquid retained in meat was reported as water holding capacity (ml) per 15 g of meat.

$$\text{WHC} = \frac{Before\ centrifuge - After\ centrifuge}{Before\ centriguge} \text{ x } 100 \quad \textbf{(5)}$$

*2.2.1.5 Determination of meat water absorptive capacity*

Thirty grams of meat from the breast muscles was cut out and blended with 20 ml of distilled water for 2 minutes to obtain water absorption capacity (WAC) of meat samples. Homogenized mixture was poured and rinsed with 20 ml of distilled water into a pre-weighed centrifuge tube, then centrifuged using the procedure detailed by Arganosa *et al*. (1991) (MERLIN 503, Spectra scientific Ltd, Great Britain) at 2000 rpm for 25 min. Unabsorbed residual water decanted post-centrifugation and water absorbed by meat was calculated as:

$$\text{WAC (\%)} = \frac{gram\ of\ water\ absorbed}{gram\ of\ meat} \text{ x } 100. \quad \textbf{(6)}$$

### *2.2.2. Analysis of sensory profile of chicken meat and sausage*

Cooked chicken meat and microwaved sausage samples were dissected into pieces, according to the number of panellists (20). Panellists were separately presented with coded samples to evaluate sensorial attributes ranging from appearance to pungency. Ordinary water was provided for gaggling to prevent or minimize any carry over effect. Panellists scored samples using slightly modified hedonic scale (Sanwo *et al*., 2013) by responses on a 9-point hedonic scale intensity (1 = Dislike extremely, 2 = Dislike very much, 3 = Dislike moderately, 4 = Dislike slightly, 5 = Intermediate, 6 = Like slightly, 7 = Like moderately, 8 = Like very much, 9 = Like extremely). Colour, juiciness, meat flavour, tenderness, saltiness, saltiness, pungency, overall flavour and overall acceptability were attributes judged by panellists.

## 2.3. Statistical analysis

Data obtained were subjected to a one-way analysis of variance using the generalized linear

model procedure of Statistical Package for Social Sciences version 21 (SPSS, 2012). Covariate analysis was carried out on the initial weight of drumstick prior to the determination of cooking and cooking loss percentage. Data obtained from sensory experiments (Chicken meat and Sausage) were subjected to Linear Mixed Model with panellists' effect weighted following GLM procedure of the same statistical package. Significantly different means ($p<0.05$) were separated using Duncan Multiple Range Test of the same Statistical Package.

## 3.Result and Discussion

### 3.1. Evaluation of physico-chemical and technological indices of meat from chickens fed experimental additives.

pH, refrigeration and cooking losses; refrigeration and cooking loss percentages; absorptive power; holding capacity and colour of meat from chickens fed dietary peppers is documented in Table 2. Meat final weight, refrigeration loss and loss percentage; cooking and cooking loss percentages; meat water absorptive power; lightness, yellowness and redness were affected ($p<0.05$). Final weight of meat was reduced ($p<0.05$) post-refrigeration among meat from birds given B+200WP diet than B+250WP and B+250CP diets. Meat refrigeration loss and refrigeration loss (%) values were statistically identical with more loss from meat of birds fed B+200WP diet compared to other groups. Percentage cooking loss of meat of birds fed the Basal and B+125WP+125CP diets increased than meat of birds given B+200CP diet but other groups had comparable loss percentages. Meat water absorptive power was least ($p<0.05$) among chickens offered B+250WP diet, but higher among meat of birds supplied B+200WP diet, though comparable as values obtained from B+200CP, B+250CP and B+125WP+125CP groups. Meat lightness was highest among groups supplied cayenne pepper powder additive solely at 200 and 250 g 100 $kg^{-1}$ of feed than lightness value of meat of birds offered 200 g 100 $kg^{-1}$ white pepper diet. Meat redness was pronounced ($p<0.05$) among birds in B+100WP+100CP group than all other groups, but the Basal, B+200WP and B+200CP groups were lower, while B+250CP was least. Yellowness was intensified ($p<0.05$) in meat of birds given the Basal diet than B+200CP and B+125WP+125CP, but least among B+250WP group.

**Table 2.** Physico-chemical and technological indices of meat from broiler chickens fed dietary white and cayenne pepper powders

| Parameters | Basal (B) | B+200WP | B+250WP | B+200CP | B+250CP | B+100WP +100CP | B+125WP +125CP | SEM |
|---|---|---|---|---|---|---|---|---|
| pH | 6.41 | 6.44 | 6.18 | 6.55 | 6.55 | 6.36 | 6.42 | 0.05 |
| **Refrigeration loss in Meat** | | | | | | | | |
| Final wt (g) | $114.36^{ab}$ | $110.38^{b}$ | $114.72^{a}$ | $113.46^{ab}$ | $114.46^{a}$ | $114.25^{ab}$ | $114.45^{ab}$ | 0.82 |
| Ref. loss (g) | $1.45^{b}$ | $5.05^{a}$ | $1.00^{b}$ | $1.40^{b}$ | $1.00^{b}$ | $2.00^{b}$ | $1.50^{b}$ | 0.38 |
| Ref. loss % | $1.30^{b}$ | $4.39^{a}$ | $0.88^{b}$ | $1.08^{b}$ | $0.81^{b}$ | $1.92^{b}$ | $1.36^{b}$ | 0.34 |
| **Cooking loss in Meat** | | | | | | | | |
| Initial wt (g) | 106.25 | 117.25 | 116.50 | 117.50 | 117.50 | 113.50 | 112.75 | 2.49 |
| Final wt (g) | 97.13 | 107.53 | 108.63 | 112.30 | 109.30 | 105.40 | 102.70 | 2.41 |
| Cooking loss (g) | 9.13 | 9.73 | 7.88 | 5.20 | 8.20 | 8.10 | 10.05 | 0.58 |
| Cooking loss % | $8.66^{a}$ | $8.38^{ab}$ | $6.80^{ab}$ | $4.26^{b}$ | $6.90^{ab}$ | $7.00^{ab}$ | $8.91^{a}$ | 0.51 |
| **Meat water absorptive power and holding capacity (%)** | | | | | | | | |
| WAP | $53.70^{bc}$ | $88.30^{a}$ | $39.20^{c}$ | $69.00^{ab}$ | $71.70^{ab}$ | $60.30^{bc}$ | $76.70^{ab}$ | 4.06 |
| WHC | 58.47 | 55.12 | 53.72 | 61.37 | 54.04 | 54.17 | 62.87 | 1.61 |
| **Colour indices** | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| L* | 51.42$^{c}$ | 48.54$^{d}$ | 52.82$^{b}$ | 54.16$^{a}$ | 54.08$^{a}$ | 51.76$^{c}$ | 53.06$^{b}$ | 0.50 |
| a* | 10.72$^{d}$ | 10.59$^{d}$ | 12.17$^{b}$ | 10.60$^{d}$ | 9.90$^{e}$ | 14.98$^{a}$ | 11.17$^{c}$ | 0.44 |
| b* | 12.43$^{a}$ | 7.53$^{d}$ | 3.37$^{f}$ | 10.17$^{b}$ | 8.36$^{c}$ | 4.17$^{e}$ | 10.91$^{b}$ | 0.87 |
| Chroma | 16.41 | 12.99 | 12.63 | 14.69 | 12.96 | 15.54 | 15.61 | 1.96 |
| Hue | 1.16 | 0.71 | 0.28 | 0.96 | 0.84 | 0.28 | 0.98 | 0.12 |

$^{a, b, c, d, e, f}$ - means on the same row with different superscripts differ significantly ($p$ <0.05).
CP – Cayenne pepper powder WP- white pepper powder WAP – water absorptive power
WHC – water holding capacity wt – weight L* - Lightness a* - Redness b* - yellowness

High moisture loss in meat result in low-weight product caused by increased water or moisture escape from muscle cells. Meat quality is affected as water soluble sarcoplasmic proteins, vitamins and enzymes are released. High refrigeration loss and loss percentage was recorded for meat from chickens given B+200WP diet than other groups. According to Anne *et al*. (2016) light muscle and dark muscle vary in processing attributes owing from their respective preponderances in red and white fibre contents with different functionalities within meat muscle proteins matrix, resulting in the immobilization of extraneous water into meat. The variation between gastrocnemius and pectoralis major myofibril in chicken meat (Ismail and Joo, 2017), determine the extent of enlargement that occurs, causing pull of extraneous water. With B+200WP diet fed effecting high loss, but not B+250WP; an inference posited is that the consumption of white pepper powder by broiler chickens at 250 g was not optimal to increase the amount of pectoralis major myofibril in meat. High cooking loss indicates moisture was lost during thermal treatment of meat. Meat from chickens supplied B+200CP diet had the lowest cooking loss percentage. According to Olsson *et al*. (2003), the effect of production system on cooking loss resulted in lower loss (%) in meat from animals raised on organic substances compared with that fed conventional diet. Hocquette *et al*. (2010) likewise declared that chemical composition of muscles is fairly constant but contains variable lipid composition between species, within species as well as between muscles and cuts. Such modifications brought about by peppers incorporated alter the properties of meat. The outcome of feeding B+200CP diet contradicted the report of Sanwo *et al*. (2019) possibly due to strain (Abor acres vs Cobb broiler chickens) variation. Tougan *et al*. (2013) identified the contributory role genetics and environmental factors play on muscle composition and meat quality. Notably, consumers pay premium for meat and meat products, especially those characterized by not only high flavour scores, better nutritive value and low-fat content; but rich in vitamins and minerals concentration.

Meat with the highest affinity for pull of exterior water was obtained from birds offered B+200WP diet but lowered in meat of groups supplied B+250WP diet. Protein structure and composition affect the absorptive capacity of fresh meat. Sarcoplasmic proteins bathe the myofibrillar proteins that provides needed biochemical functions such as energy, to synthesize protein and remove soluble metabolic by-products (Sanwo *et al*., 2019). Dietary white pepper powder possibly at its lowest inclusion percentage facilitated increased sarcoplasmic protein production within muscle cells that results in modified structural tissues. Marino *et al*. (2014) posited that optimum moisture absorption may occur owing to the water binding capacity of meat, as the sarcoplasmic proteins show higher affinity. Such meat will likely yield higher tenderness score – a delight to consumers who focus on marination brining as it becomes juicier (FSIS, 2013). Wang *et al.* (2006) likewise buttressed that high water absorptive power values are important to transfer dissolved ingredients from external sources into meat. On the other hand, contradictory and opposing values between

refrigeration loss (%) and water absorptive power values could be attributed to the different proteins responsible for water pull and binding (Xiong, 2005).

Instrumental colour analysis of meat of birds offered additives reveal impact of diet on lightness, redness and yellowness parameters. Haem pigment directly correlates with paleness of breast meat and pale meat in turn is negatively correlated with lightness ($L^*$) values (Boulianne and King, 1998; Karunanayaka *et al*., 2016). On the contrary, Anadon, (2002) explained that light dissipation affects meat $L^*$ value with an inverse correlation observed against heme pigment concentration with minimal impact on meat redness ($a^*$) and yellowness ($b^*$) values, while Jankowiak et al. (2021) clarified that lower pH corresponds with higher $L^*$ value. The report of the latter authors contradicts findings from this study. Feeding birds 200 and 250 g cayenne pepper per 100 kg of the Basal diet yielded numerically higher pH. Meat L* values of meat of chickens supplied cayenne pepper at 200 and 250 g 100 $kg^{-1}$ of Basal diet is in partial agreement with the findings of Tashla *et al*. (2020), whose study showed that lightness value was pronounced in meat of birds from the Control group that were fed dietary garlic, black pepper and hot red pepper as supplement. Alteration in meat lightness is dominantly caused by elevated meat colour opacity. This occurs due to the potency of dietary pepper powders to inhibit the extent of fat deposition, thus improving the lipid metabolism.

Possible dissipation of meat myoglobin result in increased lightness values but not the formation of PSE meat since canthaxanthin is the dominating red carotenoid in pigmented broilers (Hamelin and Catherine, 2012). Kim *et al*. (2010) stated that pigment content and redox state account for much of the variation in a* values since meat myoglobin presence is significantly correlated with redness (a*). All redness values for all treatment groups exceeds documented values by Akiba *et al*. (2001) and Wattanachant *et al*. (2004) for commercial broiler chickens. Though Tasla *et al*. (2020) established stronger redness intensity in thigh meat of broilers fed 1% hot red pepper; blood heamoglobin – myocytes and heme pigments transfer to the muscles were facilitated by biomolecules in peppers.

This study had birds on the Basal diet with the highest meat yellowness score. On observing high variability in meat yellowness despite homogeneous xanthophyl fed, Sirri *et al*. (2010) inferred that other factor influence meat yellowness along with pigment concentrations and this study supports the posit. All additives impacted negatively on yellow skin pigmentation of the birds with B+250WP and B+100WP+100CP diets most notable. In broilers, zeaxanthin influences the prominence of yellowness in tissues, more noticeably in the abdominal fat (Hamelin and Catherine, 2012). A likely explanation is that pepper powders suppress zeaxanthin formation and its deposition in the skin at varying rates. Alternatively, peppers may negatively impact the absorption of xanthophyl in the feed or intestine as xanthophyl is directly associated with intestinal integrity (Ortega *et al*., 2012). Our findings agree with the report of Pugliesea *et al*. (2013), that bioavailability of carotenoids is affected by dietary factors such as the nature, type and amount of carotenoid consumed or added to the diet.

**3.2. Sensory characteristics of meat from chickens supplied dietary** ***Piper nigrum*** **and** ***Capsicum frutescens***. Sensory characteristics of meat from chickens fed dietary additives is documented in Table 2. All sensory indices measured were significant ($p<0.05$) except saltiness and pungency. Meat from birds offered B+250CP, B+100CP+100WP and B+125CP+125WP diets had colour scores that were 'moderately liked' when compared against the Basal, B+200WP and B+200CP groups that were 'slightly liked'. Meat of chickens given B+200WP, B+250WP and B+125WP+125CP diets were 'moderately juicy' with higher

juiciness score than B+200CP group that had 'slightly liked' score. 'Intermediate flavour' score was ascribed to meat of birds fed the Basal and B+200CP diets but 'slightly flavourful score' was awarded to B+100WP+100CP and B+125WP+125CP groups which was significantly higher. Meat of chickens supplied B+200WP diet had statistically similar values as meat of chicken offered the Basal, B+250WP and B+125WP+125CP diets ('moderately tender') but was significantly higher than tenderness score of meat of birds fed B+250CP and B+100WP+100CP diets ('slightly tender'). Higher overall flavour score of meat obtained from birds supplied dietary B+125WP+125CP was comparable ($p<0.05$) as score from B+250CP and B+100WP+100CP groups, both labelled 'moderately desirable' by panellists. A similar trend was observed for meat overall acceptability scores as all three treatment groups previously mentioned were adjudged 'moderately liked' for overall acceptability.

Meat of birds supplied dietary cayenne peppers singly at 200 and 250 g 100 $kg^{-1}$ of the Basal feed as well as B+125WP+125CP diet had highly attractive colour score according to panellists. Inclusion of 125g WP: 125 CP powder did not negatively influence perceived score by panellists, indicating a foundation template for meat quality from birds fed dietary cayenne pepper. Xiao *et al*. (2011) associated improved meat colour L* (lightness) with the supplementation of vitamin E. Pepper carotenoids, especially capsanthin and capsorubin exhibited anti-oxidative properties.

**Table 3.** Sensory characteristics of meat from chickens given *Piper nigrum* and *Capsicum frutescens* as additives

| Parameters | Basal (B) | B+200WP | B+250WP | B+200CP | B+250CP | B+100WP +100CP | B+125WP +125CP | SEM |
|---|---|---|---|---|---|---|---|---|
| Colour | $6.19^{b}$ | $6.45^{b}$ | $6.51^{ab}$ | $6.41^{b}$ | $7.10^{a}$ | $7.09^{a}$ | $7.15^{a}$ | 0.08 |
| Juiciness | $6.76^{ab}$ | $7.18^{a}$ | $7.09^{a}$ | $6.32^{b}$ | $6.81^{ab}$ | $6.82^{ab}$ | $7.22^{a}$ | 0.08 |
| Meat flavour | $5.73^{b}$ | $5.95^{ab}$ | $5.97^{ab}$ | $5.75^{b}$ | $6.02^{ab}$ | $6.68^{a}$ | $6.68^{a}$ | 0.10 |
| Tenderness | $7.08^{ab}$ | $7.21^{a}$ | $7.03^{ab}$ | $6.59^{abc}$ | $6.48^{bc}$ | $6.26^{c}$ | $7.12^{ab}$ | 0.09 |
| Saltiness | 5.14 | 4.99 | 5.23 | 5.43 | 5.29 | 4.82 | 5.31 | 0.08 |
| Pungency | 6.56 | 6.36 | 6.33 | 6.52 | 6.32 | 6.37 | 6.44 | 0.09 |
| Overall flavour | $6.60^{bcd}$ | $6.30^{cd}$ | $6.69^{bc}$ | $6.06^{d}$ | $6.81^{abc}$ | $7.17^{ab}$ | $7.34^{a}$ | 0.08 |
| Overall acceptability | $6.53^{cd}$ | $6.18^{d}$ | $6.67^{bcd}$ | $6.42^{cd}$ | $6.90^{abc}$ | $7.26^{ab}$ | $7.46^{a}$ | 0.09 |

$^{a, b, c, d}$ – Means in the same row with different superscripts are significantly ($p$ <0.05) different.
CP – Cayenne pepper powder; WP - white pepper powder

Capsanthin accounts for approximately average of the total carotenoids found in fully ripe fruits (Mohd Hassan *et al*., 2019) while phenolic compounds (flavonoids - mainly quercetin and luteolin); phenolic acids; capsaicinoids; tocopherols; carotenoid, ascorbic acid; nitrates and nitrites (Campos *et al*., 2013) in peppers (fresh peppers and their seeds) contribute to light dispersion, pH and colour properties of meat. Meat juiciness was adjudged better for groups offered white pepper at 200 and 250 g 100 $kg^{-1}$ diets, and meat of birds fed B+125WP+125CP diet, while B+200WP diet offered yielded meat with desirable tenderness score. As Warner (2007) predicted, there is a connection between meat water holding capacity (WHC), texture and juiciness. Meat from birds given white pepper diets of 200 and 250 g 100 $kg^{-1}$ and B+125WP+125CP diet all had higher scores but the latter group had

numerically higher WHC. White pepper at low dosage moderately modified muscle fat and water proportions by depositing phytochemical that influenced meat juiciness, alongside other intrinsic and extrinsic factors such as water released from meat during mastication mixed with saliva (Oppen *et al*., 2022). Juárez *et al.* (2012) similarly alluded to the textural (tenderness) properties of meat such as water-holding capacity (WHC) and fat content. These factors contribute to the lubrication, mastication and chewing sensations experienced by panelists offered meat of birds fed B+200WP diet. Such complex sensory attributes are reportedly attached to descriptors such as fibre characteristics, cohesiveness, adhesion, mushiness, softness and amount of residual connective tissue. Flavour score of meat from chickens fed both combinations were adjudged 'moderately flavorful', having higher flavour score than meat from birds fed the Basal and B+200CP diets. Meat flavour forms during cooking owing to Maillard reaction and lipid oxidation. Amino acids, peptides and ribose - precursors contribute to meat flavour when exposure to increasing heat which triggers the Maillard reaction in combination with nucleotides and additional amino compounds, such as creatine, carnosine and creatinine (Sun *et al*., 2022). Brenes and Roura (2010) pointed out that the combined effect of additives results in complexity as a result of the quantity and variability of bioactive compounds, and mixture of essential oils present, causing feasible effects that alter the concentrations required to achieve a particular impact. Also, Arimboor, (2015) explained that ground and aqueous pepper (paprika) contributed to colour and flavour alterations in soups, stews and sausage, thus confirming the potency of aromatic constituents in pepper powders. Furthermore, lipid degradation releases compounds that transfer fatty aromas to cooked meat. Polyunsaturated fatty acids (PUFA) are sources of aromatic volatiles in cooked meat, thus contributing to flavour development. Phospholipids sucked out of meat gave meat a biscuit-like aroma that pale in comparison to the flavour sensations perceived from lipid-derived aldehydes and alcohols, accompanied by increased formation of compounds derived from maillard reaction (Mottram and Edwards, 1983; Shahidi and Hossain, 2022). For meat overall flavour, meat of chickens supplied B+125WP+125CP diet was preferred by panellists. Increased the concentration of soluble flavour components triggers a variety of chemical reactions that modify flavour properties, thus influencing the overall flavour of the meat. Also, melted fat spreads across the surface as boiling temperature increases alongside decomposition products contribute to perception of meat overall product, thereby, influencing sensations experienced such as mouth feel and juiciness. With B+125WP+125CP diet adjudged best for colour, juiciness, flavour and overall flavour, it expectedly translates to higher score for acceptability.

### 3.3. Physico-chemical properties of sausage from meat of chickens fed dietary peppers.

Physico-chemical properties of sausage formulated from meat of chickens fed dietary pepper powders is presented in Table 4. Sausage internal cook temperature and refrigeration loss percentage were significantly ($p<0.05$) different, but pH, cooking and refrigeration losses as well as cook loss percentage were not significant ($p>0.05$). Peak core temperature of sausage was recorded among the Basal, B+200WP and B+250CP groups after cooking in microwave. Low temperature was recorded for sausage from B+250WP group but other groups had intermediate ($p>0.05$) temperature values. Refrigeration loss (%) was significantly higher and lower respectively in sausages formulated from meat of birds fed B+250CP and B+200CP diets respectively, though all other groups had statistically ($p<0.05$) intermediate percentages. Sausage lightness (L*), redness (a*) and yellowness (b*) intensity values were affected but chroma and hue values were not influenced.

Intensity of sausage lightness, redness and yellowness was more vivid among sausage from meat of birds fed B+125WP+125CP diet. Least ($p<0.05$) lightness and yellowness values were observed among B+100WP+100CP group as B+200CP group had the least redness score.

**Table 4.** Physico-chemical properties of sausage from meat of chickens fed dietary pepper powders

| Parameters | Basal (B) | B+200WP | B+250WP | B+200CP | B+250CP | B+100WP +100CP | B+125WP +125CP | SEM |
|---|---|---|---|---|---|---|---|---|
| pH | 6.71 | 6.77 | 6.65 | 7.00 | 6.52 | 6.75 | 6.14 | 0.26 |
| T (°C) | 68.50$^{a}$ | 68.75$^{a}$ | 52.55$^{b}$ | 56.65$^{ab}$ | 69.60$^{a}$ | 63.90$^{ab}$ | 59.25$^{ab}$ | 2.09 |
| **Loss (%)** | | | | | | | | |
| Cooking (g) | 4.95 | 3.75 | 7.05 | 4.45 | 8.70 | 5.00 | 5.50 | 0.63 |
| Cooking (%) | 6.52 | 5.21 | 9.13 | 6.54 | 12.35 | 7.71 | 8.03 | 0.86 |
| Ref (g) | 6.40 | 4.30 | 5.55 | 3.80 | 8.70 | 5.00 | 5.50 | 0.57 |
| Ref (%) | 8.87$^{ab}$ | 6.77$^{ab}$ | 8.01$^{ab}$ | 5.06$^{b}$ | 14.19$^{a}$ | 8.42$^{ab}$ | 8.76$^{ab}$ | 1.00 |
| **Colour** | | | | | | | | |
| L* | 47.61$^{d}$ | 51.24$^{ab}$ | 47.69$^{cd}$ | 50.55$^{b}$ | 48.58$^{c}$ | 46.31$^{e}$ | 51.81$^{a}$ | 0.54 |
| A* | 2.88$^{bc}$ | 2.78$^{c}$ | 2.79$^{c}$ | 1.93$^{d}$ | 2.74$^{c}$ | 3.19$^{b}$ | 3.54$^{a}$ | 0.49 |
| B* | 34.03$^{c}$ | 37.93$^{b}$ | 34.03$^{c}$ | 35.10$^{c}$ | 37.38$^{b}$ | 30.02$^{d}$ | 40.48$^{a}$ | 0.88 |
| Chroma | 34.15 | 38.03 | 34.14 | 35.15 | 37.48 | 30.19 | 40.63 | 1.98 |
| Hue | 11.82 | 13.64 | 12.20 | 18.19 | 13.64 | 9.41 | 11.44 | 1.09 |

$^{a, b, c, d, e}$ - Means on the same row with different superscripts differ significantly ($p$ <0.05).
CP – cayenne pepper powder; WP- white pepper powder T – Internal cook temperature
Ref - refrigeration

Refrigeration loss values of sausage were modified by pepper powders fed and this could be attributed to the carry over effect from the meat. B+200CP diet had the overall lowest refrigeration and cook loss values which could be attributed to the type of protein developed within tissues of birds fed the dietary ingredient. Also, various oxidation-processing steps affect the properties of poultry meat, compromising animal growth, performance, and ultimately the quality of livestock. Protein and lipid oxidation have been recognized as a major threat to the quality of subsequently processed poultry products (Xiao *et al*., 2011). Instrumental analysis of L* and a* can easily be applied to muscle colour, but b* (blue and yellow) are not typical or intuitively related to meat (Mancini and Hunt, 2005) but can be to its products. French *et al*. (2000) observed a correlation between b* and carcass fat score when comparing finishing cattle on grass and concentrate. Carotenoid influence is exhibited by food properties which carotenoids are incorporated into as well as post-harvest handling, processing, storage and cooking practices (Pugliesea *et al*., 2013). In fact, capsaicinoid and colour intensities are considered key interwoven factors. Carotenoids are considered sources of red and yellow pigments that can inpact poultry product. Birds reportedly cannot generate these carotenoids, hence, ingredients such as red pepper, pine and corn meal when offered in the diet can be a source of supply (Marounek and Febriansyah, 2018). Sausages from meat of chickens offered B+125WP+125CP diet yielded brightest colour properties for l*, a* and b*. The interaction of both peppers in the diet and externally in chicken sausage reveal carotenoids were concentrated in the product. The pigments supplied by both peppers facilitated colourful properties that can attract customers to the product. The significance is reflected in the posit that consumer purchasing decision to a large extent depend on product colour.

**3.4. Sensory characteristics of sausage from meat of chickens fed dietary peppers as additives.** Panellist assessment of sausages obtained from meat of chickens given dietary peppers powders is presented in Table 5. Colour,

pungency, overall flavour and overall acceptability of chicken sausage were significantly ($p<0.05$) influenced. Colour score of sausage from birds given B+200WP diet was higher ($p<0.05$) and described as 'slightly liked' by panellists, but B+200CP and B+100WP+100CP groups were awarded lower score values, though other groups excluding B+200WP had similar ($p>0.05$) values. Pungency of sausage of meat of birds offered the Basal diet was labelled 'intermediate' by assessors, though pungent ($p<0.05$) compared to sausage from B+250CP and B+125WP+125CP meat. Sausage overall flavour and acceptability for the Basal and B+125WP+125CP groups was scored higher ($p<0.05$) than B+200CP and B+100WP+100CP groups though similar ($p>0.05$) as awarded for B+200WP, B+250WP and B+250CP groups. Both groups with the highest ($p<0.05$) overall favour and acceptability scores were described as 'slightly desirable' and 'slightly liked' sausages respectively by panellists. Sausage from B+200WP, B+250WP and B+250CP groups were similar ($p>0.05$) as the Basal and B+125WP+125CP groups for both overall flavour and acceptability indices. Colour modifying properties of pepper was reported by Arimboor (2015) with modifications in colour and flavour of soups, stews as well as sausage with the addition of ground and aqueous pepper (paprika) reported. Similarly, documented colour stabilization of nitrite-free dry sausages with the incorporation of paprika and tomato paste by Bázan-Lugo *et al*. (2012) further alluded to this. Peppers possess tremendous variation in colour and oxidative properties (Umoh *et al*., 2020). This study yielded sausage enhanced colour properties that appeal to panellists. For pungency, sausage pungency index decreased as the concentration of dietary pepper powders increased. Anderson *et al*. (2017) explained that while consuming soup containing cayenne pepper, the urge for salty and spicy foods significantly decreased while desire for sweet and fatty foods significantly increased accordingly, implying that altered desire exists when pungent molecules are consumed. Pungency, however is subjective to individual preference. Sausage overall flavour and acceptability were scored higher for formulations compounded from meat of chickens given the Basal and B+125WP+125CP diets. Express approval by panellists may be as a result of different perceptions of sensations and interactive complexities. Flavour of sausage for meat of birds fed B+125WP+125CP diet generated via aromatic interaction within botanicals possibly increased with mixture of peppers which may have influenced the volume of lactic acid and ions in product, contributing to perception expressed (Tikk *et al*., 2006).

**Table 5.** Sensory characteristics of sausage from meat of chickens fed dietary pepper (White and cayenne) powders

| Parameters | Basal (B) | B+200WP | B+250WP | B+200CP | B+250CP | B+100WP +100CP | B+125WP +125CP | SEM |
|---|---|---|---|---|---|---|---|---|
| Colour | $5.84^{ab}$ | $6.55^{a}$ | $6.07^{ab}$ | $5.15^{b}$ | $5.81^{ab}$ | $5.14^{b}$ | $6.18^{ab}$ | 0.15 |
| Juiciness | 4.90 | 4.90 | 4.94 | 3.79 | 3.76 | 4.79 | 5.07 | 0.17 |
| Sausage flavour | 6.41 | 5.96 | 5.96 | 5.30 | 5.62 | 5.30 | 6.33 | 0.15 |
| Tenderness | 4.65 | 5.22 | 5.97 | 5.17 | 5.18 | 5.88 | 4.65 | 0.18 |
| Saltiness | 3.24 | 2.79 | 3.22 | 3.19 | 2.91 | 2.86 | 3.31 | 0.09 |
| Pungency | $5.61^{a}$ | $5.07^{ab}$ | $4.99^{ab}$ | $4.66^{ab}$ | $4.13^{b}$ | $4.35^{ab}$ | $4.07^{b}$ | 0.17 |
| Overall flavour | $6.36^{a}$ | $6.05^{abc}$ | $6.16^{ab}$ | $5.05^{c}$ | $5.69^{abc}$ | $5.25^{bc}$ | $6.72^{a}$ | 0.13 |
| Overall acceptability | $6.40^{a}$ | $5.68^{abc}$ | $6.25^{abc}$ | $5.07^{c}$ | $5.73^{abc}$ | $5.30^{bc}$ | $6.75^{a}$ | 0.15 |

$^{a, b, c}$ – Means in the same row with different superscripts differ significantly ($p$ <0.05).

CP– Cayenne pepper powder WP- white pepper powder

Overall flavour is important for preference (Wang *et al*., 2021) considering that meat intra muscular fat play important role not just in flavour development but improved dissipation of flavour. Jachimowicz *et al*. (2022) explained that functional compounds in herbs incorporated into chicken diets can enhance sensorial perception of broiler chicken meat by altering the fatty acid content. Such alterations possibly extended to overall flavour perception by panellists for sausage from meat of birds fed B+125WP+125CP diet; supported by Lu *et al*. (2017) who explained that pepper fruit or powder incorporated as additive act as flavouring agent with aroma transmission in product. Shahidi and Hossain (2022) likewise explained that heat treatment at high temperatures released aromatic compounds by complex interaction of lipid oxidation and maillard reaction; reducing sugars and amino acids as well as volatile compounds that consequently translated into high overall acceptability scores for sausages from both the Basal and B+125WP+125CP groups. Overall increased impact from incorporation of peppers contribute significantly to wellbeing and consumer satisfaction since spiciness can be increased without negative consequences on well-being sensations (Byrnes and Hayes, 2013).

## 4.Conclusions

From this study, supplying B+200CP and B+250WP diets to broiler chickens can be explored to achieve improved physico-chemical and technological qualities of meat respectively while overall sensorial or organoleptic characteristics of meat from birds offered B+125WP+125CP diet is desirable to satisfy consumer sensorial preference. Sausage formulated with meat from the latter group had appealing colour (instrumental) values, while sausage formed from meat of birds fed B+200CP diet enhanced sausage weight post-microwave cooking. If sensorial characteristics of sausage is targeted and pungency is not desired, chicken sausage from meat of the Basal group is suggested, otherwise, sausage from meat of chickens fed B+125WP+125CP diet is recommended.

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# PHYSICOCHEMICAL, SENSORIAL AND FUNCTIONAL PROPERTIES OF ARROWROOT (*Maranta arundinacea*) FLOUR AS AFFECTED BY THE FLOUR EXTRACTION METHOD

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**ABSTRACT**

Arrowroot (*Maranta arundinacea*) could be used to substitute wheat flour in the food industry. The Wet extraction method, which extracts starch by crushing it with water, is the most prevalent method of extracting arrowroot flour. The purpose of this study was to compare the "wet method" and "dry method" of flour extraction in terms of the flour produced. Flour yield (%), sensory properties, proximate composition, and physicochemical and functional properties of the flour derived from the two methods were assessed using standard protocols. When compared to the wet method, the dry method yielded a higher flour yield. The flour from wet method resulted in better sensory properties (odour, appearance, flavour, texture, overall acceptability) than the flour from dry method. Proximate parameters, such as ash, crude fat, crude protein, and crude fiber contents of the flour from dry method were significantly ($P<0.05$) higher than those in the flour from wet method. The most prominent starch granule shapes were oval, spherical and irregular globular with no significant differences in the granule size between the two flour types. The flour densities revealed that arrowroot flours extracted using both procedures are suitable for use in the pharmaceutical industry. Viscosity, amylose content, and swelling power were significantly higher in the flour from the wet method indicating a better gel forming ability which is beneficial in food preparations. Wet extraction method was selected as the most suitable method for food preparations and for using arrowroot in the food industry.

## 1.Introduction

Plants are abundant in starch, which is a key source of energy. This carbohydrate can be utilized in the food industry as thickening agent, water retention agent, gelling agent, and colloidal stabilizers (Souza *et al*., 2019). Introduction of new starch sources with better functional characteristics has become a modern trend in the food industry, as it has the potential to influence the international markets. In recent years, starches from roots and tubers have received significant attention because of their prospective applications (Tetchi *et al*., 2007; Jyoti *et al*., 2011). Adding value to underutilized tuber crops and exploiting them for the food industry is a current trend.

Arrowroot (*Maranta arundinacea*, Family: Marantaceae) rhizome flour has a significant potential to replace wheat flour in food products as a gluten-free substitute. Colour of arrowroot flour is highly closer to white colour which will be an important fact when substituting wheat flour with arrowroot flour (Malki *et al*., 2022). Celiac disease and gluten intolerance can only be treated by the complete exclusion of gluten. Due to the lack of gluten-free alternatives, those replacement products can be relatively expensive. In this context, arrowroot rhizome flour has a high potential to succeed (Amante *et al.,* 2020).

Arrowroot is a perennial herbaceous tuber crop that originated in South and Central America but has since spread around the world, particularly in tropical regions (Deswina and Priadi, 2020). Arrowroot is known as "*Hulankeeriya*" or "*Aerukka*" in Sri Lanka. It has long been used in folk medicine as a medication. Arrowroot flour is used minimally in the local food industry due to lack of awareness and time-consuming starch extraction procedures, but rural communities consume porridges, curries, *roti* and similar products made of arrowroot flour.

Arrowroot rhizomes can result in 10 % - 20 % of starch yield from the manual wet extraction procedure. In many countries, the wet extraction method is the most popular flour extraction procedure for arrowroot, which involves extracting flour into water and allowing it to settle. The dried pellets are then crushed and sifted (Capina and Capina, 2017). Fresh arrowroot rhizome pieces are oven dried in the dry flour extraction process, and flour is prepared by grinding the dried pieces (Aprianita *et al*., 2014). The objective of this study was to determine the sensorial properties, proximate composition and physicochemical properties of arrowroot flour made by wet and dry extraction methods.

## 2. Materials and methods

### 2.1. Sample Collection

Arrowroot rhizomes at harvesting age were collected from Deraniyagala, Sabaragamuwa Province, Sri Lanka. Coconut milk powder, table salt, *Kithul* (*Caryota urens*) treacle, and commercial arrowroot powder (Brownson Pvt. Ltd., Sri Lanka) were purchased from the local market. All the chemicals used were of analytical reagent grade.

### 2.2. Wet Extraction Method of Arrowroot Flour

Arrowroot rhizomes were cleaned, scale leaves removed and rinsed under running tap water. Then, using a high-speed stainless steel blender (Preethi MG-172 E, Preethi Electrical Appliances, India), a 1:2 (w/w) ratio of arrowroot to water was crushed for 5 min, until a homogenous mass was obtained. The mixture was filtered using a double cotton cloth. For fiber separation, the mass washing operation with deionized water was done thrice. The water separation was carried out by manual flow after 12 h of flour sedimentation. The flour was then oven dried for 8 to 10 h at 60 °C with air circulation. Dried flour was ground to a fine powder using a grinder (Preethi MG-172 E). The flour was sifted through a 425 µm sieve and stored at -18 °C until further analyses. This method was developed by making slight modifications to Nogueira *et al*. (2018).

### 2.3. Dry Extraction Method of Arrowroot Flour

Arrowroot flour extraction by dry method was done as described by Aprianita *et al*. (2014) with slight modifications. Rhizomes were cleaned, scale leaves were removed, and cut into 1 cm size cubes. The pieces were oven dried at 65 °C for 24 h. The dried chips were ground to fine flour using a grinder (Preethi MG-172 E), sifted through a 425 µm sieve, and stored at -18 °C until further analyses.

### 2.4. Determination of Flour Yield

The flour yield was calculated using equation 1 (Souza *et al*., 2019).

Flour Yield (%) = [Weight of extracted flour / Weight of fresh rhizomes]*100----(1)

### 2.5. Preparation of Arrowroot Porridge and Sensory Evaluation

A porridge was prepared using arrowroot flour to determine the sensory properties of the flours resulted from the two extraction processes and compare those with a commercial arrowroot flour product available in the local market. Powdered coconut milk (65 g) was dissolved in water (1000 ml) and 200 g of arrowroot flour was added. The mixture was heated in a pan at medium heat. While stirring continuously, *Kithul* treacle (20 ml) and 1 g of table salt were added. The mixture was heated until boiling started. A trained panel of eight members carried out the sensory evaluation of arrowroot flour based porridge using a five-point hedonic scale (5 – Like very much through to 1 – Dislike very much) to rank the samples. The most consumer preferable flour type was determined and the relevant extraction method was selected for further analyses and product development.

### 2.6. Proximate Analysis

The two arrowroot flours resulting from the wet method and dry method were evaluated for their proximate compositions. Moisture content was measured by a moisture meter (Infrared Moisture Analyzer Kett FD-660, Kett, Japan). The total solid content was determined by oven drying at 105 °C for 3 h. Ash content was determined by the muffle incineration method at 540 °C, and crude protein was determined by the Kjeldahl distillation method. Crude fiber content and crude fat content were determined using a fiber analyzer (Fiber Extraction System F-6P, Spain) and the solvent extraction method (Fat Extraction System SX-6 MP, Spain), respectively according to the methods of Association of Official Analytical Chemists (2010). Total carbohydrate content and total gross energy were determined as described by Diddana *et al.* (2021).

### 2.7. Determination of Flour Colour and Starch Granular Morphology

The colour of arrowroot flour was assessed using the colourimeter (PCE-CSM 2, PCE Instruments, Unites States). The colourimeter was calibrated, and colour coordinates were recorded using the included calibration disc. The samples were placed in a watch glass and their surfaces flattened. Measurements were taken by setting the measurement head of the colourimeter on the prepared samples after covering them with a piece of clear polythene. The measurements were taken at three different locations on the samples. Colour characteristics were determined using the L* a* b* colour coordinates. a*(-a* for greenness and +a* for redness), L*(L* = 0 for black and L* = 100 for white), and b*(-b* for blueness and +b* for yellowness).

Starch granular morphology of flour samples was determined according to Wijesinghe (2015). A starch suspension was prepared to mix flour 1:1 (w/v) with a mixture of distilled water and glycerine (1:1 v/v). A thin smear was prepared on a glass slide with a coverslip after staining the starch solution with a 1.0 % iodine solution. Starch granules were observed under a light microscope (OPTICA Microscope B-290, Italy) at 40 x magnification and the images were captured with Optica Pro View digital camera software. The length, width, and shape of the starch granules were assessed. Dry arrowroot flour was applied as a thin layer on adhesive metallic support and then sputter-coated with gold to examine under a scanning electron microscope (SU 6600, HI-2108-003, Japan) operating at 5 kV (Horovitz *et al*., 2011).

### 2.8. Determination of Physicochemical Properties

#### *2.8.1. Determination of Least Gelation Concentration (LGC)*

Flour suspensions (2–10 % w/v) were made with distilled water (5 ml). The flour suspensions were mixed well for 5 min. After being heated for 30 min at 80 °C in a water bath, the test tubes underwent rapid cooling under flowing cold tap water for 2 h. When the sample from the inverted test tube did not drop, it was identified as the least gelation concentration for flours (Awokaya *et al.,* 2011).

### *2.8.2. Determination of Flour Density*

Density tests were performed according to Musa *et al*. (2011).

*Bulk Density* – The occupied volume of a glass graduated cylinder with a 100 ml capacity was measured after 20 g of flour samples were weighed and poured into it.

*Tapped Density* – A graduated cylinder containing 20 g of flour was dropped 50 times from a height of 20 mm on a bench, and the volume of each drop was recorded.

*Carr's Index* – The ratio was calculated by dividing the difference between the tapped density and the bulk density by the tapped density and expressing it as a percentage.

*Hausner Ratio* – The Hausner ratio was obtained by dividing all of the samples' tapped density by their bulk density.

### *2.8.3. Determination of Flour Viscosity*

The viscosity of arrowroot flour was measured using a digital viscometer (VISCOTM-6800, ATAGO, Japan). The flour was mixed with distilled water to create a starch suspension (10 %) and heated up to 78 °C. The viscosity (cP) of the flour solution was recorded at 3 minute intervals while rotating at 20 rpm (Sopade and Kassam, 1992).

### *2.8.4. Determination of Swelling Power and Solubility*

With a few minor modifications, methods of Leach *et al*. (1959) were used to measure swelling power and solubility of arrowroot flour samples prepared using the wet method and the dry method. With constant stirring, 0.25 g of arrowroot flour was heated with 10 ml of distilled water at 78 °C for 30 min. The slurry was centrifuged for 15 min at 3000 rpm after being cooled to room temperature. Starch sediment was measured after the supernatant was properly removed. The supernatant was transferred to a pre-weighed petri dish, evaporated for 2 h at 130 °C, and then weighed. The amount of starch dissolved in water was represented by the residue left over after drying the supernatant. The outcome was given as equations (2) and (3);

$$\text{Solubility (\%)} = [(Wss \times 100) / Ws] \text{------(2)}$$

$$\text{Swelling Power (g/g)} = [(Wsp \times 100)/(Ws \times (100 - \text{solubility \%})] \text{------(3)}$$

Where; Wss – weight of the soluble starch, Ws – weight of the sample, Wsp – weight of the sediment paste

### *2.8.5. Determination of Moisture Sorption Capacity*

Arrowroot flour (2 g) was spread evenly in a pre-weighed petri dish. The petri dish was put in a desiccator at room temperature with a relative humidity of 98 % until a constant weight was reached. The moisture sorption capacity was determined by calculating the percentage increase in weight (Shiihii *et al*., 2011).

### *2.8.6. Determination of Amylose Content*

The amylose content of flour was determined according to Juliano (1971). Arrowroot flour (1 g) was mixed with 1 ml of 95 % (v/v) ethanol and 9 ml of 1 N NaOH. The mixture was heated for 10 min and volume up to 100 ml. From the sample suspension, 5 ml was mixed with 50 ml of distilled water, 1 ml of 1 N acetic acid, and 1.5 ml of iodine solution. The suspension was volume up to 100 ml and held for 20 min. The absorption at 620 nm was measured using a UV visible spectrophotometer (JENWAY 6305, Cole-Parmer Ltd, United Kingdom) and the standard curve was generated using pure potato amylose.

## 2.9. Experimental Design and Statistical Analysis

The experiments were arranged in a Completely Randomized Design (CRD). Measurements for physicochemical analyses were performed on triplicate samples. Results of the sensory evaluation (non-parametric data) were analyzed using the Kruskal Wallis test. Analysis of Variance (ANOVA) was used to analyze parametric data using MINITAB (version 19) statistical software.

## 3.Results and discussions

### 3.1.Effect of Extraction Method on Flour Yield

Arrowroot flour yields from two flour extraction methods are shown in Table 1. The dry method resulted a significantly higher (24.38 %) flour yield compared to the wet method (17.10 %) because the entire rhizome was used for flour production in the dry method. In addition, the wet method caused a greater removal of fibers from the rhizome. Consequently, a lower flour yield resulted in the wet extraction method. The current result of flour yield in the wet method complies with previous studies which suggest that the wet extraction process produces a flour yield of 10 – 20 % (Capina and Capina, 2017).

**Table 1.** Variation of arrowroot flour yield from dry method and wet method

| Flour preparation method | Flour yield (%) |
|---|---|
| Dry method | 24.38±2.36[a] |
| Wet method | 17.10±2.32[b] |

*Mean±SD; n = 3*

*Between rows, mean values followed by different superscript letters are significantly different at p=0.05*

### 3.2. Effect of Flour Extraction Method on Sensory Properties of Arrowroot Flour Based Porridge

Results of sensory evaluation revealed that the porridge made of arrowroot flour from the wet method (T1) gained the highest mean scores for the sensory attributes of odour (5.00), appearance (4.75), flavour (4.50), texture (4.00), and overall acceptability (5.00) (Figure 1). All sensory attributes differed significantly ($P<0.05$) among the three treatments. The porridge made of arrowroot flour from the dry method (T2) received lower mean scores for colour (2.00), appearance (3.50), flavour (2.50), texture (3.00), and overall acceptability (3.00). Inclusion of high fiber in the flour from the dry method has caused undesirable organoleptic properties of the product made using that flour. Therefore, the wet extraction method is more appropriate for arrowroot flour preparation and product development.

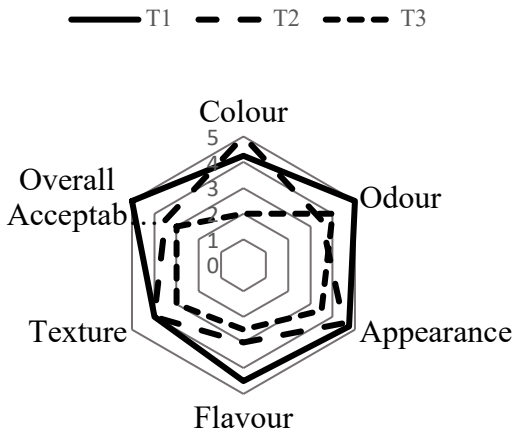



**Figure 1.** Mean scores obtained for the sensory attributes of arrowroot flour incorporated porridge

*T1 –Flour from wet method, T2 – Flour from the market (commercial product), T3 – Flour from dry method*

### 3.3. Effect of Flour Extraction Method on Proximate Composition of Flours

The moisture content of arrowroot flour from the dry method (12.27 %) was significantly higher than that of the flour from the wet method (10.60 %; Table 2). However, this difference could have been caused by the variation in preparation and drying procedures which were employed according to the extraction process. The total solid content was not significantly different between the two flour types. Dry method has resulted a higher ash content in the flour (2.32 %). However, the ash content in the flour from wet method in the current study (0.38 %) is higher compared to previous findings of ash level in arrowroot flour (wet method) from the Philippines (0.16 %; Capina and Capina, 2017). The crude fat content of flour from the dry method (0.71 %) was significantly higher than that of the wet method (0.43 %). The crude fat content of arrowroot flour from the wet extraction process was previously reported to be in the range of 0.25 % to 1.0 % (Erdman, 1986;

Madineni *et al*., 2012; Capina and Capina, 2017).

**Table 2.** Variation of proximate compositions of arrowroot flour extracted from dry method and wet method

| Parameter | Flour from dry method | Flour from wet method |
|---|---|---|
| Moisture % | 12.27±0.25[a] | 10.60±0.52[b] |
| Total Solid % | 91.68±1.54[a] | 89.40±0.52[a] |
| Ash % | 2.32±0.11[a] | 0.38±0.03[b] |
| Crude Fat % | 0.71±0.04[a] | 0.43±0.06[b] |
| Crude protein % | 5.50±0.35[a] | 0.64±0.09[b] |
| Crude Fiber % | 2.52±0.03[a] | 1.02±0.40[b] |
| Carbohydrate% | 87.20 | 87.95 |
| Gross Energy (kcal/ 100 g) | 377.19 | 358.23 |

*Mean±SD; n = 3*

*Between columns, mean values followed by different superscript letters are significantly different at p = 0.05*

Flour from the dry method reported a higher crude protein content (5.50 %) than the wet method (0.64 %). Aprianita *et al.* (2014) reported that arrowroot flour from the dry process had a protein content of 7.70 % wet method had a protein content of 0.60 %.

The reduced protein content of flour from the wet extraction process is due to the starch isolation process (Aprianita *et al*., 2014). The crude fiber content of flour from the dry method (2.52 %) was significantly higher than that of the wet method (1.02 %). This could be related to the removal of fiber during the wet extraction process. Previous studies have reported a soluble fiber level of 1.70 % in arrowroot flour from the wet method (Madineni *et al*., 2012).

With regard to the carbohydrate content of the flour, there was no difference between the two extraction processes. The flour from the dry method reported a higher gross energy content in comparison to the flour from the wet method (Table 2).

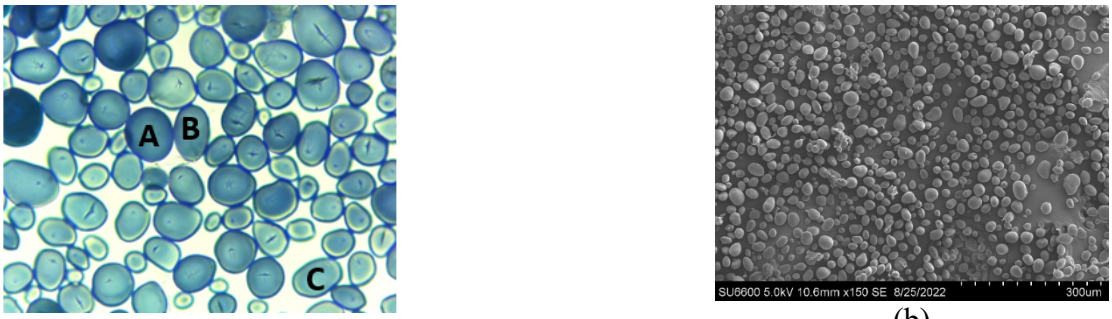
### 3.4. Starch Granular Morphology and Colour of Flour

**Table 3**. Variation in arrowroot starch granule shape, dimensions, and flour colour as resulted by wet method and dry method of flour extraction

| Parameters | Dry method | Wet method |
|---|---|---|
| *Granule shape and dimensions:* Spherical shape | 32.53±8.20[a] | 30.50±8.43[a] |
| Oval shape | 43.46±12.29[a] | 50.28±10.63[a] |
| Irregular Globular shape | 24.00±6.51[a] | 19.20±2.74[a] |
| Length (µm) | 44.99±2.44[a] | 49.40±2.77[a] |
| Width (µm) | 28.99±5.32[a] | 30.31±0.93[a] |
| *Flour colour:* L* | 82.46±1.19[b] | 92.31±0.44[a] |
| a* | 4.39±0.23[a] | 1.36±0.06[b] |
| b* | 16.28±0.23[a] | 7.37±0.05[b] |

*Mean±SD; n = 3*

*Between columns, mean values followed by different superscript letters are significantly different at p = 0.05*




(a) (b)

**Figure 2**. Arrowroot starch granule morphology under the (a) light microscope and (b) scanning electron microscope (A – Oval shape, B – Spherical shape, C – Irregular globular shape)

Oval, irregular globular, and spherical were the most commonly identified starch granule shapes. Ovoid and globular shapes were identified as the most prominent granule shapes for arrowroot by Perez *et al*. (1997). Cicular shaped or oval starch granules have been observed by Nougeira *et al*. (2018). However, the distribution of starch granule shapes (as percentages) was consistent between the two flour types. Fissures on the surfaces of granules were observed indicating the smoothness of arrowroot starch granules (Nougeira *et al*., 2018). The granule morphology under a light microscope and scanning electron microscope (SEM) are shown in Figure 2.

The length and width of the starch granules obtained by the two flour preparation methods did not differ significantly. The most prominent diameter of arrowroot starch granules, according to Souza *et al*., 2019, is between 20 and 35 µm. In the present study, the width of starch granules in both flour types ranged from 28.99 µm to 30.31 µm (Table 3).

The flour colour resulted from the two extraction methods was different in terms of lightness (L*), redness (a*), and yellowness (b*). While the flour from the dry method had much higher red and yellow colour tones, flour made using the wet method recorded significantly higher values for lightness. The dry method of making arrowroot flour results in a mixture of flour, fiber, and other residues, whereas the wet method involves washing the slurry to extract the flour which could have caused a higher lightness in it. The results indicated that arrowroot flour made using the wet method is relatively closer to white colour compared to the flour from the dry method, which is closer to reddish and brownish colour. It is indicated by higher a* and b* values of the flour from the dry method.

### 3.5. Results of Physicochemical Properties

#### *3.5.1. Results of Least Gelation Concentration (LGC)*

Gelation characteristics of the arrowroot flour are shown in Table 4. For the wet and dry extraction methods, the least gelation concentration (LGC) was 8 % and 10 %, respectively. This number is important for the determination of the ideal water to flour ratio for the desired texture in food processing operations based on flour. This is a significant consideration while preparing food because it is based on the structural components of flour, such as protein, carbohydrates, and lipids (Abbey and Ibeh, 1988). The length of the chain, the presence of phosphate ester in the remaining glucose, and the residual α-1, 6 linkages are among the factor that has the impact on the least gelation concentration, according to Klaochanpong *et al*. (2015). Arrowroot flour from the wet extraction method produces a more viscous gel from the least flour concentration that the dry method because flour from the dry method is containing more fiber content than the wet method flour.

**Table 4.** Gelation properties of arrowroot flour resulted from wet and dry methods

| Concentration (%w/v) | Status of dry method | Status of wet method |
|---|---|---|
| 2 | Viscous liquid | Viscous liquid |
| 4 | Moderately soft gel | Soft gel |
| 6 | Soft gel | Soft gel + Firm gel |
| 8 | Soft gel + Firm gel | Firm gel |
| 10 | Firm gel | Very firm gel |
| Least gelation concentration (%w/v) | 10 | 8 |

### *3.5.2. Results of Moisture Sorption Capacity*

Results of moisture sorption capacity revealed that the flour produced by the dry method was more sensitive to atmospheric moisture when compared to the wet method (Table 5). When arrowroot flour from the dry method is exposed to the atmosphere it will absorb atmospheric moisture than flour from wet method and gain more moisture in it. This property determines the physical ability of starch when formulated into a tablet, which is especially important in the pharmaceutical industry (Musa *et al*., 2011). Accordingly, arrowroot flour from the wet method has more potential to be used in the pharmaceutical industry. Moisture sorption capacity represents the amount of water absorbing molecules than starch. When the moisture sorption capacity is high starch polymer structure is looser in that starch while polymer while it is more compact for starches with low moisture sorption capacity. Moisture sorption capacity depends on the harvesting time, geographical location, or botanical source. Starches with high moisture sorption capacity easily get softened and are easy to digest but they tend to spoil faster (Aidoo *et al*., 2022). Arrowroot flour from dry method had the highest moisture absorbance. Moisture absorbance increases with the presence of fiber (Azima *et al*., 2020) and it has been proved by this study.

**Table 5**. Variation in moisture sorption capacity of arrowroot flour from dry method and wet method

| | Moisture sorption capacity (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 h | 2 h | 3 h | 6 h | 24 h | 48 h | 72 h |
| Wet method | 4.65± 1.12$^{a}$ | 6.04± 1.51$^{a}$ | 7.00± 0.72$^{a}$ | 8.56± 134$^{b}$ | 15.89± 1.75$^{b}$ | 18.39± 1.08$^{b}$ | 21.19± 2.89$^{b}$ |
| Dry method | 7.23±1.47$^{a}$ | 7.39± 0.78$^{a}$ | 7.85± 1.54$^{a}$ | 14.47± 0.27$^{a}$ | 20.17± 0.64$^{a}$ | 33.76± 0.81$^{a}$ | 38.62± 1.50$^{a}$ |

*Values are Mean ± SD;*

*The same superscript letter in each row represents values not significantly different from each other at p = 0.05*

### *3.5.3. Results of Flour Densities, Amylose Content, Swelling Power and Solubility*

The bulk density and tapped density of the two flour types were significantly different ($p<0.05$) but the Carrs index and Hausner ratio were not different (Table 6). When the Carrs index exceeds 23 % and the Hausner ratio exceeds 1.2, that flour does not have a good flow or compressibility (Muazu *et al*., 2011). Both flour types were in an acceptable range with improved compressibility when considering the Carrs index, which will be useful in the pharmaceutical industry. However, the Hausner ratio of the flours from the dry method and wet method were 1.26 and 1.29, respectively (Muazu *et al*., 2011).

The viscosity of arrowroot flour prepared by the wet method was higher than that prepared by the dry method. Less inclusion of fiber in the flour from the wet method has increased its purity and could have resulted in increased viscosity (Table 6). Arrowroot flour from wet method is having better gel forming ability than flour from dry method. The viscosity of the flours has a significant impact on their integrity and texture. Method of flour preparation and interactions between starch and hydrocolloids have an impact on viscosity. The stability of flours with higher viscosities is essential when they are used as thickeners or stabilizers. As a result, arrowroot flour from wet method produces more viscous gels, increasing its potential as an ingredient in foods (Aidoo *et al*., 2022).

The swelling power of the flour from the wet method (16.02 g/g) was significantly higher than that from the dry method (5.78 g/g; Table 6). Swelling power is positively correlated with viscosity (Singh *et al*., 2006) and it was evident in the present study. When the temperature increases, swelling of starch granules increases and it indicates the strength of internal forces of starch granules which maintains the granule structure (Hoover *et al*., 2010). When the temperature of water is continuously increased, it causes the molecules in starch granules to vibrate vigorously, breaking the intermolecular hydrogen bonds in amorphous regions. Due to swelling and partial solubilization of polymers, particularly amylose, water molecules connect to exposed hydroxyl groups of amylose and amylopectin by hydrogen bonding, increasing the granule size (Hoover, 2001). The Higher swelling power of the flour from wet method could be caused by the higher level of purity (low fiber) in that flour. Nogueira *et al*. (2018) reported the swelling power of arrowroot starch from wet extraction as 11.32 ± 0.53 g/g and it is in between the values obtained for two processing methods in the current study.

The solubility of arrowroot flour from the wet method flour was higher (10.67±2.31 %) than that of from the dry method (6.13±1.98 %) although the difference was not significant. According to previous studies, the solubility of arrowroot starch has been recorded as 17.22 % and 13.22% (Perez and Lares, 2005; Nogueira *et al*., 2018) and they are more compatible with the results obtained for flour from the wet method.
The amylose content of the flour wet method was higher (28.47 %) than that from the dry method (16.20 %; Table 6). This difference could have been caused by the variation in flour composition under the two processing methods. Erdman (1986) recorded the amylose content in arrowroot as 19.9 % and which was lower than the result of the present study. Moorthy (2002) reported a range of 16 – 27% for the total amylose content of arrowroot starch. According to several studies (Tharanathan, 2003; Li *et al*., 2011; Fakhoury *et al*., 2012; Romero-Bastida *et al*., 2015), the high amylose content of arrowroot of starch would enable its use in the production of films with better technological properties, particularly when it comes to mechanical resistance and barrier properties. Thus, the arrowroot flour from the wet method will have a higher potential for film preparation.

**Table 6.** Variation in flour densities, viscosity, amylose content, swelling power and solubility for arrowroot flour from dry method and wet method

| Parameter | Dry method | Wet method |
|---|---|---|
| Bulk Density (g/ml) | 0.55±0.00[a] | 0.39±0.00[b] |
| Tapped Density (g/ml) | 0.70±0.01[a] | 0.51±0.01[b] |
| Carrs Index (%) | 20.74±1.28[a] | 22.66±1.78[a] |
| Hausner ratio | 1.26±0.01[a] | 1.29±0.02[a] |
| Viscosity (cP) | 6338±505[b] | 7802±531[a] |
| Amylose content (%) | 16.20±0.60[b] | 28.47±0.34[a] |
| Swelling power (g/g) | 5.78±0.28[b] | 16.02±0.70[a] |
| Solubility (%) | 6.13±1.98[a] | 10.67±2.31[a] |

*db - Dry basis*, Mean±SD; n = 3*

*Between columns, mean values followed by different superscript letters are significantly different at p=0.05*

## 4. Conclusions

The study assessed the properties of arrowroot flour produced by two distinct preparation procedures. In the wet extraction process, fiber removal is done to a significant extent. The dry extraction process resulted in a higher flour yield, but when sensory properties were considered, the wet method resulted the flour with preferred sensory attributes for the food industry. The dry method produced arrowroot flour with significantly higher ash, fat, protein, and fiber contents compared to the wet method. The least gelation concentration of the flour from the wet method was 8% indicating its better gel-forming ability. Both flour types are suitable for use in the pharmaceutical industry, according to the results of flour densities. The flour colour in wet method was closer to the white colour while the flour from dry method was slightly reddish in colour. Viscosity, amylose content, and swelling power were significantly higher in the wet method. The wet extraction method could be adopted as the most acceptable procedure for arrowroot flour production for the industry because of its potential to use as thickeners, stabilizers or food ingredient.

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# IMPACTS OF HEAVY METALS IN SEED CROPS AND OIL SEED ON HUMAN HEALTH: A TREAT TO FOOD SAFETY- REVIEW

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**ABSTRACT**

The dramatic distribution of heavy metals in the 21st century is one of the most important concern in human health and the environment. A wide range of heavy metals has been detected in various environmental and food matrices due to their bio-accumulative and persistent properties. To prevent any health problems and toxicity from heavy metals in human due to their worldwide consumption, guidelines on the maximum residue level (MRL) in oilseeds have been established. This review has been done to assess vegetable and seed oil/fat and their benefits, nutritional value and applications, as well as sources and occurrence of heavy metals in vegetable and seed oil/fat, effects of heavy metals on nutrient quality of oilseed and impact on human health. The most common heavy metals, which can found in oil and oilseeds/fats, are arsenic (As), lead (Pb), cadmium (Cd), mercury (Hg), nickel (Ni) and chromium (Cd). Coexistence of heavy metals in the food commodities may lead to synergistic toxic interactions among heavy metals. In this regard, to minimize the health effects of heavy metals by consumption of plant and seed oil/fat, besides increasing regular surveillance and monitoring, revising the maximum permissible level of heavy metals is necessary.

## 1. Introduction

### 1.1. Major concern of toxic metals in foods

Food contamination has come to be a major concern and importance in food safety for human health. Food contamination can result from a number of events occurring among the food chain such as external environmental pollution, naturally occurring phytotoxins , bacterial toxins, the addition of chemicals during processing techniques, as well as emerging chemical hazards such as the potentially toxic elements (PTEs) (Kuswandi, Futra, & Heng, 2017). Heavy metals come in elemental, organic and inorganic forms. The

exposure of heavy metals has become a global concern in recent years due to the global, stability, non-biodegradability and bioaccumulation potential. The presence of these metals in agricultural soils and consequently their accumulation in plants are considered one of the most serious environmental problems in the world. Undeniably, the distinguished less amount of some metals as essential metals is important for biological activity; and they play important roles in vital metabolic pathways such as copper (Cu), iron (Fe), manganese (Mg) and zinc (Zn) but exposure to more amount of these metals might be highly toxic. Also, the presence of non-essential metals such as arsenic (As), cadmium (Cd), lead (Pb), nickel (Ni), chromium (Cr) and mercury (Hg), which have been identified as contaminants by the world organizations, is not only needed for natural biological function, but also rapidly lead to toxicity (Rai, Lee, Zhang, Tsang, & Kim, 2019).

Heavy metals can be released into environments from either natural sources such as explosive volcanoes and burning forests or through human activities (Zhang, Huang, Dong, Hu, & & Akhtar, 2017). Obviously, human resources have a greater share of these contaminants. Human activities include mining, steel and iron metallurgy industries, vehicular pollution, applying the fertilizer and pesticides in agriculture, mercury-cadmium or cadmium-nickel batteries, lead-containing ceramics and glass, mercury thermometers, etc (El-Kady & Abdel-Wahhab, 2018). Characterizations of metals, they have adverse effects on human and vital organisms (their non-biodegradable characterization, their long biological half-lives and accumulation in body organs) (Tang, Huang, & Pan, 2014). It seems mitochondrial dysfunction and oxidative stresses are suggested as important mechanisms of metal toxicity and triggering the programmed cell death pathway (Hassani, et al., 2015; M-J Hosseini, et al., 2016; Mir-Jamal Hosseini, Shaki, Ghazi-Khansari, & Pourahmad, 2013). As dietary intake is the main route of exposure to heavy metals for most people via handling and processing of foods as well as from the farm to the point of consumption, maximum levels for heavy metals in foodstuffs have been set by different regulatory international organizations such as European Union (EU), Codex Alimentarius Commission (CAC) and U.S. Food and Drug Administration (FDA). These regulations establish maximum permission levels (MLs), tolerable weekly intake (TWI), or provisional tolerable weekly intake (PTWI) for these metals in a type of vegetable oils and fats. Heavy metal uptake by oil seeds might pose a threat to human health. The objective of the present study is to highlight the impact of heavy metals in plant and seed oil/fat and human health. In this regard, this paper covers plant and seed oil/fat and their benefits, nutritional value and applications, sources and occurrence of heavy metals in them. Furthermore, the effects of heavy metals on nutrient quality of seed oil/fat and impact on the health of human are discussed.

### 1.2. Plant and Seed Oil/Fat in Human Diet

Edible oils that are originated from plants and seeds including coconut oil, corn oil, cottonseed oil, olive oil, palm oil, peanut oil, rapeseed/canola oil, safflower oil, sesame oil, soybean oil and sunflower oil are generally used in industrial food manufacturing and household consumption universally. Edible oil of seeds and plants are known as important in the food technology. The statistics show that global production of vegetable oils has been increasing from since the early 2000s and reached about 207.5 million tons worldwide in 2019-2020. However, as with all food products, there is the possibility for potentially toxic contaminants to occur in oils such as heavy metals.

## 2. Can Heavy Metals Accumulate in Plant and Seed Oils/Fat?

Oil of seeds and plants have well-organized mechanisms to absorb essential nutrients from the soil. The root system of plants, along with chelating agents, can dissolve and absorb micronutrients and heavy metals even at very low concentrations.

Transmission of pollutants is diffused through the process of evapotranspiration and translated from the roots to other parts. Heavy metals uptake by the plant use four mechanisms of phytoestruction, phytoacetylation, rhizofiltration and phyto-volatilization (Salido, Hasty, Lim, & Butcher, 2003). Heavy metal accumulation occurs more often in the edible parts of plants, which may reduce yield and quality of crops and is dangerous to human and animal health. Some heavy metals such as Pb, Al, and Cd are suggested to cause damage in some body organs even at very low concentrations, and healthy plants may sometimes contain heavy metal concentrations that are toxic to mammals (F. Chen, et al., 2007). Plant tolerance for various heavy metals involves processes of detoxification of internal metals that may be through intracellular or complex with cellular ligands such as organic acids, cysteine and low molecular weight thiols (Mnasri, et al., 2015). Species and cultivar characteristics have been studied in the process of uptake and distribution of heavy metals in plant organs. Like the accumulation of more Pb in the root than in the stem and leaves in the sunflower or the accumulation of Cd and Zn in the leaf more than the seed, but the opposite holds for Cu and Ni (Dhiman, et al., 2017). In another study, Cu, Zn, Cd and Pb were higher in sunflower seeds and in another study of heavy metal contamination, the amount of Cd in sunflower seeds was higher (Dhiman, et al., 2017). Several studies reveal that oilseeds might be an accumulator for some toxic trace elements which could enter into plants and their oil (Manzoor, Sharma, & Wani, 2018). The origin of metals in the oil could be from plant growth media, like heavy metal contaminated soil. Heavy metals with natural source are not available to the plant, however, excess heavy meals due to presence of industrial factories, near agricultural area and disposal of industrial wastes, pesticide contamination residues, sewage sludge are available to living organisms (Rizwan, et al., 2017).

In recent years, there has been a significant increase in the study of the effects of soil contamination on plants as well as the effect of production stages on heavy metal content in plant and seed oil. Indisputably, the high exposure of these metals have been confirmed to have negative effects to human health. In one of the first studies conducted by Garrido et al. (1994) on level of metals (Na, K, Ca, Mg, Cu, Zn and Fe) in some Spanish edible oils, the samples showed higher values than the maximum permitted by FAO/WHO (for Cu and Fe, respectively, 18.3% and 2.8%)(Garrido, Frías, Díaz, & Hardisson, 1994). Mendil et al. (2009) investigated the concentration of some metals (Fe, Mn, Zn, Cu, Pb, Co, Cd, Na, K, Ca and Mg) in olive oil, hazelnut oil, sunflower oil, margarine, butter and corn oil samples produced in Turkey by using Atomic absorption spectrometry (AAS) technique. The concentrations of trace element in the samples were found to be 291.0-52.0, 1.64-0.04, 3.08-1.03, 0.71-0.05, 0.03-0.01, 1.30-0.50, 84.0-0.90, 50.1-1.30, 174.2-20.8 and 20.8-0.60 µg/g for Fe, Mn, Zn, Cu, Pb, Co, Na, K, Ca, and Mg, respectively. Cd was found to be 4.57-0.09 µg/kg. The high heavy metal and mineral accumulation levels in the samples were found in olive oil for Cu, Pb, Co, margarine for Fe, K, corn oil for Zn, Mn, butter for Na, Mg, sunflower oil for Ca and hazelnut oil for Cd, respectively (Mendil, Uluözlü, Tüzen, & Soylak, 2009). In another study, Szyczewski et al. (2016) evaluated the level of Cu, Zn, Mn, Fe, Pb and Cd in linseed oil, rapeseed oil and soybean oil. In addition, the effect of technological production processes on metals concentration was also investigated. The significant concentrations of Fe, Mg and Zn were observed in oilseeds. Determination of the concentrations of some toxic elements in plant and seed oil in Zaria, northern Nigeria have shown the level of metals (Na, Cd, Pb, Cr, Al, Cu, Mn, and Ni) in the different varieties of oils. The highest and lowest metal concentrations were observed in Na and Cu in all samples respectively. The concentrations ranged from 19.10-110.6, 0.34-2.77, 0.01-0.34, 0.05-0.84, 0.02-0.25, 0.01-0.08, 0.14-0.91, 0.34-0.97 mg/kg of Na, Cd, Pb, Cr, Al, Cu, Mg and Ni

(Ogabiela, et al., 2010). Pehlivan et al. (2008) evaluated some inorganic metals (Cu, Fe, Mn, Co, Cr, Pb, Cd, Ni, and Zn) in edible oils by inductively coupled plasma atomic emission spectroscopy (ICP-OES). The highest metal concentrations were measured as Cu in almond oil (0.0850 mg/kg), Fe in corn oil (0.0352 mg/kg), Mg in soybean oil (0.0220 mg/kg), Co in sunflower oil and almond oil (0.0040 mg/kg), Cr in almond oil (0.0010 mg/kg), Pb in virgin olive oil (0.0074 mg/kg), Cd in sunflower oil (0.0045 mg/kg), Ni in almond oil (0.0254 mg/kg) and Zn in almond oil (0.2870 mg/kg) (Pehlivan, Arslan, Gode, Altun, & Özcan, 2008). Farzin and Moassesi (2014) have determined the metal contents of four edible oils (olive oil, canola oil, and sunflower oil and soybean oil) produced in Iran by using microwave-assisted acid digestion. The results have shown that the concentration of Ni, Mg, Zn, Cu, Fe, Ca and Mg were in the range of 0.91–2.17, 0.14–1.76, 3.58–9.54, 0.18–0.68, 7.78- 28.93, 21.42–78.52, 5.34–36.49 µg/g, respectively. Result of Pb and Cd were found to be 4.56– 15.82 and 1.87–8.58 µg/kg (Farzin & Moassesi, 2014). Mohammadpourfard et al. (2015) have analyzed the heavy metals in apricot and almond oils. The results showed the average of the most important toxic metals detected in apricot oil samples was as follows 721.72 µg/kg for Al, 15 µg/kg for Cd, 18 µg/kg for Pb, 14 µg/kg for As and <1 µg/kg for Hg. Furthermore, the average of heavy metals detected in almond oil samples was as follows 1019.73 µg/kg for Al, 10 µg/kg for Cd, 21 µg/kg for Pb and 11 µg/kg for As and <1 µg/kg for Hg. Also, in the studied samples, Al had the highest concentrations among all metals (Mohammadpourfard, Shariatifar, Jahed-Khaniki, & Ebadi-Fathabad, 2015). Ashraf and Khobar (2014) determined Cu, Zn, Fe, Mn, Cd, Pb and As in different varieties of edible oils consumed in Saudi Arabia. The concentrations of Cu, Zn, Fe, Mg, Pb and As were observed in the range of 0.035 - 0.286, 0.955 - 3.10, 17.3 - 57.8, 0.178 - 0.586, 0.011 - 0.017 and 0.011 - 0.018 µg/g, respectively. Cadmium was found to be in the range of 2.36 - 6.34 ng/g (Muhammad Waqar Ashraf & Khobar, 2014). Torki et al. (2018) investigation in seeds of Iran from 331 seed samples such as sunflower, pumpkin, watermelon, & jabooni (red watermelon seeds) revealed that that Pb was detected in 33% of the samples with a median and mean level of 66 and 77 ± 28 µg/kg, respectively. The level of Pb in 39% of positive samples was higher than 100 µg/kg. The highest frequency of lead contamination was detected in the pumpkin seeds (52%), followed by the sunflower seeds (30%), the watermelon seeds (25%), and the jabooni seeds (15%) (Torki, Mehrasebi, Nazari, Kamali, & Hosseini, 2018). Furthermore, Cd was found in 17% of the samples with an average level of 264 ± 177.3 µg/kg. In addition, none of the watermelon and jabooni seed samples was contaminated with Cd. The highest concentration (731 µg/kg) and incidence (35%) of Cd were recorded in the sunflower seeds (Torki, et al., 2018).

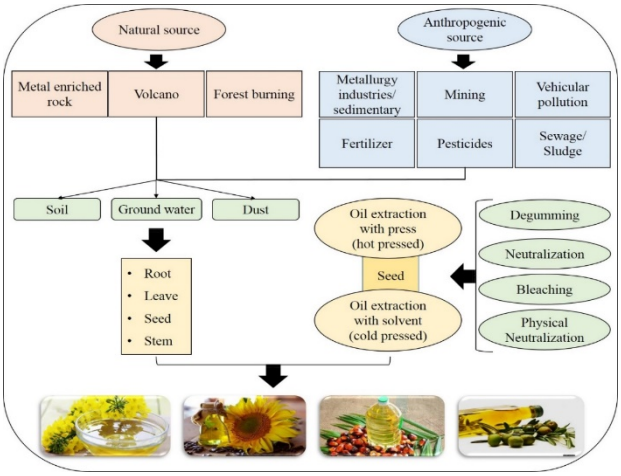


**Figure 1.** Sources of heavy metals contaminant in oilseed and mechanism entrance

In the following, more details about the source of pollution and toxicity of some of the most important heavy metals will be given. In addition to natural agents, oil production processes can also increase or decrease the amount of heavy metals in the final product. Refining edible oil treated processes, including degumming, neutralization, bleaching, and deodorizing and inevitably contact with the corrosion milling equipment surface at high

temperature, storage and packaging excess oil heavy metal contents (Ansari, et al., 2009) (**Figure 1**).

### 2.1. Lead in Seed or Edible Oil

According to European Food Safety Authority (EFSA) in 2012, the mean concentration of Pb in poppy, sesame, sunflower and pumpkin seed samples was reported between 37-42, 21-30, 34-39 and 32-40 µg/g, respectively (EFSA, 2012b). Another study in the Swedish market showed mean concentrations of Pb in sunflower and pumpkin seeds were 3.1 and 7.9 µg/g, respectively (Rodushkin, Engström, Sörlin, & Baxter, 2008). In Brazil, Pb level in cotton, sunflower and soybean seeds were 22±1, 11±3 and 20±8 µg/g respectively (Chaves, et al., 2010). Another study in Bulgaria showed Pb mainly accumulates in peanut and corn seeds especially in regions around a metal smelter with higher concentration range between 5200 and 9600 µg/g (Stefanov, Seizova, Yanishlieva, Marinova, & Popov, 1995). The result data of research by Chen et al. (2010) showed a different Pb level in seeds with the highest and lowest amount in sunflower and cotton seeds, respectively (Z. F. Chen, et al., 2010). Pb concentration in 9 varieties of edible oils in China was reported in ranges of 9-18 µg/g. Also, Pb concentration in soybean, corn, peanut, sesame, cottonseed, olive and sunflower oil was 15±1,9 ±1, 12 ±1, 18 ±2,11±1,13±1, 10 ±1 µg/g, respectively (Zhu, Fan, Wang, Qu, & Yao, 2011). In Turkey, Pb level in sunflower, hazelnut, canola, corn and olive oils was reported 99-134 µg/kg (Bakircioglu, Kurtulus, & Yurtsever, 2013). One more investigation in some oil samples from Spain and Morocco showed different levels of Pb in two countries in the range of 3.1-92.5 µg/kg (Bakkali, Martos, Souhail, & Ballesteros, 2012) (**Table 1**).

### 2.2. Cadmium in Seed or Edible Oil

The results of one investigation reported that the mean Cd concentration in soybeans was 0.006–0.028 mg / kg [34]. The average concentration of Cd in the olive oil, corn oil and sunflower oil-containing food product was between 1.1-7.1, 4.0-5.9 and 4.1-7.2µg/kg respectively (Mataveli, Pohl, Mounicou, Arruda, & Szpunar, 2010). Determination of Cd concentration in edible oil produced in Turkey by Bakircioglu et al. (2013) show that the concentration of chromium concentration is between 0.030 to 0.053 mg/kg for Hazelnut and Sunflower (FDA, 2020). Other studies have been performed on Cd in oilseeds and edible oils, which can be regarded in **Table 1**. In the study in Brazil, mean concentrations of Cd in cotton, sunflower , Tung and soybean seeds were reported <0.006 µg/kg, 0.038 µg/kg, <0.006 and <0.006µg/g, respectively (Chaves, et al., 2010).

### 2.3. Arsenic in Seed or Edible Oil

The investigation of EFSA, in 17 European countries, showed that mean concentrations of inorganic As in 119 samples of linseed, 90 samples of poppy seed, 139 samples of sesame seed, 170 samples of sunflower seed, 200 samples of rape seed, 15 samples of mustard seed and 129 samples of pumpkin seed were 20.8, 32.6, 20.1, 25.1, 33.2, 33.7 and 10.5 µg/g, respectively. In Swedish market, mean concentrations of As in sunflower, pumpkin seeds and peanut were 6.1, 5.1 and 6.6 µg/g, respectively (Rodushkin, et al., 2008). The compression of suggested previous studies showed the different levels of arsenic contamination in seeds in different regions and countries, which could relate to the use of agricultural, fertilize industrial waste, mining activities and pesticides. The mean occurrence level of inorganic As content in olive oils and other edible oil was 0.8 µg/kg (Cubadda, D'Amato, Aureli, Raggi, & Mantovani, 2016). Determination of metal content in oil samples in Spain and Morocco shows As concentration range is between 0.56-6.2 µg/kg (Bakkali, et al., 2012) (**Table 1**).

### 2.4. Mercury in Seed or Edible Oil

Mercury (Hg) has long been recognized as a dangerous metal related to its naturally occurrence in the environment, bioaccumulation

and transportation in a variety of foods (EFSA, 2012c). Although major Hg has been detected in marine foods, which sometimes contain small amounts of inorganic Hg. In several countries, foodstuffs have shown that plant samples typically contain low concentrations of Hg (Khanna, 2011). From 2002 to 2011, 20 European countries submitted approximately 60,000 analytical results of Hg concentrations; 98 % of the data were on total Hg with mean concentrations 3.7 µg/g in 556 samples of Oilseeds (Alexander, et al., 2012). Rodushkin et al. (2008) result on the Swedish market showed that mean concentrations of Hg in sunflower and pumpkin seeds were 0.13 and 0.19 µg/g, respectively (Rodushkin, et al., 2008) (**Table 1**).

### 2.5. Chromium in Seed or Edible Oil

EFSA database in 2012 supposed that a concentration of Cr (III) was 214 -227.3 µg/g in 455 oilseed samples (EFSA, 2014b). Also, the study in 2008 on the Swedish market, reported the mean concentrations of Cr in sunflower, pumpkin seeds and peanut were 5.3, 11 and 12 µg/g, respectively (Rodushkin, et al., 2008). Average concentration of Cr in soybean based edible product was between 560-5880 µg/kg (Barbosa, et al., 2015). Determination of Cr concentration in edible oil that was produced in Turkey by Bakircioglu et al. (2013) shows that chromium concentration range is between 0.126 and 7.106 mg/kg (Bakircioglu, et al., 2013) (**Table 1**).

### 2.6. Nickel in Seed or Edible Oil

The results of investigation in 15 different European countries showed the high mean levels Ni were in 'Legumes, nuts and oilseeds' (~ 2000 µg/g), certain types of chocolate (Cocoa) products (3800 µg/g), and 'Cocoa beans and cocoa products' (9500 µg/g) among 18885 food and 25700 drinking water samples (Davide. Arcella, Gergelova, Innocenti, López-Gálvez, & Steinkellner, 2019). In Swedish market, mean concentrations of Ni in sunflower, pumpkin seeds and peanuts were 2500, 3100 and 850 µg/g, respectively (Rodushkin, et al., 2008). In the study in Brazil, mean concentrations of Ni in cotton, sunflower and soybean seeds were reported 310±20, 1010±380 and 1640±1070 µg/g, respectively (Chaves, et al., 2010). Determination of nickel in Nigerian foods shows Ni content in some oils (groundnut oil and vegetable oil) was (570 and 330 µg/kg). It is related to food processing, the natural geochemistry of the soils and chemicals used as fertilizer and pesticide (Onianwa, Lawal, Ogunkeye, & Orejimi, 2000). Bakkali et al. (2012) performed a study for heavy metal determination in vegetables and oils and Ni content in oils was between (1.0-25.6 µg/kg) (Bakkali, et al., 2012) (**Table 1**).

## 3. Co-existence of heavy metals and anions

Epidemiologic studies have shown that co-exposure to heavy metals has been investigated by different researches. It is reported that renal dysfunction of Cd and Pb co-exposure can induce additive or synergistic interactions or even new adverse effects which are not observed following single exposure of toxic metals on occupational workers (X. Chen, et al., 2019). Agrawal et al. (2015) concluded that co-exposure of mercury, arsenic and lead resulted in a significant increase in oxidative stress in kidneys and liver (Agrawal, Bhatnagar, & Flora, 2015). The finding of data in animal study indicated co-exposure to non-toxic levels of Cd and fluoride can potentiate their individual hepatotoxicity through disruption of the cellular redox status, inflammation, and apoptosis pathway (Arab-Nozari, et al., 2020).

**Table 1.** Mean concentration of heavy metals in seeds and oilseeds

| Heavy Metals | Seed/Oils | Mean Concentration | Range (µg/kg) | Country | Year | Ref. |
|---|---|---|---|---|---|---|
| Pb | Olive oil | 29.2 µg/kg | 4.4-92.5 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Corn oil | 8.52 µg/kg | 3.1-13 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Sunflower oil | 18.42 µg/kg | 6.4-39.4 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Types of vegetable oils | 0.003 mg/kg | - | French | 2012 | (Arnich, et al., 2012) |
| | Sunflower oil | 0.056 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Hazelnut oil | 0.059 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Canola oil | 0.073 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Corn oil | 0.048 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Olive oil | 0.071 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Soybean | - | <29–110 | Brazil | 2015 | (Barbosa, et al., 2015) |
| | Cotton seed | 0.022µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Sunflower | 0.011µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Tung | <0.006 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Soybean | 0.02 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Curcas bean | 0.062 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Fodder turnip | 0.025 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Castor bean | 0.043 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Olive oil flavored with pepper | 0.984 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with fungi | 12.33 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with vegetable | 10.76 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil | 10.111 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Vegetable oils | - | 11-17 | KSA | 2014 | (M. W. Ashraf, 2014) |
| | Rapeseed oil | 0.09 mg/kg | - | Poland | 2016 | (Szyczewski, et al., 2016) |
| | Soybean oil | 0.08 mg/kg | - | Poland | 2016 | (Szyczewski, et al., 2016) |
| | Linseed oil | < 0.06 mg/kg | - | Poland | 2016 | (Szyczewski, et al., 2016) |
| Cd | Olive oil | 23.35 µg/kg | 1.1-7.1 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Corn oil | 3.62 µg/kg | 4.0-5.9 | Spain | 2012 | (Bakkali, et al., 2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Sunflower oil | 5.5 µg/kg | 4.1-7.2 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Oilseeds | 371 µg/kg | - | Europe | 2012 | (EFSA, 2012a) |
| | Types of vegetable oils | 0.0014mg/kg | - | French | 2012 | (Arnich, et al., 2012) |
| | Sunflower | 0.053 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Hazelnut | 0.030 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Canola | 0.040 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Corn | 0.036 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Olive | 0.036 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Soybean | - | <6–28 | Brazil | 2015 | (Barbosa, et al., 2015) |
| | Cotton seed | <0.006 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Sunflower | 0.038 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Tung | <0.006 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Soybean | <0.006 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Curcas bean | <0.006 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Fodder turnip | 0.053 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Castor bean | <0.006 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Olive oil flavored with pepper | Not detected | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with fungi | 1.004 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with vegetable | 0.078 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil | 0.096 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Vegetable oil | | 2.36 - 6.34 | KSA | 2014 | (M. W. Ashraf, 2014) |
| | Rapeseed oil | 0.03 mg/kg | - | Poland | 2016 | (Szyczewski, et al., 2016) |
| | Soybean oil | 0.01mg/kg | - | Poland | 2016 | (Szyczewski, et al., 2016) |
| | Linseed oil | 0.03 mg/kg | - | Poland | 2016 | (Szyczewski, et al., 2016) |
| **As** | Olive oil | 51.25 µg/kg | 0.38-7.2 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Corn oil | 4.1 µg/kg | 5.1-6.2 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Sunflower oil | 15.62 µg/kg | 2.3-6.5 | Spain | 2012 | (Bakkali, et al., 2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Types of vegetable oils | 0.014 mg/kg | - | French | 2012 | (Arnich, et al., 2012) |
| | Soybean | - | <7–40 | Brazil | 2015 | (Barbosa, et al., 2015) |
| | Olive oil | 0.8 ng/ g | - | Italy | 2016 | (Cubadda, et al., 2016) |
| | Vegetable oils | 0.8 ng/ g | - | Italy | 2016 | (Cubadda, et al., 2016) |
| | Olive oil flavored with pepper | 0.00020 | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with fungi | 0.00040 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with vegetable | 0.00030 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil | 0.00040 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Vegetable oil | - | 11-18 | KSA | 2014 | (M. W. Ashraf, 2014) |
| **Ni** | Olive oil | 25.57 µg/kg | 3.4-17.9 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Corn oil | 32.72 µg/kg | 2.3-25.6 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Sunflower oil | 9.82 µg/kg | 1.0-21.5 | Spain | 2012 | (Bakkali, et al., 2012) |
| | Types of vegetable oils | 0.039 mg/kg | - | French | 2012 | (Arnich, et al., 2012) |
| | Soya oil | 4.462 µg/kg | - | EFSA | 2019 | (Davide. Arcella, et al., 2019) |
| | Sunflower oil | 1.566 µg/kg | - | EFSA | 2019 | (Davide. Arcella, et al., 2019) |
| | Rape seed | 762 µg/kg | - | EFSA | 2019 | (Davide. Arcella, et al., 2019) |
| | Linseed | - | 0–300 | EFSA | 2019 | (Davide. Arcella, et al., 2019) |
| | Sunflower | 1.490 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Hazelnut | 1.420 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Canola | 1.097 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Corn | 0.772 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Olive | 0.836 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Soybean | - | 740–4780 | Brazil | 2015 | (Barbosa, et al., 2015) |
| | Cotton seed | 0.31 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Sunflower | 1.01 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Tung | 1.97 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Soybean | 1.64 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Curcas bean | 0.90 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Fodder turnip | 0.60 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Castor bean | 1.30 µg/kg | - | Brazil | 2010 | (Chaves, et al., 2010) |
| | Olive oil flavored with pepper | Not detected | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with fungi | 10.230 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil flavored with vegetable | 12.340 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| | Olive oil | 14.180 µg/g | - | Iran | 2019 | (Ziarati, et al., 2019) |
| **Hg** | Edible oils | 0.005 mg/kg | - | French | 2012 | (Arnich, et al., 2012) |
| | Sunflower | 0.13 µg/g | - | Sweden | 2008 | (Rodushkin, et al., 2008) |
| | Pumpkin | 0.19 µg/g | - | Sweden | 2008 | (Rodushkin, et al., 2008) |
| **Cr** | Sunflower | 2.780 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Hazelnut | 0.428 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Canola | 0.450 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Corn | 0.224 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Olive | 0.646 mg/kg | - | Turkey | 2013 | (Bakircioglu, et al., 2013) |
| | Soybean | - | 1040-1120 | Brazil | 2015 | (Barbosa, et al., 2015) |
| | Olive oil | 2. 85 µg/kg | - | Spain | 2012 | (Bakkali, et al., 2012) |
| | Corn oil | 5.05 µg/kg | - | Spain | 2012 | (Bakkali, et al., 2012) |
| | Sunflower oil | 5.32 µg/kg | - | Spain | 2012 | (Bakkali, et al., 2012) |

## 4. Regulation, Incidence and Human Health Effects of Heavy Metals in Vegetable Oils/Fats

The ability of heavy metals to accumulate in seeds can pose serious health risk issues, such as gastrointestinal cancer (El-Kady & Abdel-Wahhab, 2018), immunological mechanisms, mental retardation (Raj & Maiti, 2019) and malnutrition (Dickin, Schuster-Wallace, Qadir, & Pizzacalla, 2016), as well as accumulated through diet in bones or adipose tissue, leading to the loss weakened immunological defenses (Davide. Arcella, et al., 2019). It is also reported, that some heavy metals (e.g. Al, Cd, Mn and Pb) are capable of causing intrauterine growth retardation (A. Khan, Khan, Khan, Qamar, & Waqas, 2015). In this regard, maximum residue limited levels (MRLs) have been developed by various organizations for the maximum permission level of heavy metals in oils (**Table 2**).

**Table 2.** Maximum permission level of heavy metals in vegetable oils

| Heavy Metals | Codex Alimentarius Commission | Ref. | U.S. Food and Drug Administration | Ref. | European Commission Regulation | Ref. |
|---|---|---|---|---|---|---|
| **Pb** | 0.1 mg/kg | (Commission, 1999) | 10 mg/kg (Rapeseed oil standard) | (FDA, 2020) | 0.1 mg/kg wet weight | (EFSA, 2012a) |
| **Cd** | 0.05 mg/kg | (Mir Mohammad Makki & Ziarati, 2017) | 10 mg/kg (Rapeseed oil standard) | (FDA, 2019) | - | - |
| **As** | 0.1 mg/kg | (Commission, 1999) | 3 mg/kg (Rapeseed oil standard) | (FDA, 2019) | - | - |
| **Hg** | - | - | 10 mg/kg (Rapeseed oil standard) | (FDA, 2019) | - | - |
| **Ni** | Maximum content in Hydrogenated food oils: 0.2 to 1 mg/kg | (Commission, 2012) | 0.5 mg/kg (Menhaden oil standard) | (FDA, 2019) | Maximum content in hydrogenated vegetable oils/fats: 20 mg/kg | (Davide. Arcella, et al., 2019) |

## 5. Heavy Metal Impacts on Human Health

Cadmium (Cd) is a natural source in the environment lying under volcanos and rock weathering. In addition, anthropogenic activities increase Cd levels in agricultural soils, air and water. Cd is usually classified as a carcinogenic metal and is usually not found in the pure form, and high concentrations of Cd are found in association with Pb and Zn ores (Satarug, Vesey, & Gobe, 2017). Industrial wastes, rechargeable nickel-cadmium batteries, tobacco and phosphorus fertilizers are major sources of Cd in soil and crops, and industrial wastes are a way for Cd to enter into the aquatic environment and then be accessed by fishes (El-Kady & Abdel-Wahhab, 2018). It is suggested air born Cd is the dominant source of crop contamination around pyrometallurgic smelters (Smolders, 2001) and sewage sludge (D Arcella, Cappe, Fabiansson, di Domenico, & Furst, 2012). Soil contamination by Cd result in increasing Cd uptake by some plants, depending on inherent genetic and physiological characteristics and soil pH (D Arcella, et al., 2012). Some plants such as sunflower accumulate cadmium more than other crops (Vanderpool & Reeves, 2001). Exposure to Cd inhalation stimulates the respiratory tract, which is much higher in tobacco than in cigarettes. But, food is the main source of Cd exposure in the non-smoking general population (Schwarz, Lindtner, Blume, Heinemeyer, & Schneider, 2014). Although Cd absorption through dietary exposure is relatively low (3-5%), it has a very long biological half-life (7-17 years) (D Arcella, et al., 2012). Then, prolonged intake of Cd specifically, accumulates in liver and kidney (Vanderpool & Reeves, 2001). This compound is a non-essential element in foods and exposure may pose adverse health effects, including kidney damage and possibly also bone effects and fractures and increases the risk of lung, endometrial, bladder, and breast cancer (D Arcella, et al., 2012). Cd is classified as 'carcinogenic to humans' (group I) by the International Agency for Research on Cancer (IARC) (EFSA, 2012a). In 2010, the Joint FAO/WHO Expert Committee on Food Additives set a provisional tolerable monthly intake of 25 μg/g body weight for Cd several

studies determined Cd levels in food stuff (D Arcella, et al., 2012). In terms of the target tissue, this kidney is the most important target tissue in the kidney and it is done by protein proteinuria. Threshold of Cd level that has been reported, is about 200 µg (Zang & Bolger, 2014). Thus, long-term Cd consumption, mainly through foods, leads to serious problems including kidney failure. Consumption of Cd-contaminated food products can cause bone pain (such as Itai-Itai disease), cardiovascular disease and kidney damage (EFSA, 2012a). Osteoporosis, bone malformation, and ultimately kidney dysfunction are symptoms of the disease associated with the mining industry. However, the liver also plays an important role in minimizing Cd toxicity (Baba, et al., 2013). On the other hand, in the long-term exposure, this process reduces metallothionein activity, thereby increasing ROS levels and inducing oxidative stress in brain cells (El-Kady & Abdel-Wahhab, 2018).

Lead (Pb) is a natural environmental contaminant as a result of wide spread anthropogenic activities such as mining, smelting, battery manufacturing. Pb is naturally present in the earth's crust and is used in industry due to its physicochemical properties such as softness, corrosion resistance (Wani, Ara, & & Usmani, 2015). Lead oxide (PbO) is used as a waterproofing layer in earthenware and porcelain, becoming a source of contamination in reaction to acidic foods. In addition, lead acetate is used as a pesticide and color product (Asaduzzaman, et al., 2017). Also, lead nitrate has many applications in packaging, for example, textile printing, oxidizing, sensitizing, and rodenticides (García-Lestón, Méndez, Pásaro, & Laffon, 2010). Pb is now used as a stabilizer in pipes and is a major contributor to the total daily intake of lead in the United States in drinking water (El-Kady & Abdel-Wahhab, 2018). Besides, Pb from fossil fuels and vehicles and its diffusion into the air and into the surface and gradually different parts of plants are other factors in the exposure of this metal. General population exposure to Pb occurs via food, drinking water, air, soil and dust. Although food is the main source of Pb exposure through processing, handling and packaging process chronic toxicity of Pb because of long half-life in the body is of most concern because of the human health risk (EFSA, 2010). The IARC classified inorganic lead as probably carcinogenic to humans (Group 2A) in 2006. In children, there is an association between blood level of Pb and reducing Intelligence Quotient (IQ) score and functions of cognition in a dose-dependent manner. There is an association between elevate blood pressure and kidney dysfunction at relatively low blood lead level (EFSA, 2010). Pb enters the bloodstream after the process of digestion, absorption and accumulates throughout the body organs and soft tissues of the brain. According to previous studies, due to the similarity of $Pb^{2+}$ and $Ca^{2+}$, Pb is substituted in bone (Tahir, et al., 2017). As a result, Ca deficiency causes more Pb to accumulate in the bone. Elevated levels of Pb in the blood have been observed in children with neurological symptoms, such as lack of concentration and difficulty in communication skills. Pb contamination negatively affects mental development and neurological and cardiovascular diseases in humans, especially children. Also, Pb can accumulate in the bone and can lead to gastrointestinal colitis, leukemia, hypertension, kidney damage, brain disorders and thrombotic diseases (Fang, et al., 2014). High concentrations of Pb (N-methyl-D-aspartate) also play an important role in cognitive functions and eventually impair memory and learning. Pb is also said to be effective in anemia (El-Kady & Abdel-Wahhab, 2018). In addition, Pb has a toxic effect on the reproductive system with increasing abnormal sperm count in males (Brochin, et al., 2008). Besides, lead reduces antioxidant activity (El-Kady & Abdel-Wahhab, 2018). There is a strong association between accumulation and elevated lead levels in humans and the risk of diseases associated with central nervous system disorders such as Parkinson's disease and multiple sclerosis (MS) (Ghoreishi, Mohseni, Amraei, Mirza Alizadeh, & Mazloomzadeh, 2015).

Inorganic arsenic (As) is a human carcinogen and is classified in group I of carcinogenic compounds in humans (EFSA, 2009). The most important pathway of As in humans is contaminated food and drinking water. For example, rice, which is the dominant food in some countries contamination with mineral As, can be a serious risk for contamination with this metal (Praveena & Omar, 2017). Groundwater and contaminated water, obviously, are one of the main ways to absorb the metal in contaminated plants. The toxicity of As depends on its chemical form, route and duration of exposure. The mineral forms of As consist of arsenite, As (III) and arsenate, As (V) are highly toxic compared to organic forms (Feldmann & Krupp, 2011).

Chronic oral exposure to inorganic As causes cancers and a wide range of adverse health effects in many organs. Neurotoxicity, immunotoxicity, skin lesions, cardiovascular diseases, toxic effect on developmental stages of life abnormal glucose metabolism, type II diabetes appear to be related to chronic ingestion of As (EFSA, 2014a). Methylation of As exposure is produced and has a direct effect on oxidative stress following exposure (Kesici, 2016). Inorganic, reduced glutathione, glutathione peroxidase and glutathione synthase are released. ROS mitochondrial membrane potential in As poisoning decreases and Ca balance within the cell is disrupted. Thus, it opens the membrane pores and increases the release of cytochrome c, leading to apoptosis (Gao, Li, Xu, Liu, & Liu, 2018).

In various forms of mercury (Hg), half-life of elimination depends on dose, species, strain and sex are different. Absorption and retention have great rates in neonates and children. Methyl mercury in food after absorption in the intestine, binds to plasma proteins in the bloodstream and accumulates in the human brain (EFSA, 2012c). In 2003, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the provisional tolerable weekly intake (PTWI) for $CH_3Hg$ in the range of 1.6 µg/kg body weight. Then, in 2010, the JECFA revised the PTWI of 4 µg/g/body weight for inorganic Hg (Arnich, et al., 2012; EFSA, 2012c).

Chromium (Cr) is available to the environment in three states: elemental chromium, trivalent chromium ($Cr^{3+}$); and hexagonal chromium ($Cr^{6+}$) (El-Kady & Abdel-Wahhab, 2018). Cr is ubiquitous in food such as oils, fat, meat and cereals (EFSA, 2014b). The panel considered health outcomes of Cr intake and concludes in healthy subjects, Cr has no beneficial effects. Natural presence or emissions from anthropogenic activities are a different environmental source of Cr in food. Additional source of Cr in food could be preparation of food with stainless steel containers, food processors and utensils. Groundwater contamination with chromium is contaminated by hexagonal chromium due to health risks during the process of stainless steel production, textile dyes (Kaprara, et al., 2015). Therefore, irrigation with the wastewater of these industries is the main source of contamination of this heavy metal in the soil. This increases the level of Cr in the soil and subsequently increases its uptake by plants (Liao, et al., 2011). IARC has classified chromium VI as group I of carcinogenic compounds in humans and animals while chromium III was not classifiable as a carcinogen compound to humans (Group III) (Hartwig, 1998). Cd can lead to human cancer through inhalation. $Cr^{6+}$ by NADPH produces hydroxyl radical (OH), which increases p53 elimination in epithelial cells, resulting in apoptosis. Cr is distinct from other metals owing to its capability to interact mainly and directly with DNA to form DNA-DNA cross links and DNA-protein (Nickens, Patierno, & Ceryak, 2010). Target organ for chrome are liver, kidney, spleen with Half-life 35-40 hours. The EFSA panel on contaminants in the food chain established a tolerable daily intake (TDI) of 300 µg Cr (III)/kg body weight per day from the lowest No Observed Adverse Effect Level (NOAEL) identified in a chronic oral toxicity study that amounted to 286 in rats (EFSA, 2014b).

Nickel (Ni) is a widespread natural component of the crust of earth to about 0.01%.

Nickel emission into environment is due to anthropogenic activities, industrial processes and technological sources. Deposition of air born nickel-bearing particles on soil and surface water result in accumulation in plant and animal. In the general population food is the main exposure route for Ni with an average daily intake of about 0.1-0.3 mg (Schaumlöffel, 2012). It is not essential for human, although it is higher in plants and in some animal species is an essential micronutrient (EFSA). Ni toxicological effects to organisms are dependent on species form of diet composition and fasting status as well as exposure way and concentration (Schaumlöffel, 2012). The most stable of the oxidation state forms are divalent form in food and drinking water and mono form of Ni. Ni compounds are classified by IARC as carcinogenic to humans (Group 1) (Chervona, Arita, & Costa, 2012). The WHO established a Tolerable Daily Intake (TDI) of 11 μg Ni /kg body weight per day. (Davide. Arcella, et al., 2019).

## 6. Effects of heavy metals on nutrient quality of oilseed

Recent studies suggest that seed oils are adversely affected by the heavy metal exposures oil content decrease and fatty acid composition changes in edible oil (R. Khan, Srivastava, Abdin, & Manzoor, 2013). The presence of heavy metals in plant oils may have a negative influence on the quality of oils, causing changes to their taste and smell. Such changes in oil quality, called “taste reversion,” are caused by the occurrence of the following metals: Fe, Cu, Cr, Zn, and Mn, which, through their ability to form radicals, facilitates the process of oxidation of fats (Zioła-frankowsk, Frankowski, & Szyczewski, 2011). Metal contamination of plants leads to oxidative damage of proteins, lipids and nucleic acid, which in turn is responsible for various physiological diseases. The potential toxicity of heavy metals even at low concentrations is due to the stability of their organic and inorganic ligands (Fergusson, 1990).

## 7. Conclusions

Food safety is a major public concern worldwide and food consumption has been identified as the major pathway for human exposure to contaminants. Vegetable oils and fats are widely used in cooking and alimentary, cosmetic, pharmaceutical and chemical industries, which are beneficial and popular due to their cholesterol-lowering effect. Oilseeds might concentrate some toxic metals, which could enter into vegetables and their oil. The main source of contamination of plant and seed oils and fats with heavy metals is their direct migration from arable land to oil plants. Moreover, during technological processes, the enrichment of oils with heavy metals can occur. The factors that influence the content of heavy metals in plants and seeds also include individual properties of plants used in oil production. Occurrence of heavy metals in food commodities and their uptake can pose toxic effects in human. Hence, the incidence of heavy metals in different vegetable oils and fats was observed practical way to reduce the consumer intake of heavy metals is to increase monitoring. Besides, the co-occurrence of some heavy metals, or heavy metals and some anions in a single food, which can lead to synergistic interactions, indicates the necessity for further investigation of mechanistic toxicity, also, revising the international regulations in foods to minimize the potential health threats through vegetable oils and fats consumption.

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# THE RELEASE CHARACTERISTICS OF EPIGALLOCATECHIN GALLATE FROM THE GEL WERE AFFECTED BY ITS REACTION WITH WHEY PROTEIN ISOLATE

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**ABSTRACT**

The interaction between epigallocatechin gallate (EGCG) and whey protein isolate (WPI) under various pH and ion concentrations conditions was examined by ultraviolet-visible spectrophotometer and fluorescence spectra. The results showed that EGCG with WPI was linked mainly by electrostatic force, and EGCG caused the fluorescence quenching of WPI through a static quenching. Furthermore, in vitro static release results showed that the release process of EGCG from EGCG-WPI gel in release medium of different pH and ion concentrations follows the Korsmeyer-Peppas model and conform to the non-Fick diffusion law. Finally, the binding constant KA and the constant release Kapp kinetic model were established. The model followed the equation $LnK_A = A - \frac{1}{n} LnK_{app}$.

## 1.Introduction

Whey protein is a by-product of cheese production, and it is mainly composed of β-lactoglobulin (β-Lg), α-lactalbumin, (α-La), bovine serum albumin (BSA), and immunoglobulin (Ig) (Mehla et al. 2020). The functional properties of whey protein such as solubility, gelation, emulsification, foaming, etc. make it widely in food industry application (Minj and Anand 2020). Furthermore, due to its high nutritional value, it also has become one of bioactive supplements for the human body (Marcelo and Rizvi 2008). However, protein gel can be used as a carrier to disperse some functional components (Qiu and Park 2001). There was found that protein gel can not only protect biological activity but also regulate release rate of bioactive compounds (Chen, Remondetto and Subirade 2006) (Gunasekaran, Ko and Xiao 2007, O'Hagan, Singh and Ulmer 2006). Thus, improve the bioavailability of functional components. Previous studies showed that biological activity of VC was improved by encapsulation with WPI gel by controlling condition factors including pH value, temperature, EGCG loading, and ion concentration (Betoret et al. 2011).

EGCG is one of bioactive compounds, it is prepared mainly from green tea and it is contained 50-60 % catechin (Higdon and Frei 2003). The biological activities of EGCG including anti-oxidation, anti-tumor, anti-inflammation activities, thus, it has critical role in prevention of cardiovascular and cerebrovascular diseases. However, EGCG application in food and medicine industries is limited due to its poor stability (Dai et al. 2020).

In this regard, the stability of EGCG by reaction with WPI in the protein gel has not been evaluated. Therefore, the stability of EGCG in the composite gel of EGCG-WPI was evaluated. Furthermore, interaction between the EGCG and WPI was studied.

## 2. Materials and methods

### 2.1. Materials and instruments

Whey protein isolates powder (> 95% protein content) and EGCG (98%) were purchased from Le Sueur Cheese CO., USA and Chengdu Purifa technology development CO., LTD, respectively. Other chemicals were bought from Tianjin Fengchuan chemical reagent technology CO., LTD. pH meter (FE20, Mettler Toledo Instrument Shanghai CO., LTD.), visible spectrophotometer (WFJ7200, Uniko, Shanghai instrument CO., LTD.), fluorescence spectrophotometer (Shanghai Lingguang technology CO., LTD.), high-speed centrifuge (Shanghai Anting Scientific instrument factory), multi-head magnetic stirring heater (Changzhou Guohua electric appliance CO. LTD).

### 2.2. Methods

#### 2.2.1. *Sample preparation*

Firstly, the stability of EGCG of was examined under several pH value by mixing several volumes of (10-300 μL) of EGCG solution (1.2mg/mL), separately with 3 mL of pH phosphate buffer, and then the pH value was adjusted to 2.0 - 9.0, and the ion concentration was set to 0.05 mol/L. After that, 2 mL of WPI solution (1 mg/ mL) was added. Secondly, the stability of EGCG of was examined under difference ion concentration by mixing several volumes of (10-300 μL) of EGCG solution (1.2mg/mL), separately, with several volumes' ion concentration (0.10, 0.15, 0.20 mol/L), 3 mL of phosphate buffer and 2 mL of WPI solution (1 mg/ mL, were added respectively and pH value of 3.0. To prepare the EGCG-WPI gel: the WPI and of EGCG mixtures with difference pH values or ion concentration were prepared as mentioned above. After stirring for 2 - 3 h, the mixtures were stored at 4 °C for 12 h, and then the mixtures were heated at 85°C for 30 min using water bath. After that, the prepared gel was cooled rapidly in the ice water bath.

### 2.3. *Ultraviolet absorption and Fluorescence spectra analysis*

The absorbance of WPI in EGCG-WPI solution was determined within the wavelength range of 260- 340nm. For fluorescence spectra analysis, the excitation wavelength of fluorescence spectrum was 280 nm, and the scanning emission spectrum range was 280–340 nm (Zhang et al. 2012).

### 2.4. *In vitro release experiment*

Briefly, 1 ml of prepared gel in 50 mL phosphate buffer solution with difference pH values and ion concentrations was prepared as mentioned above. During the incubation for 360 min at 25°C, the EGCG concentration in mixtures was determined every 20 min by mixing samples mixture with Folin phenol reagent and sodium carbonate (7.5%, w/v), and then the absorbance was measured at 765 nm after reaction at 25 °C for 1 h.

### 2.5. *Statistical analysis*

Data were analyzed by one-way ANOVA using 25.0 SPSS Statistics (SPSS, Inc., Chicago, IL, USA) and Microsoft Excel 2010 was used for significance analysis. The significance level was $P < 0.05$, and the results were formulated as follows:$\bar{x} \pm s$.

## 3.Results and discussions

### 3.1. Interaction between EGCG and WPI

#### 3.1.1. *Ultraviolet absorption and Fluorescence spectra*

The UV absorption of WPI with EGCG concentration within 260 ~ 340nm is shown in Figure 1. At pH 7.0, WPI native had the maximum absorption peak at 280nm. The absorption of WPI was increased successively after mixing EGCG (0.15mol/L), as well as blue shift occurred from 280.0nm to 276.4nm Figure 1 (a and d). In addition, UV absorption spectra of WPI with EGCG concentration at pH values or ion concentrations presented the same trend (data not shown). Likely, with the increase of EGCG concentration in the WPI solution, the

absorption peak position of the WPI derivative UV spectrum also shifted Figure 1 (b, c, e and f) at the same pH (7.0) and ion level (0.15mol/L), these results indicated that the interaction between EGCG and WPI was occurred.

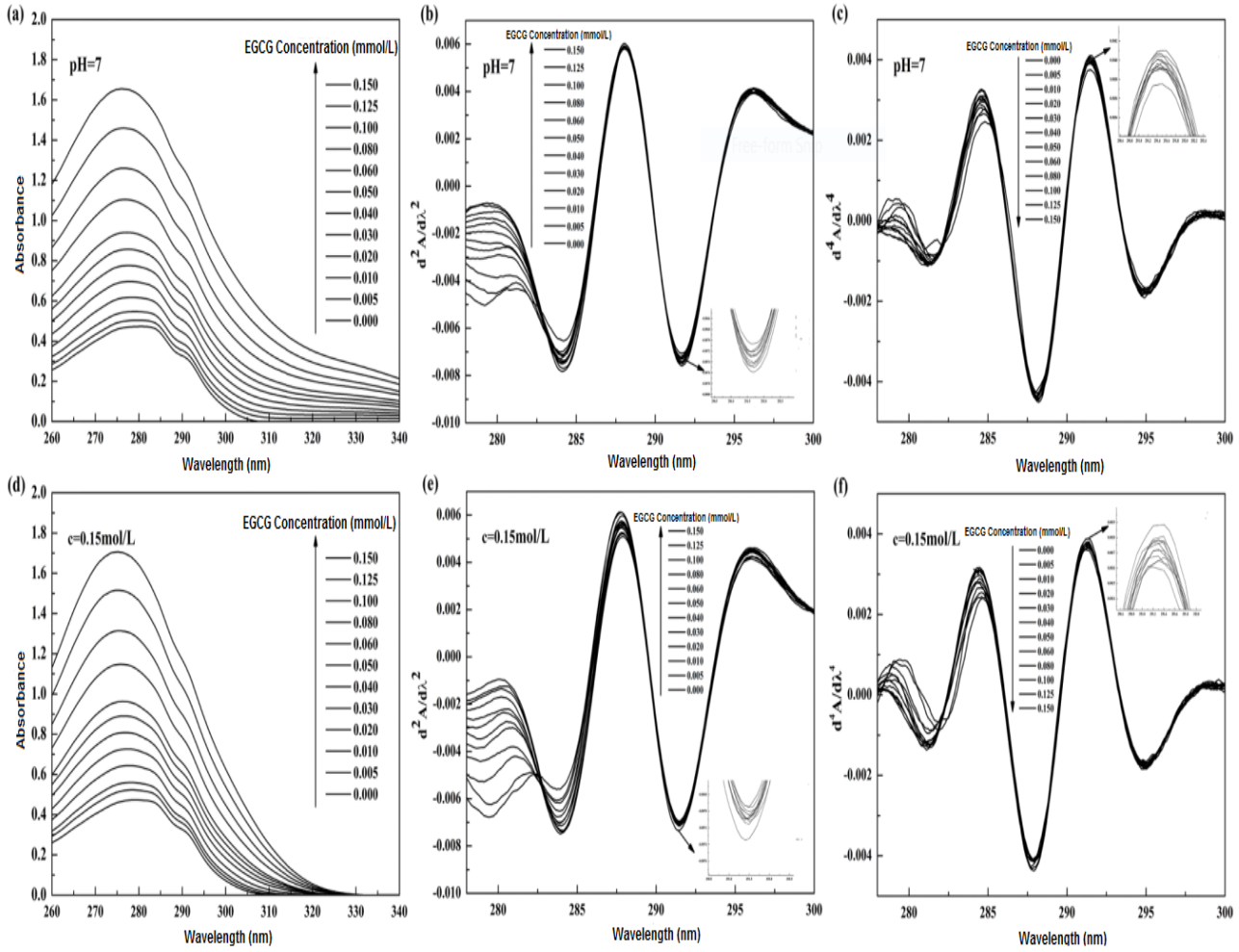


**Figure 1.** Influence of the concentration of EGCG on the UV absorption spectra of the WPI solution.

### 3.1.2.*Fluorescence spectrum*

Some amino acid residues include tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) that can emit fluorescence in the protein. Trp and Tyr are usually excited at 280 nm or longer wavelength, while Phe is excited at 295 nm or longer wavelength. Thus, the natural fluorescence and its variation value of this protein can directly reflect the changes of Trp, Tyr, and Phe residues in the protein and their surrounding microenvironment (Hemar et al. 2011).

The fluorescence spectra of WPI with EGCG concentration under experiment condition (pH 7.0, ion level, 0.15mol/L). AS displayed in Figure 2(a and b), at an excitation wavelength of 280 nm, the fluorescence spectrum of WPI solution had the maximum absorption peak near 330 nm. And this fluorescence spectrum did not change with the concentration of WPI, while fluorescence spectrum was gradually increased after added various concentrations of EGCG into WPI solution. Furthermore, the fluorescence intensity of the WPI solution was decreased and slightly redshifted, indicating that EGCG had a quenching effect on the fluorescence of WPI solution. In addition, similar phenomena were observed with other pH

values of 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0 and ion levels of 0.10 and 0.20 mol/L. These results further indicate the interaction between EGCG and WPI was occurred.

Additionally, at the excitation wavelength of 280nm, WPI emitted fluorescence at 330 nm in all pH values (2.0, 3.0, 4.0, 6.0, 7.0, 8.0, 9.0) except for the pH value of 5.0 because the pH environment affects on the solubility of WPI by changing the surface charge distribution. As known, β-Lg is the main component of WPI, and its isoelectric point is around 5.2 (Mantovani, Cavallieri and Cunha 2016).When the ambient pH value is close to the isoelectric point of WPI, the net charge on the surface of WPI molecules is about 0, and the intermolecular repulsion is the minimum. Thus, makes them easy to aggregation (Salminen and Weiss 2014), which is reflected in the fluorescence spectrum and causes the maximum difference in emission wavelength.

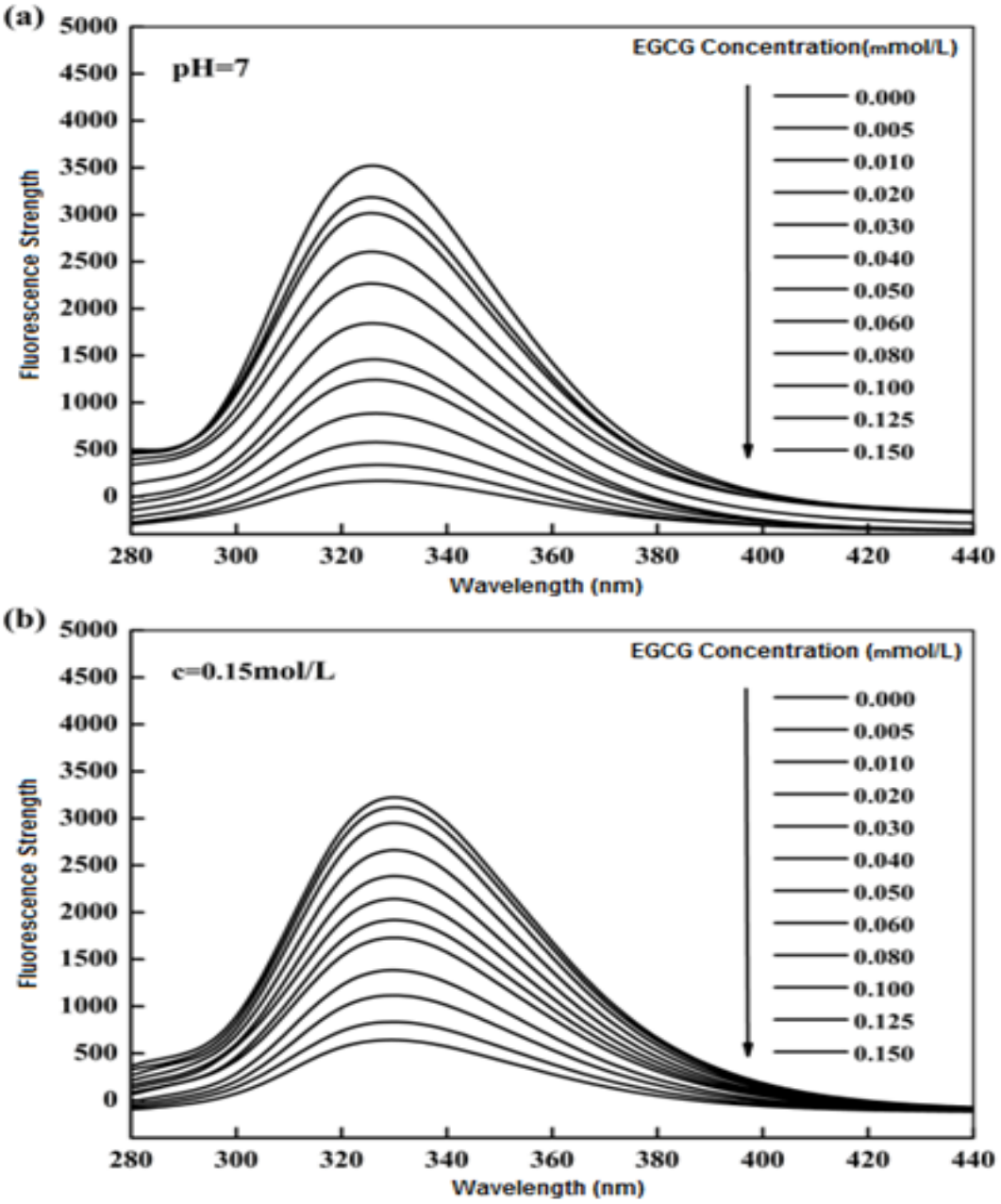


**Figure 2.** Influence of the concentration of EGCG on fluorescence emission spectra of WPI solution.

### 3.2.Fluorescence quenching mechanism analysis

The quenching mechanism for the system of biological macromolecules can be determined by comparing the quenching constant with the maximum quenching constant of biological macromolecules(Yang et al. 2018),the fluorescence quenching process caused by the collision of WPI as a fluorescent substance in the solution with the quenching agent EGCG can be analyzed by Stern-Volmer equation (Trnková et al. 2011):

$$F_0/F = 1+K_q\tau_0[Q]=1+K_{sv}[Q] \quad (1)$$

Where, $F_0$ and $F$ is the fluorescence intensity of WPI solution without and with EGCG, respectively.[$Q$] is EGCG concentration; $K_{sv}$ is Stern-Volmer quenching constant ($K_{sv}=K_q\times\tau_0$) ; $K_q$ is the rate constant of double molecular quenching;$\tau_0$represents the average fluorescence life of substances without quenching agents, and the average life of biological macromolecules is$1x10^{-8}$s (Yu et al. 2011).

Thus, $K_q$ was much higher than the limiting diffusion rate constant of the biomolecules $2\times10^{10}$ $(mol\cdot L^{-1})^{-1}\cdot s^{-1}$, it indicated that the WPI and EGCG interaction was mainly static quenching. For static quenching, the binding constant ($K_s$) was calculated using nonlinear least-squares fitting of the experimental data, as shown in Eq. (2) (Hasni et al. 2011):

$$Log\ [F_0 - F/F\ ] = logK_A + m\times log[Q] \quad (2)$$

where $m$ represents the kinetic reaction order (molecularity in $Q$). $M$ measures the number of $Q$ molecules that interact simultaneously with each site and does not express the number of independent and equivalent binding sites to analyze the reaction mechanism between EGCG and WPI, fluorescence quenching spectral data were combined with Eq. (1) and (2) to calculate the relevant characteristic parameters under different pH values and different concentrations (Table 2).

**Table 1.** Characteristic parameter in the interaction between EGCG and WPI at different pH values.

| pH value | $K_{sv}$(×10$^5$L/mol) | $K_q$(×10$^{13}$ L/mol·s) | $R_1^2$ | $K_A$ (×10$^5$ L/mol) | $n$ | $R_2^2$ |
|---|---|---|---|---|---|---|
| **2.0** | 2.022±0.082 | 2.022±0.082 | 0.996 | 2.383±0.142 | 1.152±0.079 | 0.985 |
| **3.0** | 1.751±0.062 | 1.751±0.062 | 0.998 | 2.123±0.193 | 1.094±0.101 | 0.975 |
| **4.0** | 1.662±0.066 | 1.662±0.066 | 0.998 | 1.835±0.101 | 1.044±0.060 | 0.984 |
| **5.0** | 1.371±0.073 | 1.371±0.073 | 0.997 | 1.527±0.099 | 0.989±0.064 | 0.563 |
| **6.0** | 2.025±0.098 | 2.025±0.098 | 0.997 | 1.653±0.359 | 1.067±0.178 | 0.973 |
| **7.0** | 2.063±0.123 | 2.063±0.123 | 0.998 | 2.221±0.259 | 1.034±0.136 | 0.967 |
| **8.0** | 3.172±0.147 | 3.172±0.147 | 0.998 | 3.423±0.180 | 1.045±0.094 | 0.984 |
| **9.0** | 4.133±0.190 | 4.133±0.190 | 0.999 | 5.389±0.103 | 1.076±0.054 | 0.985 |

**Table 2** Characteristic parameter in the interaction between EGCG and WPI at different ion concentrations.

| Ion concentrations (mol/L) | $K_{sv}$(×10$^5$ L/mol) | $K_q$(×10$^{13}$ L/mol·s) | $R_1^2$ | $K_A$ (×10$^5$ L/mol) | *n* | $R_2^2$ |
|---|---|---|---|---|---|---|
| **0.10** | 1.884±0.069 | 1.884±0.069 | 0.998 | 2.504±0.053 | 1.111±0.030 | 0.996 |
| **0.15** | 1.979±0.076 | 1.979±0.076 | 0.985 | 4.315±0.017 | 1.355±0.010 | 0.999 |
| **0.20** | 2.779±0.145 | 2.779±0.145 | 0.995 | 6.882±0.037 | 1.303±0.022 | 0.998 |

In this study, the $K_q$ parameters were used to determine the quenching process, As shown in (Table 1 and 2), under different pH values and ion concentrations. The $K_q$ values found from the reactions were much higher than the limiting diffusion rate constant of the biomolecules2.0×10$^{10}$L/mol.s, indicating that EGCG could successfully quench the WPI fluorescence via static quenching, which was induced mainly by the formation of WPI-EGCG complex. Besides, the interaction between EGCG and WPI was affected by pH values and ion concentrations, since when the pH value was set as 5.0, and the $K_A$ presented the law of first decreasing and then increasing as well as $K_A$ was increased with the increase of ion concentration (0.10 ~ 0.20 mol/L), indicating the interaction between EGCG and WPI was influenced by pH value via affecting the ionization degree of Trp, Tyr, and Phe in WPI, isoelectric point, charge WPI, and $K_A$ value. However, increasing ion concentration led to protect the electrostatic repulsion, since the $K_A$ value is significantly increased when the ion concentration in the environment is increased. Suggesting the EGCG and WPI were linked mainly by electrostatic force.

### 3.3.Gel release characteristics of EGCG and WPI

A kinetic model is usually used to describe the release kinetics mechanism to analyze the release process. The ideal model for analysing the release of active ingredients in porous materials is the Korsmeyer-Peppas model (Korsmeyer et al. 1983):

$$X=Kt^n = m_t/m_0 \quad (3)$$

where , $m_0=A_{total}\frac{V_{disk}}{V_{total}}$ (4)

Whereas $X$ is the cumulative release amount of active ingredients at time $t$; $K$ is the kinetic constant; n is the diffusion constant; $m_t$ is the number of active components released at the time of $t$. $m_0$ is the maximum release amount of active components. $A_{total}$ is the total amount of active components in the gel. $V_{disk}$ is the volume of the gel block used in the release process; $V_{total}$ is the volume of EGCG-WPI solution required for gelation. The kinetic mechanism was determined by $n$ at that time $n\leq0.45$, the release mechanism of active components was consistent with the Fick diffusion mechanism; at that time,$0.45<n<0.89$, it was consistent with the non-Fick diffusion mechanism, the coexistence of diffusion and dissolution. At that time, $n\geq0.89$ followed the mechanism dominated by skeleton corrosion.

EGCG-WPI composite gels prepared under different conditions were respectively placed in phosphate buffer under identical conditions. According to Eq. (3), the EGCG cumulative release concentration changed with time was examined. As shown in (Table 3,6) the correlation coefficients $R^2$ of dynamic fitting was above of 0.90 in all tested samples, indicating a high degree of model fitting. The release rule of EGCG was similar under different conditions. thus, the release

concentration of EGCG from the gel gradually increased with the increase of release time.

**Table 3.** Characteristic parameter of EGCG release from WPI-EGCG at different pH values.

| Release medium pH | $K(\times 10^{-3})$ | $n$ | $R^2$ |
|---|---|---|---|
| **2.0** | 2.310±0.238 | 0.589±0.018 | 0.989 |
| **3.0** | 2.878±0.544 | 0.485±0.034 | 0.945 |
| **4.0** | 1.978±0.321 | 0.497±0.030 | 0.961 |
| **5.0** | 1.415±0.217 | 0.550±0.027 | 0.973 |
| **6.0** | 1.859±0.311 | 0.518±0.030 | 0.963 |
| **7.0** | 1.917±0.282 | 0.519±0.026 | 0.971 |
| **8.0** | 2.372±0.347 | 0.484±0.026 | 0.966 |
| **9.0** | 2.217±0.298 | 0.557±0.024 | 0.980 |

**Table 4**. Characteristic parameter of EGCG release from WPI-EGCG gel with drug loading.

| EGCG loading (mg/mL) | $K(\times 10^{-3})$ | $n$ | $R^2$ |
|---|---|---|---|
| **6** | 0.239±0.041 | 0.656±0.031 | 0.977 |
| **12** | 0.672±0.112 | 0.593±0.030 | 0.973 |
| **18** | 0.942±0.200 | 0.609±0.038 | 0.961 |
| **24** | 2.844±0.546 | 0.487±0.034 | 0.944 |

**Table 5.** Characteristic parameter of release EGCG-WPI composite gel at different pH values.

| Gel inside pH | $K(\times 10^{-3})$ | $n$ | $R^2$ |
|---|---|---|---|
| **3.0** | 2.878±0.545 | 0.485±0.034 | 0.945 |
| **4.0** | 2.897±0.306 | 0.431±0.019 | 0.976 |
| **5.0** | 1.627±0.190 | 0.519±0.021 | 0.981 |
| **6.0** | 1.972±0.245 | 0.488±0.022 | 0.975 |
| **7.0** | 2.160±0.198 | 0.479±0.016 | 0.986 |
| **8.0** | 2.808±0.359 | 0.468±0.023 | 0.971 |

**Table 6.** Characteristic parameter of EGCG release from WPI-EGCG gel at different ion concentrations.

| Ion concentrations. (mol/L) | $K(\times 10^{-3})$ | $n$ | $R^2$ |
|---|---|---|---|
| **0.05** | 1.478±0.232 | 0.568±0.028 | 0.973 |
| **0.10** | 1.713±0.257 | 0.552±0.027 | 0.974 |
| **0.15** | 1.821±0.281 | 0.550±0.028 | 0.972 |
| **0.20** | 2.578±0.355 | 0.494±0.025 | 0.971 |

The release fitting curve of EGCG-WPI gel prepared at a pH of 3.0 and an ion concentration of 0.05 mol/L is shown in Figure 3 (a and b). When the pH value is 2-5, the concentration of EGCG released gradually was decreased as the pH value of the medium releasing increases, while in the pH range of 6~9, the EGCG released gradually was increased as the pH of the medium releasing increases.

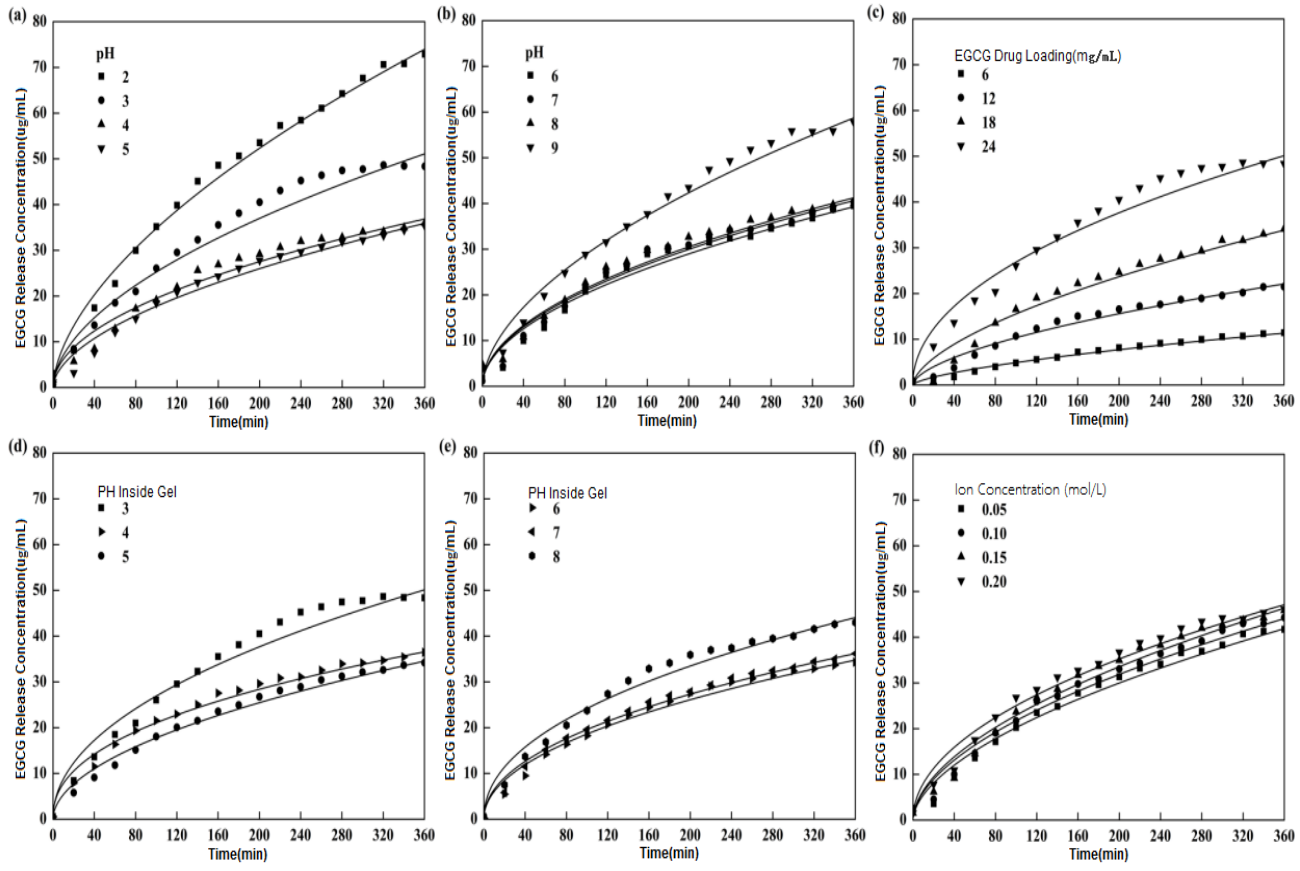


**Figure 3.** Kinetic fitting of the release of EGCG at different conditions.

Swelling is an important factor due to its effect on release system of the protein gel (Wang et al. 2008). The swelling property of the protein gel depends on the gel density and ionic charge of protein molecules (Caillard, Mateescu and Subirade 2010). In this regards, WPI gel contains a large number of carboxyl and amino groups. With the change of pH value, these groups will ionize, receive, or give protons, resulting in changes in the charge inside and

outside the gel. With high pH value, the ionization of these groups results in the formation of gel macromolecules with positive or negative charges. It makes the positive-positive or negative-negative charges on the WPI gel repel each other, and the gel expands, resulting in the release of EGCG from the gel. As shown in Figure 3C**,** when the gel EGCG level was 6mg/ml, the minimum concentration of EGCG released from the gel was 11.56mg /mL, while the loading increased to 24mg/mL, the release concentration of EGCG was 48.97mg /mL, with the increase of EGCG loading, indicated the positive correlation between the loading EGCG concentration and average releasing. due to gel swelling, the infiltration rate of water molecules into the gel is accelerated, resulting in the smooth release of EGCG (Shin et al. 2016).

At a pH 3.0, and ion concentration of 0.05mol/l, the release concentration of EGCG was the smallest (41.95 μg/mL). while, when the ion concentration increased to 0.20M, the release concentration of EGCG reached the maximum value of 48.09 μg/mL Figure 3f. It might be because the increase of ion concentration in the releasing medium increases osmotic pressure, leading to the rapid dissolution of the gel matrix and the exosmosis of water inside the gel (Jalil and Ferdous 1993), leading to the homeopathic increase of EGCG release concentration.

The relevant fitting parameters in the release process of EGCG from EGCG-WPI composite gel follows the Korsmeyer-Peppas model and conforms to the non-Fick diffusion mechanism under different pH (Table 3) and under different EGCG loading **(**Table 4**)**, release EGCG-WPI composite gel at different pH values (Table 5), and ion concentration release conditions (Table 6).

Kinetic analysis of the influence of EGCG and WPI interaction on the gel release process according to the equation:

$$LnX=LnK+nLnt \quad (5)$$

The release constant *K* and release exponent *n* of EGCG in release medium with different pH values, and ion concentrations can be calculated (Table 7 and 8), respectively.

**Table 7.** Release constant *K* and release index *n* of EGCG release from WPI-EGCG gel at different pH and ion concentrations.

| Release medium pH | $K(\times 10^{-5})$ | *n* | $R^2$ |
|---|---|---|---|
| **3.0** | 0.508±0.006 | 0.658±0.013 | 0.985 |
| **4.0** | 0.704±0.013 | 0.540±0.019 | 0.994 |
| **5.0** | 0.760±0.015 | 0.501±0.017 | 0.982 |
| **6.0** | 0.825±0.012 | 0.490±0.014 | 0.986 |
| **7.0** | 0.815±0.009 | 0.500±0.011 | 0.992 |
| **8.0** | 0.723±0.017 | 0.557±0.023 | 0.968 |

**Table 8.** Release constant *K* and release index *n* of EGCG release from WPI-EGCG gel at different ion concentrations.

| **Ion concentrations. (M)** | ***K*(×10$^{-5}$)** | ***n*** | **R$^2$** |
|---|---|---|---|
| **0.05** | 0.411±0.008 | 0.647±0.020 | 0.983 |
| **0.10** | 0.453±0.009 | 0.641±0.023 | 0.980 |
| **0.15** | 0.371±0.010 | 0.684±0.027 | 0.971 |
| **0.20** | 0.466±0.021 | 0.691±0.038 | 0.950 |

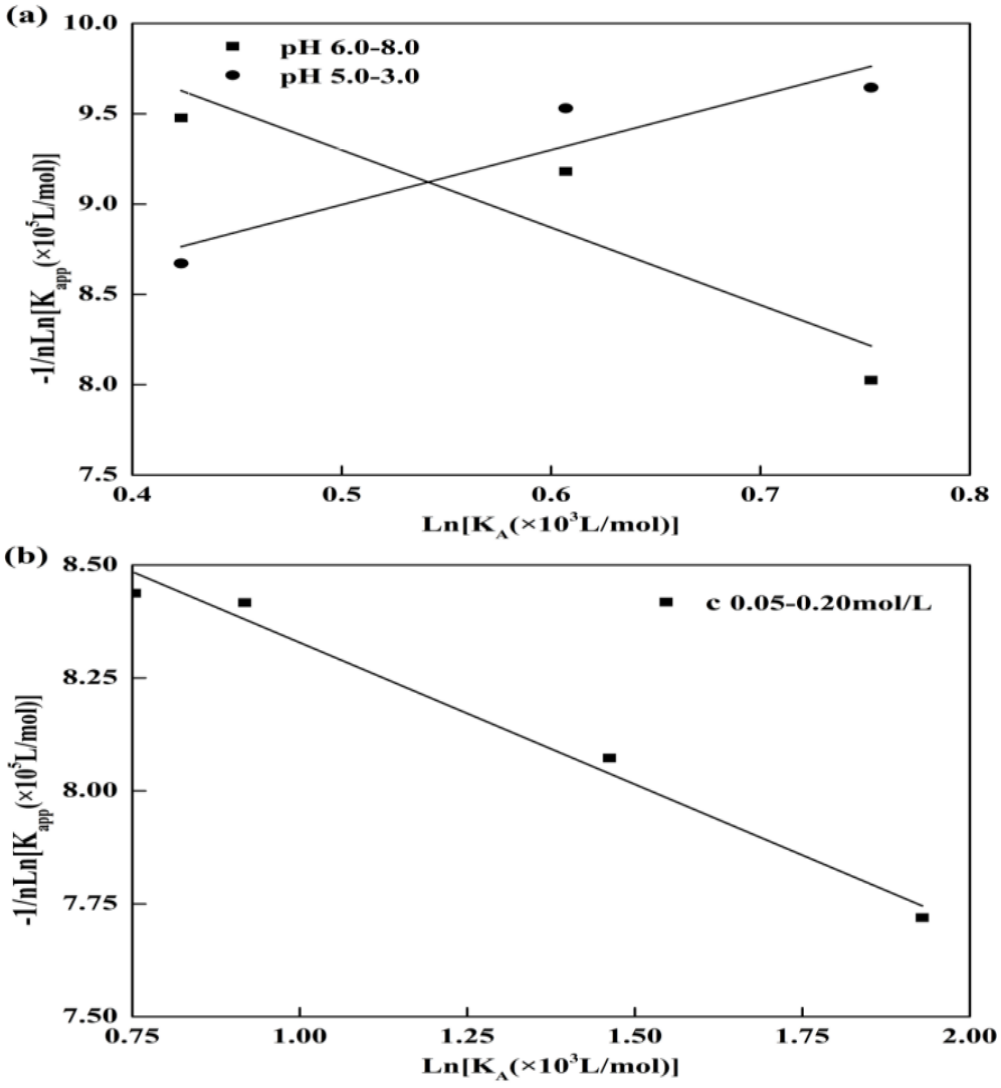


**Figure 4.** Binding constant $K_A$ dependence of the release constant $K_{app}$ at different conditions.

According to the quenching reaction

$$nQ+B \leftrightarrow Q_nB \quad (6)$$

the instantaneous equilibrium equation can be obtained

$$[Q_nB]=K_A[Q]^n[B_0] \quad (7)$$

According to the mass transfer equation of the existing chemical reaction process, the following equation can be obtained:

$$\frac{\partial C}{\partial t}=D\frac{\partial^2 C}{\partial x^2}-\frac{\partial}{\partial t}[Q_nB]=D\frac{\partial C^2}{\partial x^2}-K_An[B_0][Q]^{n-1}\frac{\partial}{\partial t}[Q \quad (8)$$

According to equation (3), we get

$$X=K_{app}t^n \quad (9)$$

$$K_{app}=K\left[\frac{D}{1+K_AD}\right]^n \quad (10)$$

Where, $K_{app}$is the apparent release constant, and $D$ is the mass transfer coefficient, while n≈1,

$$\frac{1}{K_{app}}=\frac{1}{K}\left[\frac{1}{D}+K_A\right] \quad (11)$$

$$\frac{1}{K_{app}}\propto K_A \quad (12)$$

Simultaneous Eq. (5) and (11) that is a dynamic model equation:

$$LnK_A=A-\frac{1}{n}LnK_{app} \quad (13)$$

Formula $LnK_A$ a $-\frac{1}{n}LnK_{app}$ to obtain the relationship between the interaction binding constant $K_A$ and the apparent release constant$K_{app}$ Figure 4a. It can be seen from Figure 4a that in the range of pH value 5.0 ~ 3.0, with the decrease of pH value, $LnK_A$ and $-\frac{1}{n}LnK_{app}$is in direct proportion. In the range of pH value 6.0 ~ 8.0 and ion concentration 0.05 ~ 0.20mol/L, with the increase of pH value and ion concentration, $LnK_A$ and $-\frac{1}{n}LnK_{app}$ represent the inverse proportional relationship Figure 4b.

The binding constant $K_A$ reflects the degree of EGCG binding with protein which has correlation with degree of EGCG binding with protein. When the pH value was 3.0 ~ 5.0, $K_A$ *was* decreased as the pH value increased, also the released EGCG from the gel was increased. Whereas, at pH values 6.0 ~ 8.0, as the pH value increases, $K_A$ *was* increased, and EGCG release from the gel was decreased, due to electric point of WPI, which is close to the mentioned pH value, leading to decrease in surface charge of WPI molecules, and the binding degree between the WPI molecules and EGCG was weakened, thus increase of the released EGCG. On the other hands, $K_A$ was increased with the increase of ion concentration, while the released EGCG was decreased. Increasing ion concentration, resulting in an increase in the binding degree between EGCG and WPI.

## 4.Conclusion

Briefly, the results of this study showed that reaction between EGCG and WPI was occurred mainly on the electrostatic interaction. The release process of EGCG confirmed Korsmeyer-Peppasmodel from EGCG-WPI gel. Furthermore, a kinetic model of $K_A$ and $K_{app}$ between EGCG and WPI at different pH values and ion concentrations was established, which was consistent with the kinetic equation $LnK_A=A-\frac{1}{n}LnK_{app}$. However, our results provided a further theoretical basis for applying WPI as functional ingredients in the food industry, especially in the development of novel edible gel.

The fitting curve of EGCG-WPI gel prepared at 0.05 mol/L ion concentration and different pH values was released into phosphate buffer release medium under identical conditions, as shown in Figure3(d and e). According to mentioned results in this study, the release concentration of EGCG was the smallest in the release process of gels prepared at pH values of 5.0 and 6.0, respectively 35.15 and 34.89 μg/mL. During the release of gels prepared at pH values of 3.0 and 8.0, EGCG was released at the highest concentrations of 48.97 and 44. 99μg/mL, respectively.

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# STUDY OF CYTOTOXIC AND ANTIBACTERIAL POTENTIAL OF VARIOUS VARIETIES AND POLARITIES OF EXTRACTS OF UNRIPE BANANAS

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**ABSTRACT**

Different ethnic communities traditionally use herbal medicines to treat different curable and incurable diseases. The main purpose of this research was to assess the antibacterial and cytotoxic potential of different polarities extracts of unripe bananas collected from Omani farmers. The antibacterial and cytotoxic potential of the prepared extracts were assessed against the gram + and - antibacterial strains and brine shrimp lethality (BSL) bioassays. All prepared extracts at dissimilar concentrations exhibited acceptable antibacterial potential against two Gram-positive and two Gram-negative bacteria strains with a diameter of inhibition range of 0-11.5 mm. The maximum antibacterial activity among the plant extracts was obtained from hexane extract against *E. coli*. The cytotoxic results exposed that all polarities unripe banana extracts at each concentrations lethaled the shrimp larvae at all applied concentrations. Among the six polarities extracts of unripe banana from Sohar and Dhofar, the butanol and hexane extracts exhibited significant cytotoxic potential with an $LC_{50}$ value of 20.12±0.10; 26.19±0.57 and 22.39±0.11; 27.88±0.17 µg/ml. The lesser potential found in ethyl acetate extract among the six extracts with an $LC_{50}$ value of 36.68±0.22 and 49.32±0.16 µg/ml. The results displayed that non-polar unripe banana extract has substantial antibacterial and cytotoxic potential. Therefore, the significant extract from the unripe banana can be used further for isolation of biologically active ingredients against a number of ailments.

## 1.Introduction

Plants are considered as an alternative source of antibiotics or chemotherapy agents (Marjorie, 1999). They play a significant role in drug discovery due to their medicinal values to prevent diseases (Marjorie, 1999; Adinarayana & Babu, 2011). Some of them were already detected and characterized to be the best source of antibacterial products (Cintia et al., 2013). Among them, many species all over the world still remain unexplored (Cintia et al., 2013). In most of the cases, the less developed countries rely only on plant based medicine, which is available locally as a primary form of the health care system. All plant based medicines spread quickly in the form of herbal safe medicine in developed countries due to their active ingredients (Ahmed et al., 2016). Plants, fruits, vegetables and their prepared extracts are more

active than chemically prepared medicines with limited side effects (Al-Matani et al., 2015). Several evidences suggested that increased consumption of fresh unripe and ripe fruits have a lower risk of cancer (Chen et al., 2004). Several chemical ingredients like phenol derivatives, heterocyclic nitrogenous compounds and cardiac glycoside derivatives are vital part any diet (Latifa & Hossain, 2019). In addition, the mentioned groups of ingredients show a variety of pharmacological activities. The pharmacological and biological functions in the human body are directly related to the interference in different stages of malignancy (Debabandya et al., 2010). Bananas are the most popular fruits available everywhere based on nutritive values. Numerous varieties and species are cultivated in different countries including Oman. Banana is a rich nutritional source belonging to Musaceae family. It is originated in Southeast Asia and the South Pacific around 8000 to 5000 BC. From the literature, this fruit is one of the oldest cultivated crops (Khair, 2013). Long-time, most tropical countries commercially produce the fruit on a regular basis. Banana is used as an important source of instant energy as well as an important element of a healthy diet all over the world (Khair, 2013). It is the fourth cultivated agricultural crops and most priority traded crops in the world market (Khair, 2013).

The banana plant is about 12 to 15 feet in height and the leaves are spirally arranged. The whole plant:stems, leaves and fruit have significant medicinal values. There are numerous literature data showing that banana extracts are a rich source of different acids such as fatty acids, linoleic, linolenic, mannose and oleic acids and other active compounds including sterols and steryl esters, fructose, xylose, galactose and glucose (Liu, 2004; Mallikarjuna & Jyothirmay, 2011; Natcharee & Sudip 2011). Recently, some other rare ingredients have been found in bananas, which are responsible for biological activities (Nessma, 2015; Oliveira et al., 2008). Currently, researchers have found that chemical ingredients existing as a mixture in the extracts of banana are pharmacologically more significant than the individual ingredients to protect malignance due to its synergistic effect (Pereira & Maraschin, 2015). Traditionally, the stem juice of the plant is used to treat acute diarrhea and dysentery, epilepsy and hysteria (Oliveira et al., 2008). The flower extract of the plant is used to treat acute bronchitis, dysentery and ulcers (Liu, 2004; Mallikarjuna & Jyothirmay, 2011; Natcharee & Sudip 2011) and the syrup of cooked flowers is used for acute dysentery and diarrhea, fever, diabetes, epilepsy, leprosy, hysteria and hemorrhages (Liu, 2004; Mallikarjuna & Jyothirmay, 2011; Natcharee & Sudip 2011). The paste made of leaves is used for the treatment of burns, various infections, dysentery, diarrhea and ulcers (Liu, 2004; Mallikarjuna & Jyothirmay, 2011; Natcharee & Sudip 2011). In addition, the paste made of roots is used for the treatment of dysentery and other chronic diseases (Liu, 2004; Mallikarjuna & Jyothirmay, 2011; Natcharee & Sudip 2011). In Oman, local communities use different herbal preparations from bananas for the treatment of indigestion, constipation and diarrhea. Still, there is no preliminary work conducted on the unripe local species. Therefore, it is necessary to determine the antibacterial and cytotoxic activities of unripe bananas that are available in Oman. In this regard, this study was to prepare the crude extracts from different varieties and determine the antibacterial and cytotoxic activities of the prepared extracts.

## 2.Materials and methods

### 2.1. Materials

All solvents and chemicals such as hexane (purity 98.3%), ethyl acetate (purity 96.9%), butanol (purity 99.1%), methanol (purity 95.55%), chloroform (purity 89.45%) and acetone (purity 97.01%) were bought from Sigma Aldrich Company, Germany. The reagents and chemicals for this experiment, especially NaCl and $Na_2SO_4$ were bought from BDH, UK. The broad spectrum antibiotic levofloxacin as well as dimethyl sulphoxide (DMSO) were bought from E. Merck, Germany.

In this experiment, all the glassware used were from Brosil, India.

### 2.2. Microorganisms

Two gram-positive antibacterial strains such as *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneunoniae* (*S. pneunoniae*), and two gram-negative *Escherichia coli* (*E. coli*) and *Haemophilus influenza* (*H. influenza*) used in this experiment were obtained from local Hospital, Nizwa, Oman in January 2017.

### 2.3.Sample collection

The unripe bananas were collected from gardens in Dhofar and Sohar regions, Oman. The samples were collected from farmers in December 31, 2016. The samples were carried home for the necessary steps of extraction. The identification of unripe bananas was done by the local people and match with the website (Latifa & Hossain, 2019).

### 2.4.Preparation of extracts

The selected unripe banana samples of approximately the same size were cleaned by water and sliced for drying. The sliced fruit was dried under the sun for 5 days. During the drying, the samples were turned over every day to avoid antibacterial contamination. After completely dried, the samples were ground into a coarse powder. The coarse powder sample (350 gm) was packed in white cloth and kept into the two-liter capacity amber beaker for extraction. Methanol (1 L) was added to the beaker and left it for 48 hours. After 48 hours, the coarse powder samples were filtered by vacuum filter and the filtrate was evaporated at 22$^{o}$C. The methanol free extract (20 gm) was liquefied in water and fractionation with different polarity of solvents starting from non-polar hexane (Rao et al., 2012) to give the corresponding extracts. The extraction process was repeated twice and the mother solvent was evaporated from each extract by using the same way and all dried organic extracts were used to assess the antibacterial and cytotoxic potential.

### 2.5.Antibacterial potential

The six extracts with different polarity were used to determine their antibacterial potential against two Gram positive bacteria: *S. aureus* and *S. pneunoniae* and two Gram negative bacteria: *E. coli* and *H. influenza* by using modified gel diffusion assay (Rao et al., 2016; Reinisalo et al., 2015). Both antibiotic levofloxacin and DMSO solvents were used as controls. The concentration of levofloxacin was 0.5 mg/ml in DMSO. Filter paper (Whatmann) as disc (diameter 6 mm) was used in the present study, which was prepared by a punch machine. The six polarities extracts at four concentrations such as 2, 1, 0.5 and 0.25 mg/ml were used to assess the antibacterial potential. The discs were initially disinfected and soaked for 30 minutes with each concentration of extract and apply on the inoculated agar plates. All plates with samples were hatched at 37$^{o}$C for 24 hours (Latifa & Hossain, 2019). The zone of inhibition as diameter was measured by scale against the tested antibacterial strains. It was repeated three times for average zone of inhibition. The antibacterial potential was evaluated of each concentration of all extracts by using the reputable formula.

Antibacterial potential = Diameter zone of inhibition of the sample/Diameter zone of inhibition of the standard.

### 2.6.Cytotoxic potential

The cytotoxic potential of the extracts was analyzed by the BSL method reported (Latifa & Hossain, 2019). The artemia cysts were incubated in a duo compartment plastic container containing artificial seawater (250 ml) for 24 hours. After 24 hours hatching, the live nauplii were transferred from one compartment to the opposite compartment. In this present experiment, those live nauplii were used to determine cytotoxic potential. six extracts at various concentrations (10, 100, 250, 500 µg/ml) were prepared by using $H_2O$. Each concentration of each solution, 100 µl samples were placed in the working tube containing 4.9 ml of artificial seawater with 10 live nauplii. After incubation, the surviving nauplii were calculated using a

powerful glass. The percentage of lethality of brine shrimps as well as $IC_{50}$ was calculated for each test sample by using Microsoft Excel.

## 3.Results and discussions

The banana samples were collected from Sohar and Dhofar, Oman where bananas are cultivated on a large scale. Afterwards, the unripe samples were sliced and kept under the sun for 5 days. The dried banana samples were ready for grinding.

### 3.1. Preparation of different extracts

The powder samples were used to prepare various polarity extracts by using a maceration method for several days. The prepared extracts and their mass were presented in Table 1.

**Table 1.**Yield of crude extracts of Sohar and Dhofar's unripe banana

| Extracts | Yield of extracts (gm) | | Percentage yield of extracts (%) | |
|---|---|---|---|---|
| | Sohar | Dhofar | Sohar | Dhofar |
| Hexane | 5.28 | 4.35 | 26.40 | 21.75 |
| Ethyl acetate | 4.22 | 3.82 | 21.10 | 19.10 |
| Chloroform | 3.19 | 5.13 | 15.95 | 25.65 |
| Butanol | 2.97 | 3.11 | 18.85 | 15.55 |
| Methanol | 38.7 | 13.91 | 11.05 | 6.98 |
| Water | 3.82 | 3.85 | 19.10 | 19.25 |

The values are means ± S D of three replicates

### 3.2.Antibacterial potential

The antibacterial potential of each variety and each polarity extract of unripe banana was determined by the diffusion method reported (Rao et al., 2016; Reinisalo et al., 2015). The potential of various polarities extracts was determined through the gel diffusion method against Gram (+ and -) antibacterial strains. Each extract of samples from both regions at four different concentrations was used to evaluate the antibacterial potential. All extracts of unripe bananas displayed different ranges of zones of inhibition and the results were presented in Adinarayana. Among the gram (+ and -) antibacterial strains, all gram (-) antibacterial strains gave more zone of inhibition compare to gram (+) antibacterial strains. On the other hand, non-polar extract gave the highest inhibition compared then other extracts (Table 2).

### 3.3. Cytotoxic potential

All six extracts with different polarities showed substantial cytotoxic potential against the artemia cysts reported by several authors (Rehab & Hossain, 2016). The percentage of mortality (%) and $IC_{50}$ values are shown in Table 3. Among the extracts with different polarities, the non-polar hexane extract gave more $IC_{50}$ compared to other extracts. On the other hand, non-polar extract gave the highest inhibition compared then other extracts (Table 3).

### 3.4.Discussion

Since old times, the world population has been using different herbal therapies as a safe and primary health care system to treat diverse diseases. Researchers are working on the available natural resources to search for pharmacologically active compounds, specially antibiotics and anticancer drugs. The selected banana crop is one of the most valuable nutritional agricultural crops. It gives us instant energy and has several medicinal benefits. Banana is mainly used to treat indigestion, hypertension, constipation, GI problems and diarrhea (Liu, 2004; Mallikarjuna & Jyothirmay, 2011; Natcharee & Sudip 2011; Latifa & Hossain, 2019; Serafino et al., 2008; Weli et al., 2014). Omani communities also use the fruit to treat these problems. In this context, this present study, we intend to assess the antibacterial and cytotoxic potential of various species of local unripe banana (Sohar and Dhofar).

The antibacterial potential of six extracts varied polarities at four numerous concentrations from samples from both areas was determined against four Gram (+ and -) antibacterial strains. All culture antibacterial strains were available in our laboratory. The experimental results from the six different extracts showed that all six extracts give reasonable antibacterial potential against the applied antibacterial strains at varied concentrations in the range of 0-11.5 mm.

**Table 2.** Antimicrobial activity of different crude extracts from Sohar and Dhofar unripe banana samples

| **Bacteria** | **Extract** | **Hexane** | | **Chloroform** | | **Ethyl acetate** | | **Butanol** | | **Methanol** | | **Water** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Conc.** (mg/ml) | **Sohar (mm)** | **Dhofar (mm)** | **Sohar (mm)** | **Dhofar (mm)** | **Sohar (mm)** | **Dhofar (mm)** | **Sohar (mm)** | **Dhofar (mm)** | **Sohar (mm)** | **Dhofar (mm)** | **Sohar (mm)** | **Dhofar (mm)** |
| *E. coli* (Code no. 337) | 2 | 11±0.12 | 10.5±0.56 | 13±0.14 | 9±0.42 | 9±0.12 | 7.5±0.55 | 7±0.15 | 9±0.20 | 7±0.12 | 8±0.17 | 7±0.13 | 7.5±0.07 |
| | 1 | 11±0.43 | 10±0.23 | 8±0.11 | 8.5±0.33 | 8±0.10 | 7±0.18 | 7±0.43 | 8.5±0.07 | 8±0.23 | 7.5±0.10 | 6±0.90 | 7±0.21 |
| | 0.5 | 9.5±0.89 | 7±0.78 | 8±0.09 | 6.5±0.41 | 7.75±0.11 | 6.5±0.23 | 7±0.55 | 8±0.65 | 7.15±0.15 | 6.5±0.15 | 7±0.10 | 6.5±0.57 |
| | 0.25 | 9±0.15 | 6.5±0.23 | 7±0.16 | 6±0.10 | 9±0.07 | 6±0.32 | 7±0.08 | 7±0.14 | 7±0.10 | nd | 6±0.19 | 6±0.16 |
| Control | 3 | 12±0.32 | 8±0.47 | 30±0.10 | 25±0.16 | 11±0.23 | 29±0.21 | 10±0.25 | 9±0.18 | 7±0.10 | nd | 9±0.12 | 22±0.13 |
| *H. influenza* (Code no. 236) | 2 | 7±0.76 | 8±0.28 | 8±0.19 | 7.5±0.19 | 8±0.15 | 7.5±0.10 | 8±0.18 | 7.5±0.15 | 8±0.32 | 8.5±0.09 | 9±0.15 | nd |
| | 1 | 6±0.19 | 7.5±0.26 | 6±0.22 | 7±0.31 | 8±0.78 | 7±0.19 | 8±0.42 | 7.15±0.11 | 7±0.42 | 8.15±0.54 | 9±0.08 | nd |
| | 0.5 | nd | 7±0.55 | 8±0.41 | 6.5±0.64 | 7.5±0.52 | 6.5±0.11 | 7±0.08 | 7.15±0.17 | 7±0.12 | 8±0.22 | 6±0.10 | nd |
| | 0.25 | nd | 7±0.16 | 8±0.23 | 6.5±0.55 | 7±0.10 | 6.5±0.45 | 6±0.09 | 7±0.19 | nd | nd | 6±0.54 | nd |
| Control | 3 | 33±0.18 | 26±0.21 | 28±0.32 | 27±0.14 | 27±0.10 | 27±0.56 | 28±0.23 | 27±0.09 | 29±0.15 | 33±0.32 | 30±0.87 | 25±0.23 |
| *S. aureus* (Code no. 207) | 2 | 6±0.90 | 8±0.12 | nd | 9±0.17 | 6±0.55 | 10±0.23 | 8±0.27 | nd | 8±0.10 | 10±0.10 | 8±0.10 | nd |
| | 1 | 0±0.39 | 7±0.82 | nd | 8.5±0.29 | nd | 9±0.91 | 6±0.14 | nd | 6±0.35 | 8±0.15 | 7±0.10 | nd |
| | 0.5 | 0±0.55 | 7±0.29 | nd | 6.5±0.18 | nd | 9±0.12 | 6±0.17 | nd | 6±0.34 | 7.5±0.19 | nd | nd |
| | 0.25 | 0±0.72 | 6±0.65 | nd | 6±0.15 | nd | 9±0.16 | 6±0.19 | nd | 6±0.18 | nd | nd | nd |
| Control | 3 | 24±0.15 | 31±0.34 | 26±0.17 | 29±0.10 | 29±0.23 | 29±0.10 | 27±0.10 | 25±0.89 | 27±0.13 | nd | 29±0.16 | 29±0.13 |
| *S. pneunoniae* (Code no. 257) | 2 | 8±0.82 | 7.5±0.41 | 7±0.34 | 7.5±0.06 | 7±0.11 | 8±0.22 | nd | 7±0.10 | 8±0.21 | 7±0.11 | nd | 7±0.09 |
| | 1 | 7±0.24 | 7±0.23 | 7±0.14 | 7±0.18 | 6±0.18 | 6.15±0.10 | nd | 6.75±0.22 | 7±0.19 | 7±0.10 | nd | 6.5±0.10 |
| | 0.5 | 6±0.12 | 6.5±0.24 | 6±0.10 | 6±0.52 | 6±0.25 | 6±0.09 | nd | 6.5±0.37 | 7±0.10 | nd | nd | nd |
| | 0.25 | 0±0.12 | 6.5±0.27 | nd | 6±0.21 | 6±0.72 | 0±0.65 | nd | 6±0.13 | nd | nd | nd | nd |
| Control | 3 | 31±0.12 | 27±0.56 | 27±0.18 | 34±0.32 | 33±0.12 | 40±0.15 | 31±0.22 | 34±0.17 | 26±0.12 | 30±0.17 | 30±0.12 | 26±0.19 |

nd= not detected; Each value is a mean of three biological replicates

**Table 3**. Percentage of mortality and lethal concentration ($IC_{50}$) of different polarities Sohar and Dhofar unripe banana samples

| Crude extract | Conc | Mortality (%) | | $LC_{50}$ (µg/ml) | |
|---|---|---|---|---|---|
| | µg/ml | Sohar | Dhofar | Sohar | Dhofar |
| Hexane | 500 | 100 | 100 | | |
| | 100 | 80 | 80 | 22.39±011 | 27.88±017 |
| | 50 | 60 | 50 | | |
| | 10 | 30 | 10 | | |
| | Control | 0 | 0 | | |
| Chloroform | 500 | 100 | 100 | | |
| | 100 | 70 | 80 | 29.74±0.14 | 29.16±0.10 |
| | 50 | 50 | 40 | | |
| | 10 | 20 | 20 | | |
| | Control | 0 | 0 | | |
| Ethyl acetate | 500 | 100 | 100 | | |
| | 100 | 60 | 50 | 36.68±0.22 | 49.32±0.16 |
| | 50 | 50 | 40 | | |
| | 10 | 10 | 10 | | |
| | Control | 0 | 0 | | |
| Butanol | 500 | 100 | 100 | | |
| | 100 | 90 | 80 | 20.12±0.10 | 26.19±0.57 |
| | 50 | 60 | 50 | | |
| | 10 | 30 | 20 | | |
| | Control | 0 | 0 | | |
| Methanol | 500 | 100 | 100 | | |
| | 100 | 80 | 70 | 29.15±0.18 | 27.86±0.23 |
| | 50 | 40 | 50 | | |
| | 10 | 20 | 30 | | |
| | Control | 0 | 0 | | |
| Water | 500 | 100 | 100 | | |
| | 100 | 80 | 50 | | |
| | 50 | 50 | 40 | 27.94±0.25 | 24.22±0.18 |
| | 10 | 10 | 20 | | |
| | Control | 0 | 0 | | |

Each value is a mean ±SD of three biological replicates

Among the six unripe banana extracts, only the chloroform extract showed the maximum potential in the samples collected from Sohar against *E. coli* at 2 mg/ml. The other five extracts from both regions displayed average potential against *E. coli* at several varied concentrations. Conversely, the water extract from Dhofar did not show potential against both *H. influenza* and *S. aureus* at any prepared concentrations. In addition, the chloroform extract from Sohar banana also did not show any potential against *S. aureus* at any prepared concentrations. It means that the antibacterial activity of the plant extracts largely depends on numerous factors such as chemical ingredients, doses of extract, sensitivity of the antibacterial strains and the types of antibacterial strains used in the experiment. Our experiment results indicated that all six extracts varied polarity gave dissimilar potential that means not all

polarities extracts from both types of unripe bananas contain enough number of chemical ingredients that can actively participate for antibacterial potential. Several other related reports available on antibacterial potential of various polarities extracts showed that the unripe banana extracts gave reasonable activity against almost all gram (+ and -) antibacterial strains (Latifa & Hossain, 2019). The obtained results also indicated that not all six polarities extracts are significant against the applied gram (+ and -) antibacterial strains. The reason for variation of antibacterial results could be the chemical ingredients in the extracts or the sensitivity of the applied microbes. On the other hand, the cytotoxic potential of all six polarities banana extracts did not kill all tested nauplii (mortality 100%) at the concentration of 500 ug/ml. The mortality (%) of our experiment for all six polarities extracts at all prepared concentrations was given in Table 3. The maximum cytotoxic potential was obtained in butanol extract from Sohar and the water extract of Dhofar, but less potential was obtained in ethyl acetate extract. In this present experiment, it indicates that there was a significant correlation between the concentration and mortalities. The mortality (%) is increased with the increasing concentrations of the banana extract. Our results are completely different from what has been reported on various polarity extracts of unripe banana samples collected from elsewhere (Serafino et al., 2008; Weli et al., 2014; WHO, 1993; Yusoff & Adlin, 2008; Zafar et al., 2011; Al Alawi et al., 2018). The variation of mortality (%) and $LC_{50}$ value could have been caused by the differences in the procedures for evaluating cytotoxicity. In our present study, BST assay was used however, other investigators used *in-vitro* or *in-vivo* based assay.

## 4.Conclusions

The aim of this current study was to evaluate the antibacterial and cytotoxic potential of unripe bananas from Sohar and Dhofar by gel diffusion and BSL bioassays. Six different polarities extracts showed substantial antibacterial potential against the Gram (+ and -) antibacterial strains. In addition, all six polarities extracts also indicated moderate cytotoxic potential against BSL assay. The maximum potential extracts can be used as a natural safe medicine to treat different human ailments. Further extensive studies will be planned for the isolation, characterization and evaluation of the pharmacologically active pure ingredients and their diverse action.

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# COMPARING PHYSICOCHEMICAL, NUTRITIONAL AND BIOCHEMICAL PROPERTIES OF THREE TRADITIONAL FIG VINEGARS PRODUCED IN ALGERIA

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**ABSTRACT**

Several fruits, including figs, can be used to produce vinegar, which has been recognized for its therapeutic uses. However, limited studies are available on Algerian fig vinegar production and properties, despite increased research interest in this product. The aim of this study was to compare the physicochemical, nutritional and biochemical properties of three vinegars produced from three fresh fig varieties of Beni-Ourtilane region (Setif - Algeria). Various physicochemical parameters (pH, conductivity, °Brix, etc.), nutritional properties (proteins, sugars, and lipids), and biochemical parameters (acetic acid content and alcohol) were measured. The traditional homemade vinegars produced had alcohol contents below 1% in accordance with the Codex Alimentarius standards, while the levels of acetic acid ranged between 1.05 and 2.79 °, not meeting the required standards. The chemical properties including pH value, conductivity, °Brix, moisture, dry extract, density, ash and organic matter of vinegar samples were determined as 3.9 to 4.05, 392 to 451 µS/cm, 7.6 to 10.2%, 90.29 to 93.58%, 6.42 to 9.71%, 1.01 to 1.02, 0.09 to 0.20% and 6.32 to 8.9%, respectively. Furthermore, the energy intake from the three vinegar samples was not significant as the values obtained varied between 3.54 and 4.47 Kcal per 100g of product. In conclusion, the homemade vinegars produced from three varieties of fresh figs exhibited comparable physicochemical, nutritional and biochemical properties and this transformation constitutes a new way to utilize the fruit of the fig tree.

## 1.Introduction

The fig (*Ficus carica*) is the fruit of the fig tree cultivated since the dawn of time by humans, especially in warm, dry climates and can be eaten fresh, dried (peeled or unpeeled) or as jam. Many studies show that figs are an important source of nutrient such as minerals, vitamins and dietary fiber; they are fat, sodium and cholesterol-free and contain a high number of amino acids (Solomon *et al.*, 2006; Veberic *et al.*, 2008) and bioactive molecules (Shahidi *et al.*, 2008). However, figs have a limited post-harvest shelf life, which can cause important economic losses. So, to increase the shelf life of figs, they can be dried or processed to vinegar for obtaining very special taste for flavouring. Vinegar is a liquid, fit for human consumption, produced exclusively from suitable products containing starch and/or sugars by the process of double fermentation, first alcoholic and then acetic. Acetic acid, the principal organic component of vinegar, is known for its

preservative and flavoring properties (Sholberg *et al.*, 2000). Vinegar may contain optional ingredients such as herbs, spices, fruit and honey. According to Budak et al. (2014), the Babylonians produced and sold vinegars flavored with fruit, honey, and malt until the 6$^{th}$ century. References in the Old Testament and from Hippocrates indicate vinegar was used medicinally to manage wounds. Vinegar has many health promoting effects as antimicrobial, satietogenic effect, hypolipidemic, hypoglycemic and seems to prevent atherogenic risk (Shishehbor *et al.*, 2008; Beheshti *et al.*, 2012). Several fruits can be used to produce vinegar since they all contain sugar. The traditional production of fig vinegar is based on a spontaneous fermentation. This spontaneous fermentation occurs generally for 6 to 14 weeks, according to the ambient temperature, until desired acidity (at least 4%, w/v) and flavour is obtained. The dominant component in vinegar is acetic acid. It is commonly known that the production of fig vinegar is not an easy work. Low acidic value of fig fruit (0.18 to 0.48%, w/v) provides a suitable condition for uncontrolled microbial growth during fermentation process (Sengun, 2013). To the best of the authors' knowledge, there has been no study done regarding the properties of Algerian traditional fig vinegar. Hence, the objective of this study was to investigate the physicochemical, nutritional and biochemical properties of homemade fig vinegar produced traditionally. For this, three varieties of fresh fig (Thaamriwth, Aberkan and Azandjar) from the region of Béni-Ouartiène, District of Sétif (Algeria) known for its fig culture, were chosen for this study to produce Thaamriwth vinegar (V1), Aberkan vinegar (V2) and Azendjar vinegar (V3).

## 2. Materials and methods

### 2.1.Materials

#### *2.1.1.Samples*

The fig fresh varieties used were harvested from the region of Béni-Ouartilan (Sétif) at august 2018. The varieties are Thaamriwth (green variety), Aberkan and Azandjar (purple variety) (Fig 1). For each variety, 1kg was taken for vinegar preparation.


**Figure 1.** The three fresh fig used for preparation of vinegar; a): Aberkan variety; b): Thaamriwth variety; c): Azandjar variety

#### *2.1.2. Vinegar preparation*

After washing, sorting and crushing the figs, they are fermented in appropriate containers according to the modified adapted diagram of Sungen (2013) (Fig 2). Traditional vinegar production is based on a double aerobic and anaerobic combined spontaneous fermentation. This bioconversion uses yeasts and acetic bacteria naturally present in the fig. These lead to the production of ethanol which is converted into acetic acid. The first fermentation stage (alcohol production), which took place under anaerobic conditions, lasted 15 days at room temperature. During this stage, the sugars of the figs were transformed into ethanol by the action of yeasts. The second stage (production of acetic acid), which took place under aerobic conditions lasted 30 days at room temperature. During this stage, the alcohol was converted into acetic acid by the action of acetic bacteria. After fermentation, vinegars produced were directly analyzed.

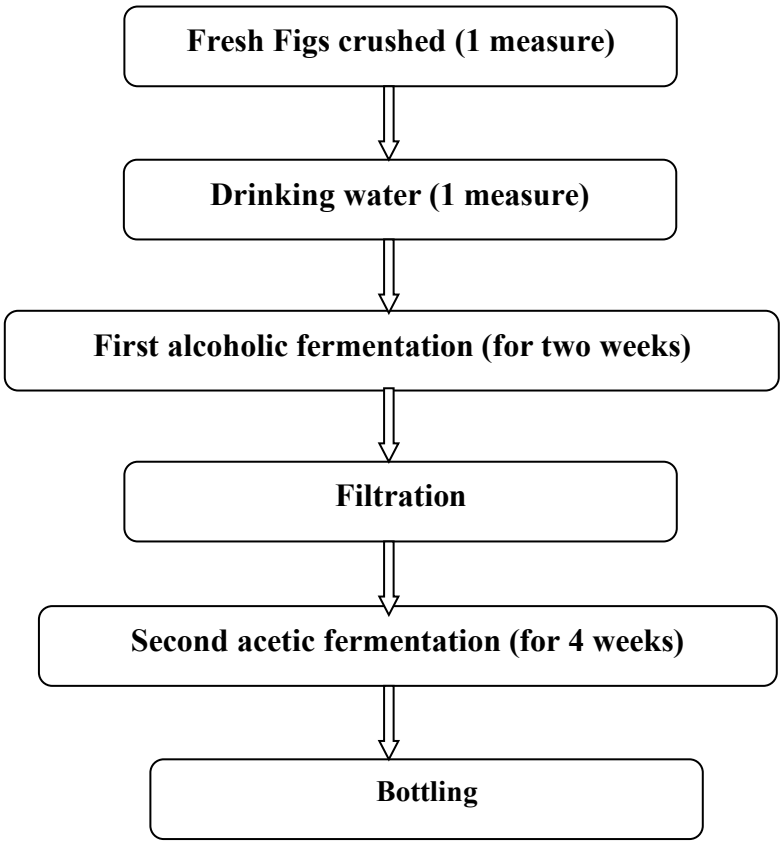


**Figure 2.** Traditional vinegar manufacturing diagram

The vinegars produced were studied to determine their physicochemical, nutritional and biochemical quality by applying protocols using classical methods. All analyses were conducted after the fermentation process.

## 2.2 Vinegar analysis

### *2.2.1. Physicochemical parameters*

The pH value of fig vinegar samples was measured by using previously calibrated pH-meter, while conductivity was measured by electrometry according to the Algerian Standard (NA 749), results were expressed as µS/cm. Brix degree (°Brix) of the samples were measured using refractometer at 20 °C and results were reported as percentage. Moisture percentage was estimated according to the method described in the Algerian Standard (NA 1132). Ash content was determined by incineration of the sample at 525°C to constant weight according to the international standard (ISO 2171) and the results were expressed as percentage. As for the dry residue, it was estimated after drying vinegar samples in an oven (105 °C) to constant weight, whereas the vinegar density was estimated using a densimeter. The organic matter content was calculated knowing the moisture and the ash content.

### *2.2.2 Nutritional parameters*

It is the determination of the rate of the energy elements content in vinegar. The dosage of the sugars was carried out according to the *Bertrand* method. Its principle is based on the reduction of CuO (cupric oxide) into small brick red grains-$Cu_2O$ (cuprous oxide). A table gives the correspondence between the mass of copper and the mass of glucose. The result was expressed as g / 100g. The protein content was estimated using the *Kjeldahl* method, which is based on the mineralization of the sample in sulfuric acid with the presence of a catalyst. During this process, the organic nitrogen in the sample is converted into ammonium. Subsequently, in an alkaline medium, the

ammonium ions are converted into ammonia. The ammonia is then released by steam and quantified using acid / base volumetric titration. The fat was determined by weighing after hot extraction with petroleum ether as solvent by Soxhlet. The result was expressed as g/100g of product. finally, energy intake is determined by the contribution of each of these elements

***2.2.3 Biochemical parameters***

It's about the determination of the two parameters characterizing a vinegar; the alcohol level, which was obtained by directly reading the alcohol content using an alcoholimeter after distillation. The result was expressed as percentage. Additionally, the acidic acid content was determined through titration, using a strong base (NaOH) to titrate the weak acid (acetic acid). The result was expressed as acetic degree.

**2.3. Statistical analysis**

Correlation among physicochemical fermentation parameters was analyzed by Pearson's correlation using the Excel 2007 software.

**3.Results and discussions**

**3.1. Physicochemical Properties of homemade fig vinegars**

Table 1 summarizes the results related to physicochemical parameters. There are a few studies considering these parameters for different kinds of vinegars, especially on the fig vinegar. From Table 1, pH values ranged from 3.9 to 4.05. This low pH makes vinegar a product with antimicrobial properties that make it useful for a number of applications. Vinegar is considered as disinfectant product since the ancient Greece era, it has been commonly used as an antifungal and antimicrobial element because of its very low pH and the presence of acetic acid as a major component (Ali *et al.*, 2017). In the work of Sengun (2013), the pH of fig vinegar produced in Turkey ranged from 3.05 to 3.73. the recorded values in the present study are slightly higher than those reported by Sengun (2013). This difference can be attributed to the vinegar preparation process, specifically, fermentation time. It has been observed that vinegars with longer fermentation times tend to have highly acidity, resulting in lower pH levels. However, the pH obtained makes it possible to inhibit the development of pathogenic microorganisms and to permit a good preservation of the product. Some other researchers reported that the pH values of different kind of vinegars ranged between 2.64 and 3.21 (Jang *et al.*, 2015) and 3.12 and 3.65 (Ould El Hadj *et al.*, 2001). However, as noted by Golivari et al. (2015), commonly commercial vinegar has pH 4.2. This value is depended on the acetic acid content.

°Brix is used as an index for the amount of soluble solid content including sugars, acids and minerals. From Table 1, vinegar V1 exhibited a significantly higher °Brix value (10.2%) compared to the other vinegars with °Brix values of 8.5% and 7.6 % for V3 and V2 vinegars, respectively. The measurement of °Brix is important in all drinks including vinegar. The °Brix of pineapple peelings vinegar was 5.3% as recorded by Sossou et al. (2009). In a study by Çaliskan and Polat (2008) that examined eight cultivars of fresh fig from Turkey ('Sarilop', 'Bursa Siyahi', 'Goklop', 'Yediveren', 'Yesilguz', 'Morguz', 'Sari Zeybek' and 'UfakYesil') °Brix values ranging from 22.7 to 27.2 % were reported. Therefore, the results of this study indicate that after approximately 30 days of acetic fermentation, the percentage of sugar in the vinegars studied decreased compared to the initial °Brix of fresh figs and juice. The recorded values ranging from 6.7 to 10.2 %, highlighting the reduction in sugar content during the fermentation process. This proves that the fermentation process runs properly. The results of this study were close to those recorded by Ould El Hadj et al. (2001) on date vinegars with values ranging from 7 to 10 %. Moreover, these differences recorded in the results are a function of the raw material used for the production of the vinegar. Indeed, according to Ait Haddou et al. (2014), the level of soluble solid (° Brix) is closely related to the content of dry matter, mineral, organic matter, total fiber, total protein, glucose and fructose.

**Table 1.** Physico-chemical composition of vinegar samples

| | pH | Conductivity (µS/cm) | °Brix | Moisture (%) | Dry extract (%) | Density | Ash (%) | Organic matter (%) |
|---|---|---|---|---|---|---|---|---|
| Thaamriwth (V1) | 4.05 | 392 | 10.2 | 90.29 | 9.71 | 1.0186 | 0.20 | 8.9 |
| Aberkan (V2) | 3.9 | 451 | 7.6 | 92.64 | 7.36 | 1.0120 | 0.09 | 7.27 |
| Azandjar (V3) | 4.01 | 432 | 8.5 | 93.58 | 6.42 | 1.0128 | 0.10 | 6.32 |

Table 1 also shows the relative density values for the produced vinegar samples: 1.02 for V1 and 1.01, for both V2, V3. The results obtained in this study closely align with the findings of Abdullah (2016) regarding some vinegars, where values ranged from 1.015 to 1.025 g/cm$^3$ and are slightly higher than those reported by Golivari et al. (2015) in their investigation of three types of Iranian vinegars (ranging from 1.004 to 1.007). This high density of solutions studied may be attributed to the presence of large amount of colloidal materials suspended in vinegars under investigation.

The percentage of moisture for samples of the produced vinegar was 90.29, 92.64, and 93.58 % for V1, V2 and V3, respectively (Table 1). The moisture content is crucial in vinegar production as water constitute the major component of this beverage. An increase in dissolved moisture enables yeasts to produce a larger amount of alcohol during alcoholic fermentation in the first stage. This, in turn, facilitate the subsequent production of a greater amount of acetic acid by the acetic acid bacteria present in the vinegar mother during the second stage of ferme,tation (Al-Asadi and Abdullah, 2005 cited *by* Abdullah, 2016). These findings closely align with the results obtained in the same study for samples vinegar produced by the malt of some varieties of maize, Zehdi dates, and grapes as values ranged between 94.721 and 96.107 % (Abdullah, 2016).

Results in Table 1 indicate that the dry extract (%) values for the produced vinegar samples were 6.42, 7.36 and 9.71 for V1, V2 and V3, respectively. The richness of these traditional vinegars in microorganisms (double fermentation) explained, in part, the significant dry residue recorded in samples. Mbungu et al. (2016) found a dry matter content of 2.27 % in their study on mangoes vinegar. Bakir et al. (2016) reported a range of values in grape vinegar (3.8 – 8.25 %) and apple vinegar (4.3 – 8.8 %) in their research.

It was also observed from Table 1, that the ash contents of the vinegar samples were 0.20 %, 0.09 % and 0.10 % for V1, V2 and V3, respectively. Furthermore, the percentage of organic matter in the three traditional homemade vinegars samples, were 6.32 %, 7.27 % and 8.9 % for samples V1, V2 and V3, respectively. Ash determination is important to support vinegar characterization and quality evaluation. Results from this study were close to the findings of Mbungu et al. (2016) as the percentage of total ash in filtered mangoes vinegar was 0.20 %. The obtained results were also close to those obtained by Abdullah (2016) in his work on some vinegars as the ash content ranged between 0.26 – 0.52 %. According to the same study, the overall percentages of ash are affected by several factors, including the nature and quality of the raw material used in production, as well as factors that impact the proportions of total solids. The rate of the organic matter recorded in our study, were higher than those reported by Akakabe et al. (2006) as they found amounts estimated to be 2.3 to 4.6 % (w/w) in bamboo vinegar. These differences may be due to the nature and components of the raw materials.

The conductivity values for the produced samples were 392, 451 and 432 (µS/cm) for samples V1, V2 and V3, respectively (Table1). Conductivity is the measure of the soluble salts

content in the product. The obtained results differ from those found by Ould El Hadj et al. (2001) in their study on three Algerian date vinegars, where values ranged between 4.88 and 6.29 (mohms/cm). The fruit used and the preparation mode of vinegars may explain these differences. Furthermore, the contribution of tap water used in the vinegar preparation should not be overlooked, as it significantly influences the product's conductivity due to its dissolved salts content.

### 3.2. Nutritional properties of homemade fig vinegar

The content of energetic elements (total sugars, proteins and fat) is shown in Figure 3.

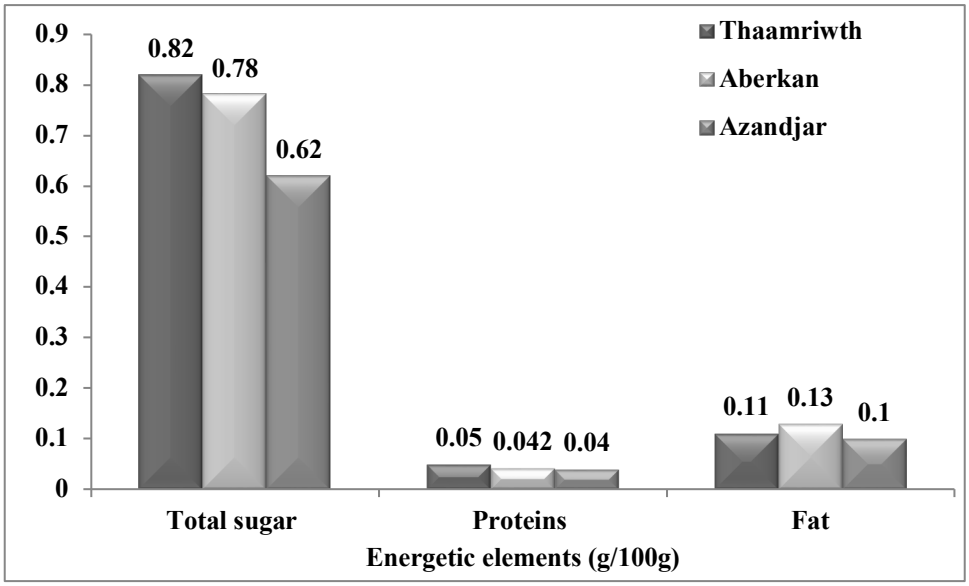


**Figure 3.** The energetic elements content of the vinegar samples

As it can be seen (Fig. 3), the protein contents of the vinegar samples, namely V1, V2 and V3 were found to be 0.05, 0.042 and 0.040 (g/100 g), respectively. The protein levels obtained were ten times lower than those recorded by Zakaria and Mokhtar (2014) for samples of Kelubi vinegar, Rambutan vinegar and Dokong vinegar as the values were 0.45 %, 0.42 % and 0.44 %, respectively. Additionally, the results also indicated lower protein content compared to the findings of Mokhtar et al. (2016) for Rambutan vinegar (0.27 %), Dokong vinegar (, 0.18 %), apple cider vinegar (0.13 %) and Nipa vinegar (0.25 %). It is worth noting that the high acidity and the presence of tannins in vinegars can potentially coagulate and denature some of the proteins.

After fermentation, the content of total sugars in vinegars were 0.82, 0.78 and 0.62 (g/100 g) for samples V1, V2 and V3, respectively (Fig. 3). It should be noted that total sugars include reducing and non-reducing sugars. The levels of sugars recorded were significantly higher when compared to the findings of Tanaka et al. (2016) on banana pulp vinegar as they found 10.27g/L and by Matloob and Hamza (2013) for artisanal manufactured and unrecorded dates vinegars where the total sugar content varied between 0.88 % and 4.07 % (w/v). These differences can be attributed to variation in raw materials used and the specific protocol followed during the vinegar production process. The low levels recorded in this study could also be interpreted by the complete utilization of sugar present is the figs by yeasts, which was subsequently transformed into ethyl alcohol. The crushing of figs during the process facilitated the efficient diffusion of sugars trapped within the cells of the pulp, making them readily accessible and usable by the microorganisms involved.

The fat contents of the samples V1, V2 and V3 were measured at 0.11, 0.13 and 0.10 (g/100g), respectively. The lipid contents in the vinegar sample are particularly low, which is expected since vinegar is primarily an aqueous product. The small amount of fat can be explained by the fruit used, which, like other fruits, is not rich in lipids. Limited data are available in the literature concerning the lipid content of vinegar, especially fig vinegar. In the study conducted by Mokhtar et al. (2016) on various vinegar, the fat content ranged between 0.07 % and 0.59 %. As stated previously, fig like most fruits, contain negligeable amount of lipids. According to Favier et al. (1993), the average lipid content of fresh figs is estimated to be around 0.2 g / 100g, which explains the low lipid content observed in fig vinegar.

Based on Figure 4, the energy intake of the three homemade vinegar samples (V1, V2 and V3) was measured at 4.47, 4.46 and 3.54 (Kcal/100g), respectively. The low content of energy elements (sugars, proteins and lipids) in vinegars results in a correspondingly low energy input, as these elements are the primary source of energy. However, the presence of acetic acid in vinegars can enhance biological energy consumption by increasing myoglobin levels and upregulating the expression of genes related to the synthesis of fatty acids (Yamashita *et al.*, 2009; Hattori *et al.*, 2010).

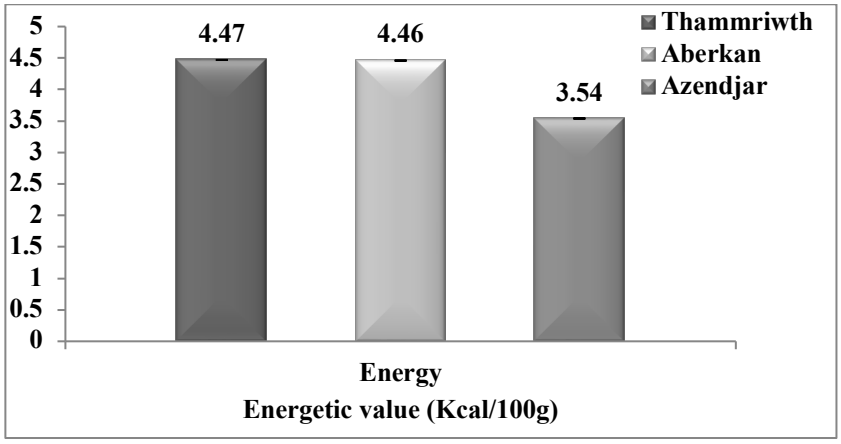


**Figure 4.** The energy value of the vinegar samples

### 3.3. Biochemical Characteristic of traditional fig vinegar

Alcohol and acetic acid contents of homemade fig vinegars are presented in Figure 5. Alcohol is the main metabolite of yeasts; it is used as a source of carbon for acetic acid bacteria during the first stage of vinegar production. Alcohol levels recorded were 0.18 %, 0.16 % and 0.17 % for vinegars V1, V2 and V3, respectively. While the conversion of sugar to alcohol occurs during the first stage of fermentation, the conversion of ethanol to acetic acid occurs during the second stage of fermentation by the action of the acetic bacteria.

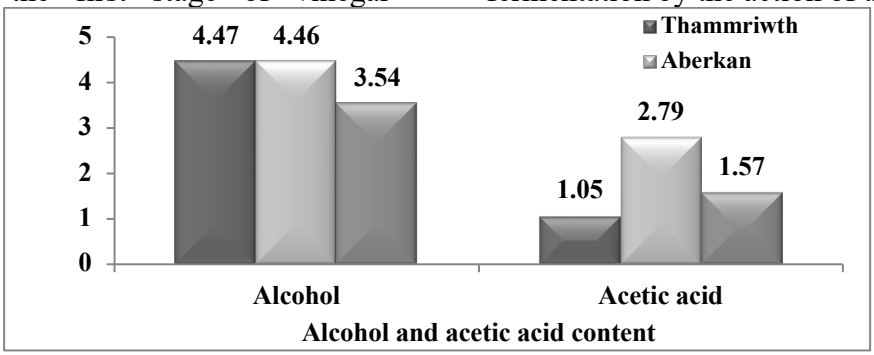


**Figure 5.** Acetic acid and alcohol content of homemade fig vinegars

Results are expressed as % for alcohol and as acetic degree (°) for acetic acid

*Saccharomyces cerevisiae* has been commonly recognized as the most prominent microorganism historically used for bioethanol production. It is capable in fermenting hexose sugars / sucrose and can yield ethanol concentration as high as 18 % in the fermentation broth. As result, it remains the preferred choice for the most of ethanol fermentation processes. *Saccharomyces cerevisiae* is also generally recognized as safe (GRAS) as a food additive for human consumption. Therefore, it is an ideal option for producing alcoholic beverages and for leavening bread (Lin *et al.*, 2006; Sossous *et al.*, 2009; Balakrishnaraja *et al.*, 2017). The alcohol content obtained corresponds to that of the unprocessed alcohol, in other words, to residual alcohol. The unprocessed alcohol level is regulated by the Codex Alimentarius (2000) standards, which stipulate that wine vinegar should not contain more than 0.5 % (v/v) residual alcohol or 1.0 % (v/v) for other types of vinegars. The results obtained in the current study were higher than those reported by Grégrová et al. (2012), who recorded residual alcohol contents ranging from 0.15 to 3.40 g/L in twenty samples of spirit vinegar. According to Jamaludin et al. (2016), after five days of fermentation, the content of ethanol in grape, apple and orange was 7.42 %, 6.53 % and 6.79 %, respectively, close to the findings of our study. Based on the same analysis, the authors found that, overall, during the initial stage of fermentation, the alcohol content is influenced by various factors, including pH, percentage of sugar and amount of acid present.

Acetic acid bacteria are group of Gram-negative bacteria that are strictly aerobic. They are well known for their ability to rapidly and incompletely oxidize carbon substrates, especially sugars and alcohols. These bacteria are widely distributed in nature and play an important role in the production of various food and beverages, such as vinegar (Gullo *et al.*, 2008; Sossous *et al.*, 2009; Gomes *et al.*, 2018).

The concentrations of acetic acid in the analyzed samples are depicted in Figure 5. The acetic acid contents in vinegar samples were found to be 1.05°, 2.79° and 1.57° for samples V1, V2 and V3, respectively. However, the levels of acetic acid obtained during acetification were significantly lower than the values recommended by the Codex Alimentarius (2000) standards which requires a minimum of 5 % or 50 g/L of acetic acid. This may be attributed to several factors. Firstly, the fermentation time may have been insufficient for the acetic bacteria to convert all the alcohol produced during the initial stage of fermentation into acetic acid. Additionally, it is possible that the production rate of alcohol was low, or the activity of yeast and/or acetic bacteria was inadequate. According to Ho et al. (2017), this low content of acetic acid might be due to inadequate oxygen levels during the acetic fermentation process. In fact, acetic acid bacteria require aerobic conditions to produce acetic acid effectively. The low oxygen concentration could influence the production of acetic acid and the speed of the fermentation process (Dabija and Hatnean, 2014). It has been reported by Buyuksirit and Kuleasan (2014) that the low production of acetic acid during the aerobic acetic fermentation could be due to the presence of toxic-secreting strains of *Saccharomyces cerevisiae* (yeast), that may inhibit the growth of Acetobacter species, the bacteria responsible for acetic acid production. Therefore, in order to enhance the acetic acid concentration, it is suggested to increase the oxygen levels through aeration during the acetic fermentation process. This can promote the production of acetic acid in the fig vinegar. Morales et al. (2001) reported a range of acetic acid levels between 71.8 and 94.4 µg/mL in their work on sherry vinegars. Similarly, Abdullah (2016), found varying values for different vinegars ranging from 27.890 µg/mL and 78.962 µg/mL.

### 3.4. Pearson correlation analysis

The correlation matrix (Table 2) reveals significant correlations between the various fermentation parameters. A high positive

correlation was observed between the pH and the alcohol level (r = 0.97). Conversely, a negative correlation was found between pH and acetic degree, representing acidity (r=-1.00). The °Brix exhibited an inverse relationship with the alcohol level (r= -0.81). Finally, a highly significant negative correlation was identified between the alcohol level and the acidity (r = -0.97). These different relationships between the parameters elucidate the progression of the fermentation. The decrease in the °Brix during the fermentation is accompanied, in fact, by an increase in the alcohol content due to yeast utilizing sugar as a carbon source to produce alcohol. This alcohol is then metabolized by acetic acid bacteria to generate acetic acid, resulting in a decrease of alcohol level and an increase in acidity. This transformation is reflected in the decreasing pH of the medium. In the study of Ho et al. (2017), it was found that a higher pH of 5.5 significantly ($p<0.05$) increase acetic acid production in vinegar, while pH did not affect significantly ($p>0.05$) the ethanol production.

**Table 2.** Correlation between the different physicochemical parameters of fermentation

| | **pH** | **Brix (%)** | **Alcohol** | **acetic acid** |
|---|---|---|---|---|
| **pH** | 1,00 | | | |
| **Brix (%)** | -0,62 | 1,00 | | |
| **Alcohol** | 0,97* | -0,81* | 1,00 | |
| **acetic acid** | -1,00* | 0,65 | -0,97* | 1,00 |

** Significant correlations marked at p <0.05.*

## 4. Conclusions

The present study is the first one that represents the initial exploration of the physicochemical, nutritional and biochemical properties of traditional homemade fig vinegar, produced in Algeria. The obtained results indicate a comparable quality profile among the three vinegar samples. The alcohol content of vinegars met the standards set by the Codex Alimentarius, while the level of acetic acid did not comply with these standards. The acidic pH of vinegars serves as a natural barrier against the proliferation of many pathogenic bacteria. Further investigations are warranted to examine the influence of factors such as raw materials, fermentation time, and fermentation temperature on the physicochemical, nutritional and biochemical properties of vinegar. Additionally, research should include the determination of bioactive molecules level, assessment of product acceptance among Algerian consumers and the evaluation of the microbiological quality under laboratory conditions.

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# FUNCTIONAL NATURAL YOGURT TABLET BASED ON MUCILAG OKRA FRUIT AND FLEXSEED POWDER AS PREBIOTIC

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**ABSTRACT**

This research highlights the potential for preparing nutraceutical yogurt using okra fruit mucilage and flaxseed powder as prebiotics and the potential of probiotics (lactic bacteria) to be preserved in these prebiotics at ambient temperature. Four yogurt formulation tablets were prepared and characterized for physicochemical, biochemical, biological, and pharmacodynamic (swelling and release of bioactive substances) properties. The primary findings showed that the yogurt formulation tablets F1 based on okra fruit mucilage and flaxseed powder are rich in flavonoids and exhibit interesting activities including antioxidant activity with% of DPPH inhibition=77.263%, antibacterial and pharmacodynamic. Okra fruit mucilage and flaxseed powder are good a source of bioactive substances, which could be further used as a natural antioxidant and as a matrix for the preservation of probiotics.

## 1.Introduction

The intestinal microbiota is crucial for the human body with its many physiological functions, among which the digestion of unassimilated food (Bourlioux, 2014). However, the disruption of this floral can cause numerous diseases (Possemiers et al., 2009) such as inflammatory bowel disease and irritable bowel syndrome (Desscoins, 2017).

Pharmacobiotic is a strategy to address intestinal balance and prevent various affections by supplying probiotic bacteria and prebiotics (Debré et Le, 2014).

Probiotics have many beneficial therapeutic qualities, including antibacterial, anti-inflammatory, and anti-diabetic effects (Ghahfarokhi et al., 2020). It has been demonstrated that adding antioxidants from

natural sources, such as plants, is an efficient strategy to act against oxidative stress. Additionally, bioactive dietary additives can improve immune system performance (Shahein et al., 2022).

Okra (*Abelmoschus esculentus* L.) is the only important vegetable crop in the *Malvacées* family (Kumar et al., 2013). The stem, leaves, fruits, seeds, and pods of this vegetable contain important bioactive substances, including flavonoids, tannins, carotenoids, and mucilage. These substances possess dietary and therapeutic properties for diseases such as type 2 diabetes, cardiovascular disease, digestive disorders, and they can also act as antioxidants, anticancer, immunomodulators, and microbicides (Gemede et al. 2018; Belgasem et al., 2019; Elkhalifa et al., 2021). Okra mucilage has also been extensively employed in medical treatments, such as blood volume expanders and plasma replacement.

Flaxseed (*Linum usitatissimum* L.) has been grown since civilization's dawn. It has high carbohydrates content and its oil is rich in essential fatty acids in w3 (44.75%) and especially in w6 with a value of 52.69% (Benahmed Djilali et al., 2022) and protective phytochemicals that protect against chronic diseases like lignin (Madhusudhan, 2009). It has been demonstrated that flaxseed mucilages help prevent intestinal inflammation, lower blood sugar, and cholesterol. In addition, they lubricate the skin, and even take the shape of artificial mucus (Fabre et al., 2014).

According to preliminary published studies, the mucilage of okra fruit affects milk clotting. However, no research work has improved the capacity of the compact to be used as a matrix of conservation of acid lactic bacteria at ambient temperature.

This study aims to prepare a functional yogurt tablet using flaxseed powder and okra fruit mucilage as prebiotics. Some biochemical, nutraceutical, biological, and functional properties of the yogurts have been investigated.

## 2. Materials and methods

### 2.1. Plant material

*Albomuschus esculentus* fruit was harvested from the southern Sahara of Algeria from Ghardaia region in September 2019. Okra fruit was open-air dried (20 to 22°C) in the shade with good ventilation. The characteristics of the dried fruits included an average weight of 38.66 ± 0.94g, humidity 10%, and an acidity of roughly 3.9±0.0001%.

### 2.2. Biological material

Three bacterial strains (*Enterococcus feacalis*, *Staphylococcus aureus* ATCC25923 and *Staphylococcus aureus* MU50) were used to test the antibacterial effect of the ethanolic extract of the elaborated yogurt formulation, okra fruit and flaxseed. These strains were provided by the microbiology laboratory of Mouloud Mammeri University of Tizi-Ouzou.
Strains mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* from pure freeze-dried commercial yogurt (CHRISTAN HENCEN, Denmark) were employed as probiotics to prepare the yogurt.

### 2.3. Methods

#### *2.3.1. Preparation of natural yogurt formulations*

Several natural yogurt formulations were prepared using probiotics, prebiotics (okra fruit infused, flaxseed powder), and milk powder; four formulations were chosen (Table 1). The fermentation temperature of these yogurt formulations was kept at 45°C. Both flaxseed and milk powders were added at different concentrations to both pre-heated milk and okra fruit infusion (45°C), and each formulation was inoculated with the probiotics (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) at a concentration of 0.0015g of starter culture (each per liter). The formulation was fermented at 45°C to reach a pH of 4.5.

#### *2.3.2. Yogurt formulation powders*

The obtained yogurt (Fig 1) was spread out in a thin layer and dried for two days at 40°C using oven drying. The obtained powders were

maintained away from light and moisture until they were compressed and characterized.


**Figure 1.** Aspect of the elaborated yogurt formulation powders

**Table 1.** Composition and coagulation time of the natural yogurt formulation

| Ingredients | F1 | F2 | F3 | F4 |
|---|---|---|---|---|
| **Okra fruit infusion (mucilage) (ml)** | 100 | 100 | 100 | 50 |
| **Pasteurized milk (ml)** | 0 | 0 | 0 | 50 |
| **Flaxseed powder (g)** | 14 | 7 | 0 | 0 |
| **Milk powder (g)** | 0 | 7 | 14 | 0 |
| **Probiotics (mg)** | 15 | 15 | 15 | 15 |
| **Coagulation time (min)** | 75 | 75 | 75 | 90 |

The obtained yogurt powders were compacted using a HERZOG manual press using round and flat punches. Each tablet is 40mm in diameter with an average weight of 4±0.05g. 160KPa is the optimized pressure applied to obtain tablets with a tolerable level of friability.

***2.3.3. Assessment methods***

Phytochemical, biochemical, and rheological data were used to identify a variety of dietary and nutraceutical compounds in the elaborated yogurt formulation, including:

- ✓ Phytochemical compounds were performed according to the standard phytochemical screening methods (Kumar et al., 2018);
- ✓ Total Polyphenols Content: was quantified using Folin-Ciocalteu technique (Adrian et al., 1995) via a spectrophotometer. The absorbance at 710 nm of the two extracts (ethanolic and aqueous) for each formulation was determined. The TPC value was given as mg of Gallic Acid Equivalent per g of dry basis (mg GAE/g db);
- ✓ Total Flavonoids Content (TFC): was evaluated using a colorimetric technique (Bahorun et al., 1996). The regression equation using quercetin as the standard was utilized to determine the TFC value, which is reported as mg of Quercetin Equivalent per g of dry basis (mg QE/g db);
- ✓ The scavenging activity with the DPPH free radical (2,2-diphenyl-1-picrylhydrazyl) was employed to assess the antioxidant activity of the prepared yogurt formulation. This technique is based on evaluating the yogurt formulation to scavenge the DPPH free radical (Kim et al., 2002);
- ✓ IR spectrometry was used to analyze the functional groupings of the probiotic (lactic bacteria) to confirm the presence

of a cryoprotection agent based on bioactive substances.

## 3. Results and discussions

### 3.1. Results of the phytochemical analysis of okra fruit and flax seed

According to the phytochemical analysis, okra fruit lacked anthocyanins, free and mixed quinones, alkaloids, and sennosides. but contained gallic tannins, saponosides, flavonoids, and coumarins. Our results agree with those of Yora and Syukur, (2018) who analyzed various okra genotypes and demonstrated the absence of quinones.

Flaxseed powder is rich in flavonoids and coumarins. However, alkaloids, sennosides, free and mixed quinones, gallic tannins, and quinones were absent.

The two plant materials under investigation contain flavonoids, specifically coumarins, which are water-soluble chemicals with antioxidant, anti-inflammatory (Goyal et al., 2014) and antibacterial properties (Tiwari et al., 2016).

### 3.2. Results of the biochemical composition of the yogurt formulation

According to our findings (Table 2), the ethanolic extracts of yogurt formulations F1, F2, and F3 have comparable total polyphenols. Formulation F4 has a higher polyphenols concentration. Besides, significant and similar concentrations of Total Flavonoids characterize the ethanolic extracts of the elaborated yogurt F1 based on the mucilage of okra and flaxseeds have higher concentration of flavonoids with a value of 6.84±0.014 (mg QE/g d.b). This yogurt showed a very high DPPH reduction percentage compared to the other formulations, which are characterized by lower percentages.

The availability of phenolic compounds is linked to the nature of the prebiotics used (okra fruit mucilage and flaxseed) and, in part, by the composition of the cryoprotective agents of the freeze-dried lactic bacteria used as probiotics. Results show that the okra fruit mucilage and flax seeds are notable for having interesting concentrations of TP with corresponding values of 0.487 mg GAE/g d.b, 0.298 mg GAE/g d.b), respectively. Our findings are similar to other studies on the antiradical activity of plant extracts conducted by Samaniego Sanchez et al. (2007) and Liao et al. (2012).

Yogurt formulations F1 and F3 and the lactic bacteria (probiotics) can be seen to contain OH, carboxylic acids, linear hydrocarburizer group and chlorophyll by looking at the Infra-Red spectrum (Fig.2) and table 3. Peaks at 1346cm-1, 1577cm-1, and 1716 cm-1 indicate the existence of Galacturonic Acid's (GalA) carboxyl group (COOH) (Kpodo et al. 2017, Benahmed Djilali et al. 2021). The signal observed at 1091cm-1 can be considered specific to the ether group which only includes the yogurt F1.

**Table 2.** Biochemical composition of the yogurt formulation

| Ingredients | F1 | F2 | F3 | F4 |
|---|---|---|---|---|
| **Total sugars** | 0 | 0 | 1.95 | 0 |
| **Sucrose content** | 0 | 0 | 1.77 | 0 |
| **Reducing sugars** | 0 | 0.10 | 0.08 | 0 |
| ***TPC (mg GAE/g d.b) Ethanolic extract*** | 0.2895±0.016 | 0.3755±0.01 | 0.296±0.02 | 0.5465±0.023 |
| ***TF(mg QE/g d.b) Ethanolic extract*** | 6.84±0.014 | 5.705±0.01 | 5.44±0.05 | 5.83±0.01 |
| ***% Inhibition of DPPH Water extract*** | 4.5±1.5 | 31.8±13.7 | 6.06±3.59 | 3.03±1.5 |
| **Ethanolic extract** | 77.263±13.77 | 39.3±12.73 | 30.3±4.5 | 40.09±14.19 |

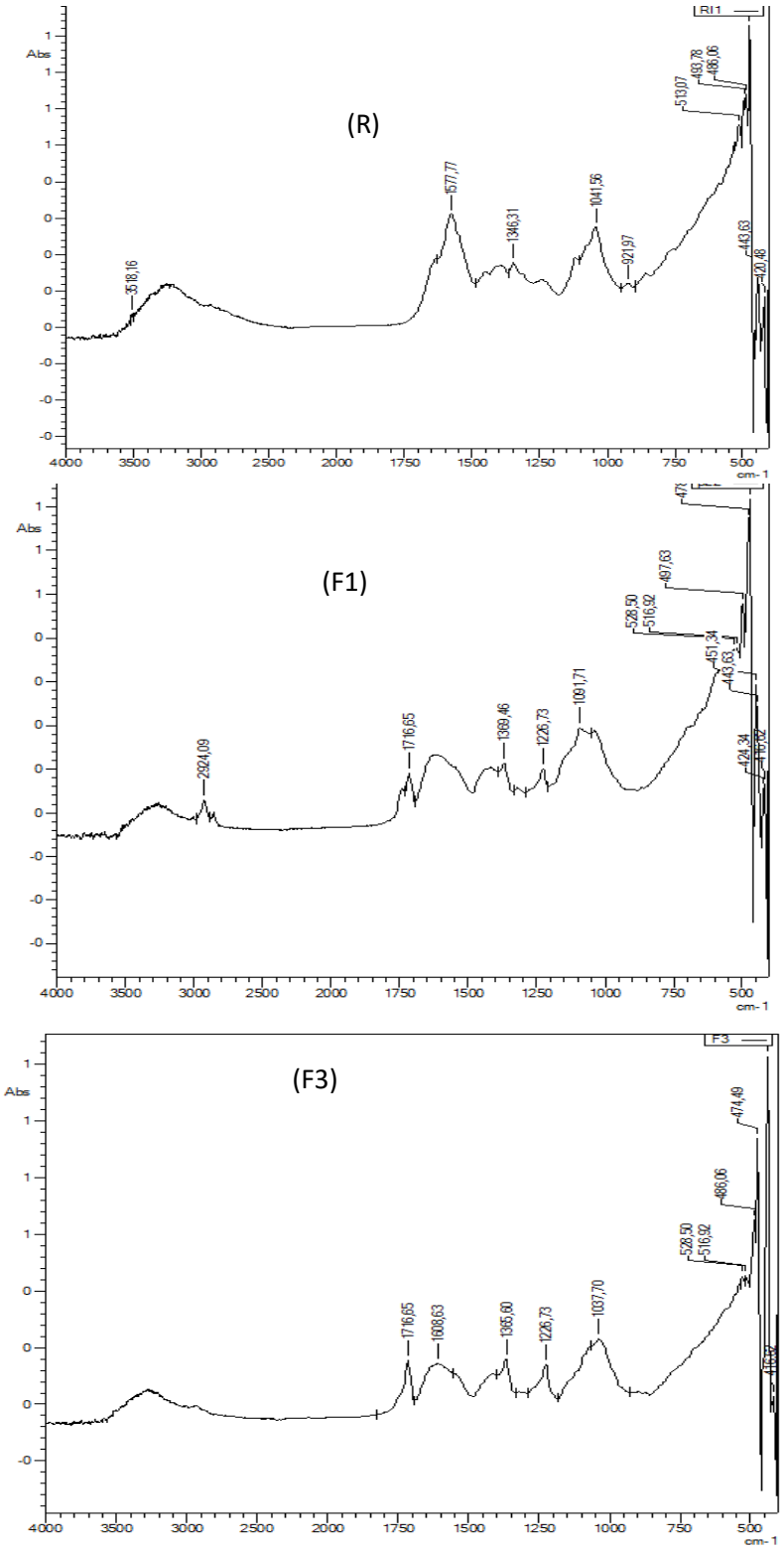


**Figure 2.** The Infra-Red spectrum of the probiotics (R), yogurt (F1) and (F3)

**Table 3.** Functional groups of yogurt formulations F1, F3 and and lactic acid bacteria (probiotics (R))

| Functional groups | Yogurt F1 | Yogurt F3 | Probiotic (R) |
|---|---|---|---|
| **Alcool (O-H)** | 2924 cm-1 | 1037 cm-1 | 1041 cm-1 |
| **Carboxylic (COOH)** | 1716 cm-1 | 1716 cm-1 | 1577-1346 cm-1 |
| **C-H** | 1365 cm-1 | 1369 cm-1 | - |
| **Amides (N-H)** | 1226 cm-1 | 1226 cm-1 | - |
| **Ether(CO-O)** | 1091cm-1 | - | - |
| **Amine NH2** | - | - | 3518 cm-1 |
| **Linear hydrocarburizer** | 497-478 cm-1 | 474-486 cm-1 | 493-486 cm-1 |
| **Chlorophyll** | 451 cm-1 | 416 cm-1 | 443-420 cm-1 |

### 3.3. Results of the antimicrobial activity of the elaborated yogurt formulation

Results of the dairy formulation's antimicrobial activity tests (Table 4) revealed that *Enterococcus feacalis* is only sensitive to the ethanolic extract of the flaxseed powder, which has an inhibition diameter of 10.66±0.02 mm. The type and amount of antioxidant nutrients, such as fatty acids, inhibit this strain. The analyzed flaxseeds oil is very rich in essential fatty acids such as linolic acid (52%), oleic acid (20.21%), and linoleic acid (15.96%). These fatty acids prevent the appearance of diseases including obesity, atherosclerosis, and some cancer (Kaleem, 2013). In addition, these acids can reduce the risk of cardiovascular disease by lowering triglyceride levels in the blood.

*Entrococcus feacalis* is resistant to infused okra fruit. Our findings support Solomon et al. (2016) who found that *Enterococcus feacalis* is resistant to okra flower extract.

*S.aureus* ATCC25923 sensitivity to the ethanolic extract of the formulation F3 with an inhibitory diameter of about 13.083mm is due to the availability of bioactive substances of okra fruit (carotenoids, mucilage, and flavonoids).

The ethanolic extracts of all formulations inhibit the *S.aureus* MU50 strain with intermediate inhibition zones. The nutraceutical compounds of okra fruit and flaxseed powder are responsible for the inhibitory activity of this strain.

**Table 4.** Inhibition diameters of ethanolic extracts of the different yogurt formulations, okra fruit infusion, and flaxseed powder (n=3)

| ***Strains*** | **F1** | **F2** | **F3** | **F4** | **Okra fruit infusion** | **Flaxseed powder** |
|---|---|---|---|---|---|---|
| ***Enterococcus feacalis*** | Abs | Abs | Abs | Abs | Abs | 10±0.66 |
| ***Staphylococcus aureus* ATCC25923** | Abs | Abs | 13±0.083 | 9±0.043 | 9±0.001 | 9.3±0.005 |
| ***Staphylococcus aureus* MU50** | 7±0.012 | 11.6±0.086 | 9±0.013 | 9±0.09 | 11±0.002 | 13±0.013 |

### 3.4. Results of some physical and rheological properties of yogurt tablet

According to the yogurt tablet formulation's rate of disintegration in distilled water (Fig 1), the tablet of formulations F1 and F2 decomposed quickly in less than 15 min. longer than 20 min, and presented low Water Holding Capacity (WHC) with a swelling index value of 4.76 and 2.66. With respect to swelling This time is based on the European Pharmacopoeia's criteria (EP, 2010). The flaxseed powder facilitates the disintegration effect.

On the other hand, both F3 and F4 yogurt tablets decomposed more slowly taking indices, formulation powders F3 and F4 exhibited higher values compared to formulation F1 and F2 with 6.33 and 5.7,

respectively. It can be explained by the herding of tablets, which is induced by the mucilage of both okra-attracting proteins and milk's calcium. Mucilage can be used as a linking agent. Our findings coincide with those of Benahmed Djilali et al. (2011) who demonstrated how date powder affects the disintegration of tablets made from a combination of date and spirulina powders.

Formulations F1 and F2 exhibit interesting rheological characteristics, including good flow, compatibility and rapid disintegration with the release of the bioactive substances.

We advise using these formulations as a therapeutic functional yogurt without sucrose, rich in prebiotics with anti-inflammatory, antioxidant, and antibacterial effects. Flaxseed powder and okra fruit mucilage can also be employed as prebiotic protection agents for probiotics (lactic bacteria).

### *3.4.1. SEM microstructure of yogurt*

The freeze-dried lactic bacteria and the complex yogurt compositions both displayed numerous irregularly shaped, non-porous particles that are similar to polymers (Fig. 3). The yogurt formulations F1 and F2 include voluminous particles and interesting porosity between the particles, whereas F3 and F4 contain high degrees of agglomeration and fewer holes between the particles. The pores affected the disintegration and release of bioactive substances.

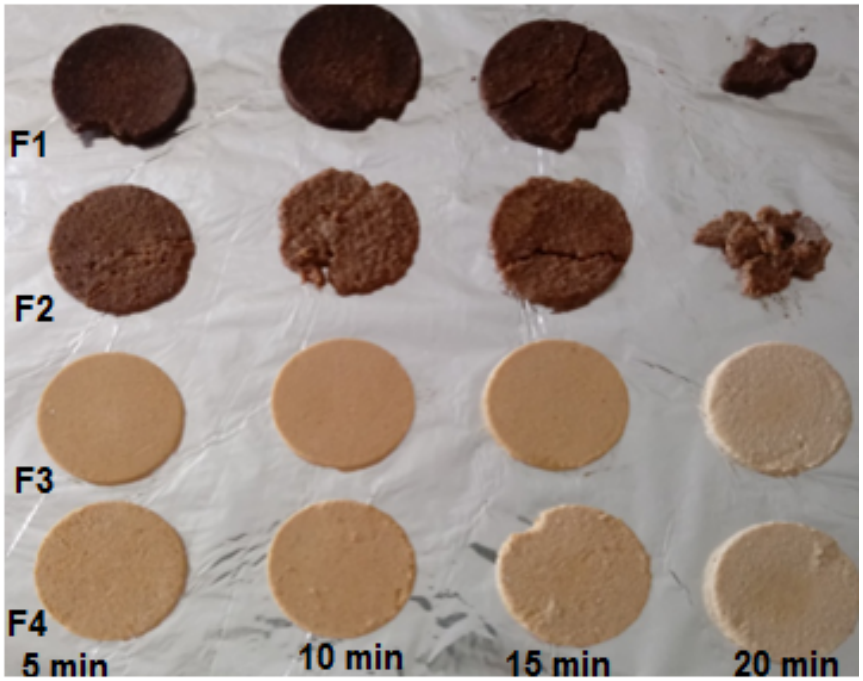


**Figure 3.** Morphological aspect of yogurt tablets as function of time of swelling in distilled water

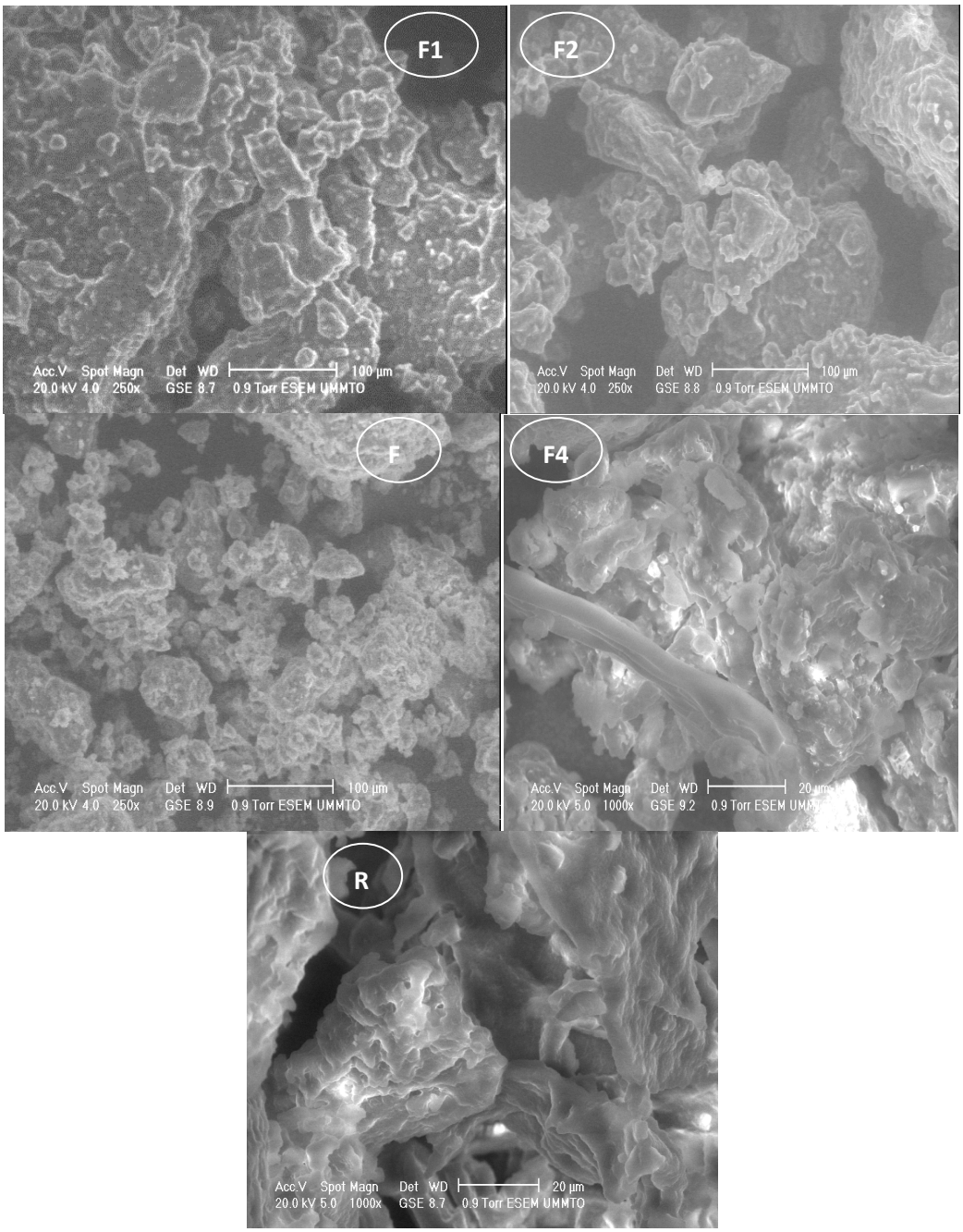


**Figure 4.** Microstructures of the elaborated yogurt formulations (F1, F2, F3 and F4) and lactic acid bacteria (R)

***3.4.2. Stability and functionality of lactic bacteria In*** order to assess the viability and functionality of lactic acid bacteria, yogurt tablets stored at 30°C for three months were used to perform milk coagulation.

The F3 tablets allowed better milk clotting time (90 min) in comparison to other yogurt tablets with a time greater than 120 min.

Figure 5 shows the obtained sour milk using F1 and F3. The combination of flaxseed powder and okra fruit mucilage must be optimized to reduce the milk coagulation time

of speed. This study is currently being published.

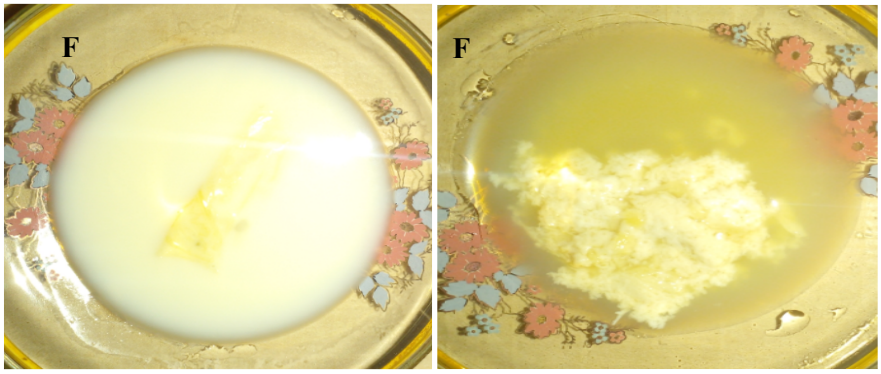


**Figure 5.** Obtained sour milk using tablets of yogurt F1 and F3 stored at 30°C for three months

## 4. Conclusions

The current study merely marks the beginning of a thorough inquiry into the medicinal and practical benefits of flaxseed powder and okra fruit mucilage, which is abundant in nutraceuticals (flavonoids and mucilage) that allow a variety of industrial uses such as agent linking, clotting milk and agent of conservation of lactic bacteria.

Further research will be required to draw additional scientific and technological information and define and apply the elaborated yogurt as a functional and therapeutic dairy product.

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# EFFECT COLD TEMPERATURE AND pH ON PERFORMANCE OF A STARCH-BASED WATERMELON SEED NANOCOMPOSITE FILM FOR LOCUST BEANS PACKAGING

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**ABSTRACT**

In this research, the performance of starch-based watermelon seed nanocomposite film was evaluated for locust beans packaging under varying pH and temperature conditions. The film was prepared by casting a blend of 15g potato starch, 0.3g watermelon seed nanoparticles with an average size 4.04 nm, 100g glycerol, and 300ml distilled water at 85ºC in 300× 150 mm plastic mold. It was characterized to determine its microstructural, thermal, and barrier properties using standard methods. Samples of the locust beans, prepared at 7.22– 11.13 pH range, were wrapped using the film and a low-density-polyethylene (LDPE) which serves as a control. The microbial loads were determined after 30 days of storage between 4– 10ºC temperatures. Results show ~ 4% loss in weight up to 300ºC, thus the film is thermally stable. The permeability coefficients were $0.7\times 10^{-10}$, $2.15\times 10^{-10}$ and $22.0\times 10^{-10}$ $cm^3$ (STP) $cm/cm^2scm$ Hg for nitrogen, oxygen, and carbon-dioxide, gases, respectively. Morphologically, the particles in the film are heterogeneously distributed within the matrix, revealing traces of elemental components with average pore size smaller than a water molecule but sufficient to allow exchange of the gases in the microsphere. There was significant difference ($p<0.05$) in the level of the microbial loads in the samples packaged using the nanocomposite film compared those packaged using the LDPE. The nanocomposite film is therefore a better packaging material than the LDPE for locust beans packaging under the same pH and temperature.

## 1.Introduction

A crucial step in the post-harvest processing of the Africa locust beans is packaging (Barak and Mudgil, 2014). This contributes to the product's containment and defense against microbial, fungal, and bacterial attack, consequently extending its shelf life (Yun *et al.,* 2022). The performance of the packaging material may be impacted by the application, the type of food being transported, the temperature, storage, and distribution, and other factors (Wu *et al.,* 2021). Typically, the type of packaging materials used for sea foods may not be suitable for other kind of foods because of the temperature and transportation effects (Alabi *et al.*, 2022). To ensure that the product is effectively secured for a prolonged shelf-life, packaging needs to be applied carefully for different foods and purposes.

Aluminum foil, paper, and leaves have all been used to contain the condiment over time to prevent it from deteriorating(Liu *et al.,* 2021). Although there has been improvement, the product still demonstrates signs of poor-quality degradation, which shortens its shelf life. Additionally, printing on these materials may be challenging, which may impair market communication between the product's makers

and consumers (Liu *et al.,* 2020). Although nylon and other standard packaging can help with the problems, their long-term use is frequently discouraged because they are not biocompatible. In the meanwhile, bio-based packages have been suggested as a solution to the issue with conventional materials. This kind of package, which is typically made from sustainable by-products of plant and animal origin, can provide the product with thoughtful and long-lasting protection (Yun *et al.,* 2022). As a result, almost all the problems with locust bean packaging can be resolved by adopting bio-based packaging. Biodegradable nanocomposite films have generally been reported for use in food packaging. For instance, Fadeyibi *et al.* (2020) reported the packaging of cucumber and garden eggs using nanocomposite films. The film has also been successfully applied for the packaging of tomatoes (Fadeyibi *et al.*, 2017), sliced okra (Fadeyibi *et al.*, 2019; Al-Naamani *et al.*, 2018), strawberry (Barikloo and Ahmadi, 2018), pears (Bodaghi and Hagh, 2019), and many other food products (Farhoodi, 2015; Huang *et al.,* 2015). However, the application of the biodegradable film for locust beans packaging is only sparingly reported. Also, there was no reported research on the synthesis of a bioplastic from the mixture of potato starch and watermelon seed nanoparticles hitherto. This research was therefore carried out to produce and test a nanocomposite film from the blend of sweet-potato starch and watermelon seed nanoparticles for locust beans packaging under varying conditions of pH and low temperatures.

## 2. Materials and methods

### 2.1. Starch preparation

A 1000 g tuber of fresh harvested sweet potatoes was bought at the main market of Malete and processed to extract the starch needed. This was done by peeling the potato and crushing it to reduce the size before grating it into a purée. The material was then passed over a prepared sieve and water was added to the filter. The filtrate was maintained intact for 1 hour to allow the starch to settle before the supernatant was decanted. The starch formed was dried in an air circulation oven for 24 hours and then packaged in a polyethylene bag for further experiment.

### 2.2. Preparation of the starch-based water-melon seed nanocomposite film

A 1000 g of watermelon fruits were bought from the Malete market, Kwara State, Nigeria. It was then divided into small pieces, crushed to make paste, then carefully cleaned with fresh water. The seeds were then extracted from the mixture and poured into a sieve. The seeds were bleached in an acid solution of 10% (w/w) hydrogen chloride. They were then thoroughly rinsed with tap water and dried in an air circulated oven for 24 hours. An electric mixer was then used for milling, and a mechanical sieve shaker was used for pruning into fine particle size. The fine materials in the shaker pan were collected and subjected to a particle size experiment with a Zetasizer (version 7.01). This gives an average particle size of 4.04 nm. Based on preliminary research and previous literature reports, the film was prepared by mixing 15 g of the sweet-potato starch, 0.3 g of the watermelon seed nanoparticles and 100 g of glycerol in 300 ml of distilled water (Farhoodi, 2015; Huang *et al.*, 2015). The mixture was placed on a burner and heated to 80°C and stirred continuously until it became gelatin-like in consistency. The gelatinous liquid was then poured into a mold that measured 300 mm by 150 mm, and it was dried for 24 hours at 60 $^{o}$C and 65% R.H. in an air-circulated oven dryer. The dried film was packed in a nylon for a later investigation.

### 2.3. Characterization of the starch-based water-melon seed nanocomposite film

The titration method described by Hadassah and Sehgal (2006) and Fadeyibi *et al*. (2023) was used to determine the methane, oxygen, hydrogen, nitrogen, and carbon dioxide gas permeabilities of the starch-based watermelon seed nanocomposite film. The method described by Kviesitis (1971) and Schöffski and Strohm (2000) was used to measure the water vapor permeability of the film. Thermal properties were determined using the procedure described

by Fadeyibi *et al.* (2017) and Shanks (2010). The procedure described by Nikov *et al.* (2020) for Scanning electron microscopy (JEOL JSM-7600F model electron microscope) in conjunction with the Energy-Dispersive x-ray Spectroscopy was used to analyze the microstructural property of the nanocomposite film at 50 and 100nm resolutions.

### 2.4. Evaluation of the starch-based water-melon seed nanocomposite film

The performance of the nanocomposite film was assessed using 1000 g of fresh locust beans, which were purchased at Malete Market. To change the pH in the range of 7.22–11.13, a buffer solution of sodium chloride at a concentration of 3% (w/w) was applied to the sample of locust beans. The products were then packaged using the film and the LDPE and stored between 4– 10$^{o}$C for 30 days. A total 12 main samples were thus obtained at the end of storage, as shown in Table 1, and the microbial loads were determined using the methods reported by Fadeyibi *et al.* (2017) and Sunmonu *et al.* (2020). The experiment was replicated three times, and the average value and standard deviation were recorded as the microbial loads of the locust beans condiment.

**Table 1:** Sample of packaged locust beans

| | | Variables | |
|---|---|---|---|
| | Sample | Temp ($^{o}$C) | pH |
| Film | A | 4 | 7.22 |
| | B | 4 | 9.34 |
| | C | 4 | 11.13 |
| | D | 8 | 7.22 |
| | E | 8 | 9.34 |
| | F | 8 | 11.13 |
| | G | 10 | 7.22 |
| | H | 10 | 9.34 |
| | I | 10 | 11.13 |
| LDPE | control 1 | 4 | 7.22 |
| | control 2 | 8 | 9.34 |
| | control 3 | 10 | 11.13 |

### 2.5. Statistical analysis

The results obtained from the barrier, mechanical, thermal, and structural characterizations were illustrated graphically. To reduce experimental error, the data were also examined in triplicate, and average values and standard deviations were established. At $p< 0.05$, a two-way multivariate analysis was utilized to determine the significance of temperature, pH, and their interaction on microbial growth in the 12 samples of packing material. We utilized the Duncan Multiple Range (DMRT) test to determine the degree of significance for each sample treatment compared to the control (LDPE) for various pH and cold storage temperatures at $p< 0.05$.

## 3. Results and discussions

### 3.1. Permeability of the starch-based water-melon seed nanocomposite film

The findings of the permeability of the starch-based watermelon seed nanocomposite film are shown in Figure 1. This shows that the film is more permeable to carbon dioxide and hydrogen gases and less permeable to oxygen, methane, and nitrogen gases. According to Fadeyibi *et al.* (2023) and Kim *et al.* (2014) locust beans typically produce oxygen, and carbon dioxide gases during packaging. Over accumulation of these gases can lead to imbalance in the storage structure thereby decreasing the shelf life. Therefore, it is essential to control the gas concentration to prevent this from happening (Ghosh and Singh, 2022; Mohammadpour and Naghib, 2021). During locust beans packaging, a stable microsphere can be created with high carbon dioxide and low oxygen and methane gas concentrations because of the inherent property of the nanocomposite film. Due to the limited amount of oxygen gas present in the microsphere, the product is likely to undergo anaerobic respiration, which will cause the concentration of the carbon dioxide to increase thereby extending the shelf life of the locust beans. This supports the findings of Qin *et al.* (2021), who used graphene oxide nanosheets to extend the shelf life of maize cellulose, and Zhang *et al.* (2020), who used

lignin to improve the water vapour transmission rate of the film by incorporating it into polyvinyl acetate. In other similar works, Raja and Xavier (2021) functionalized a silane nanoparticle on nanoclay to improve the maintaining quality of the film during packing, while Fadeyibi and Osunde (2021) added a zinc nanoparticle to cassava starch. Considering the foregoing, it follows that during food packing, the nanocomposite film can monitor and regulate the gaseous concentration within the microsphere.

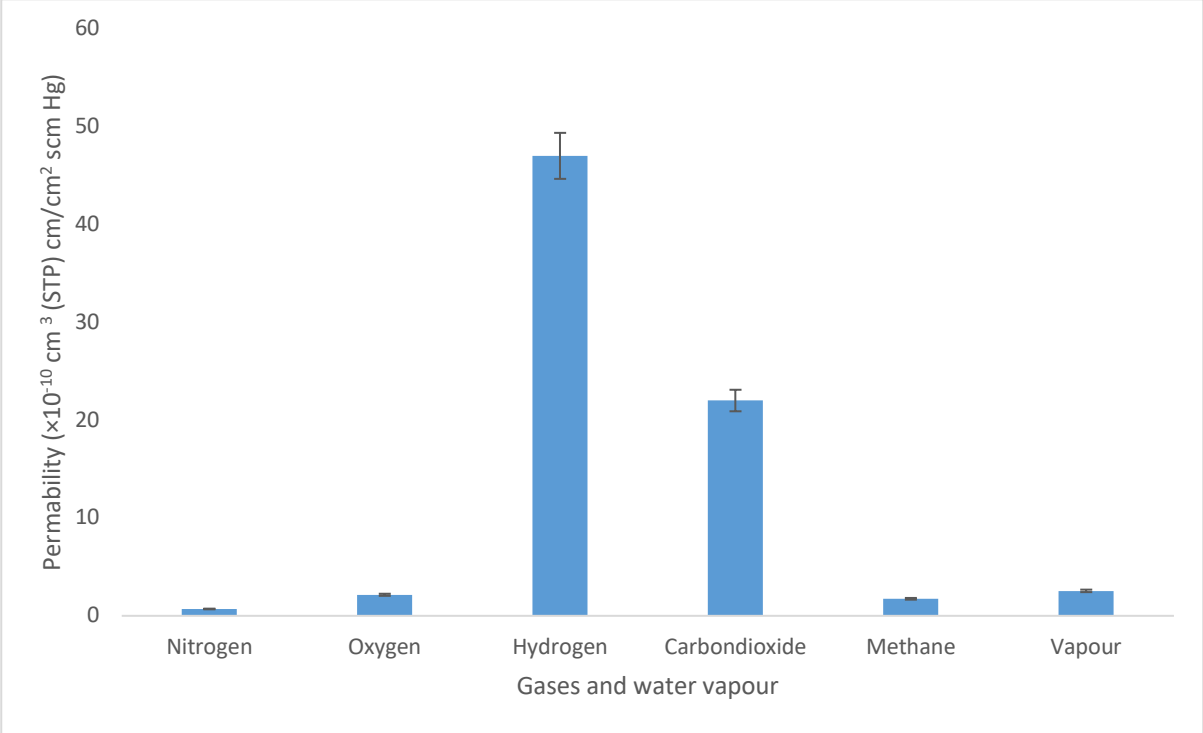


**Figure 1.** Permeability of the starch-based water-melon seed nanocomposite film

### 3.2. Microstructural properties of the starch based water-melon seed nanocomposite film

The results of the microstructural properties of the water-melon seed nanocomposite film are shown in Figure 2. A heterogeneous arrangement of the film matrix can be seen which indicates inconsistencies in the particle size between 10.55 to 11.77nm. This arrangement can be associated with the different particle sizes of the glycerol and starch molecules used in forming the film. Consequently, gas molecules smaller than 11.77 nm like oxygen, nitrogen, carbon-dioxide, and methane can easily permeate the film due to their inherent smaller molecular sizes in comparison with the pore sizes in the film. Thus, this allows gaseous exchange when applied for food packaging. In other related investigations, Merritt *et al*. (2020) reported that a polystyrene encapsulation will enhance the gas permeability of graphene-based nanocomposite film. Also, Figure 2 shows the EDS profile of the watermelon seed-based nanocomposite film with a high signal for oxygen gas (30.3% by weight). This means high oxygen and other gas permeability for the film. The findings of Nikov *et al.* (2020) on the SEM characterization of a polyvinyl alcohol and clay-based nanocomposite film agree with the present research.

The results of the microstructural properties of the nanocomposite film as viewed from the TEM micrograph (50nm and 100 nm resolutions) are shown in Figure 3. White and dark clusters of particles are seen converging around some spots within the film matrix. Areas

with dark clusters depicts the spots where the watermelon seeds are mostly concentrated while the areas with white patches show the spots where the nanoparticles are less concentrated. This variation can be associated with the high surface area of the nanoparticles which provided the bonds needed to concentrate the particles around some spots more than the others (Chenwei *et al.,* 2018; Feng *et al.,* 2018). At 50 nm resolution, this disparity is even clearly seen than at 10 nm resolution; and the pattern observed can further give information about the particle size arrangement and suggest the permeability level of the watermelon seeds nanocomposite film.

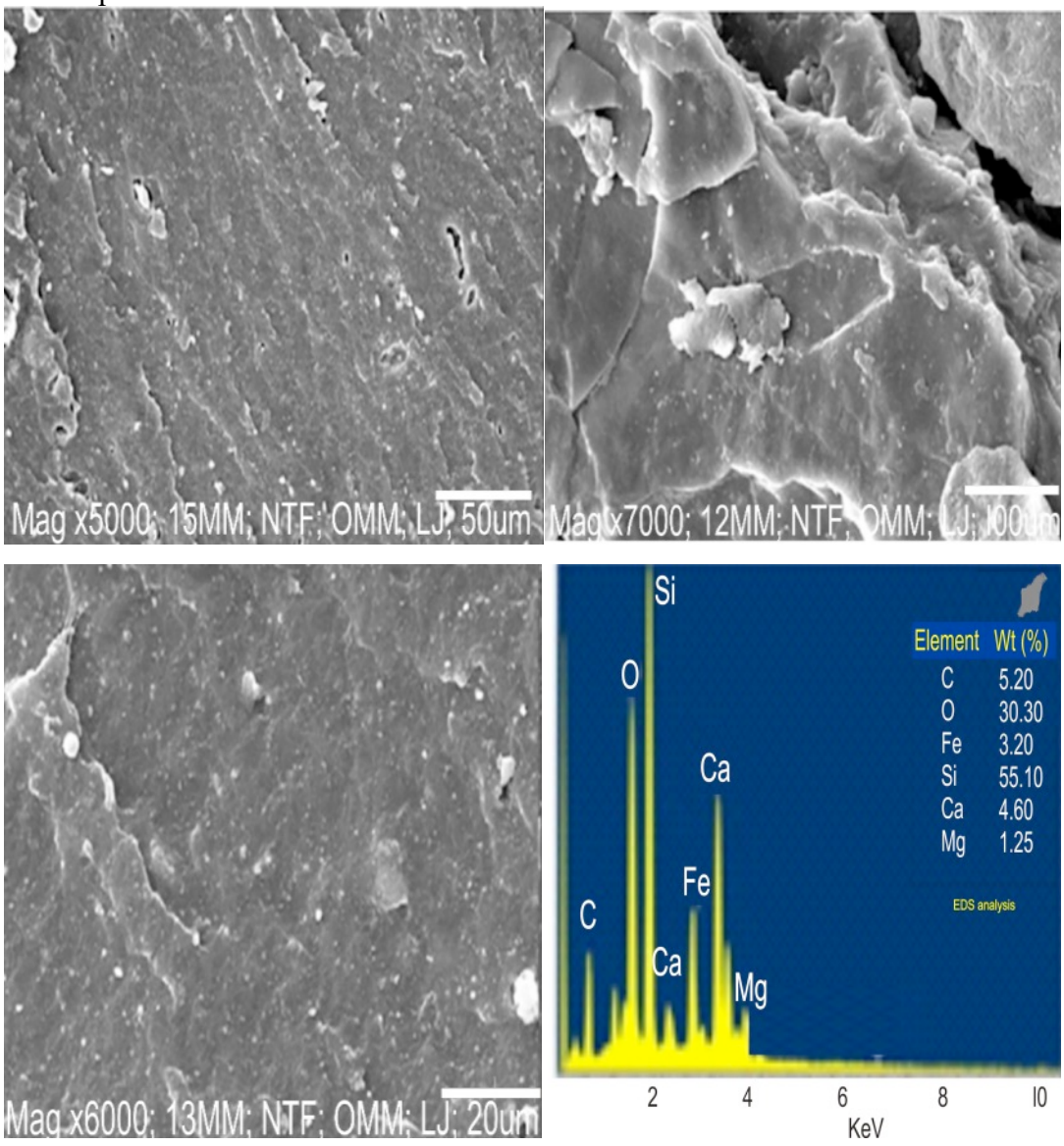


**Figure 2.** SEM micrographs of the starch-based water-melon seed nanocomposite film

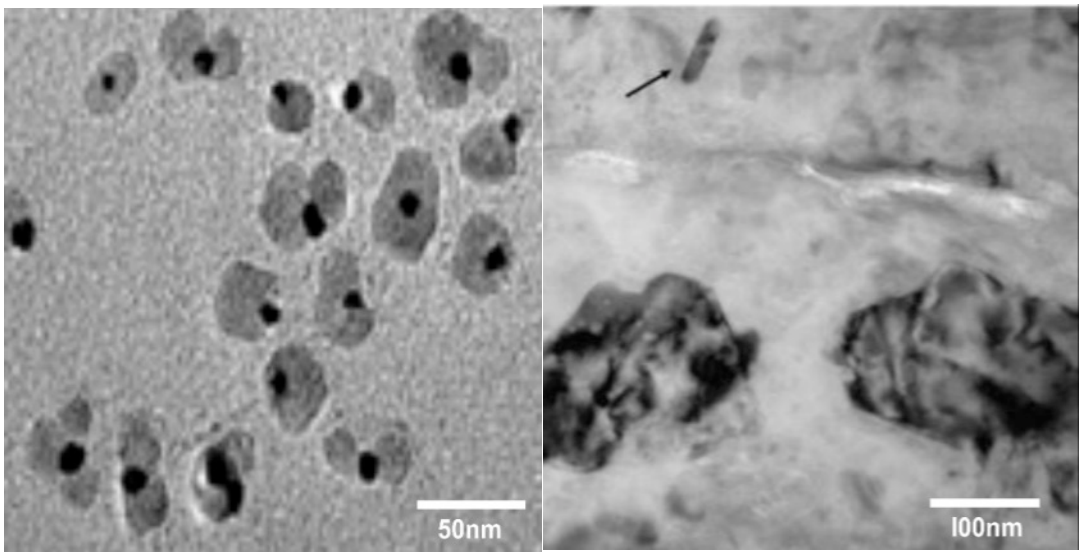


**Figure 3.** TEM microtopography of the starch-based water-melon seed nanocomposite film

### 3.3. Thermal properties of the starch based water-melon seed nanocomposite film

The results of thermal properties of the water-melon seed nanocomposite film obtained from the TGA/DTA analysis are shown in Figure 4. There was only a 4% weight degradation when the film was heated to 300°C, which correspond to the threshold or glass transition temperature where it was stable thermally. At this threshold, the heat of sublimation was 4.55 J/g which gradually increased with an increase in the temperature. Thus, above the glass transition temperature the degradation maybe enormous thereby rendering the film thermally unstable. The findings of Thomas *et al. (*2021), Manikandan *et al.* (2019) on natural rubber and nano-cellular composite films, respectively, reported high weight degradation above the glass transition temperature. Hence, the starch-based watermelon seed nanocomposite film can be recommended for packaging food at temperatures below 300°C.

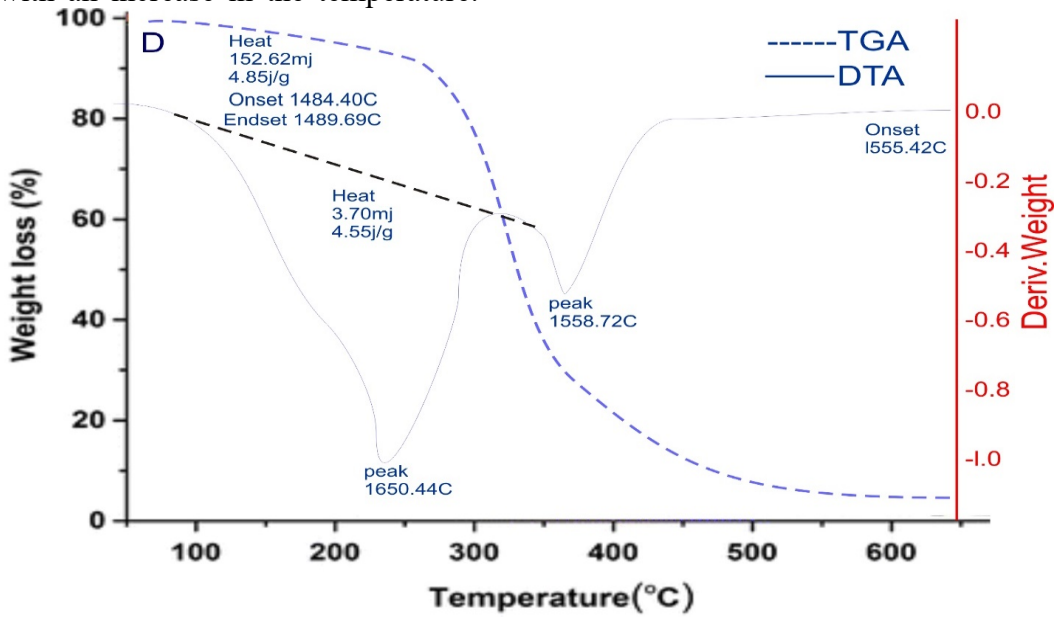


**Figure 4.** Effect of temperature on weight degradation of the starch-based water-melon seed nanocomposite film

### 3.5. Effects of cold temeprature and pH on microbial loads of packaged locust beans

The results of the influence of pH and cold temperature on the microbial load of the packaged locust beans are shown in Table 2. This shows high performance of the nanocomposite film compared with the LDPE at the same temperature and pH. There is no significant difference between the pH and temperature interaction or their individual effects on the microbial loads ($p< 0.05$) as shown in Table 3. Also, there is no significant difference in the level of the microbial loads between the packaged samples, partly due to the presence of the nanoparticles in the matrix of the film, and partly due to the surface area between the molecules in the film mix. In related studies, Fadeyibi *et al.* (2020) and Shi *et al.* (2022) reported a low level of microorganisms on some fruits and vegetables when nanocomposite films are used to package them. This means a low pH and temperature can lower the number of microorganisms in the stored locust beans. Furthermore, the DMRT in Table 4 indicates no significant different between the fungi counts in B and G samples, but A, C, D, E, F, I samples were significantly different from each other and from the control ($p< 0.05$). Similarly, all the film samples are significantly different from each other and from the control ($p< 0.05$) for the bacteria counts. Thus, the starch-based watermelon seed nanocomposite film can be suitable for the locust beans packaging at all pH and temperature.

**Table 2.** Effects of temperature and pH on the microbial loads in packaged locust beans

| | | Variables | | Microbial loads ($\times 10^5$ cfu/g) | |
|---|---|---|---|---|---|
| | sample | Temp ($^oC$) | pH | Fungi count | Bacteria count |
| Film sample | A | 4 | 7.22 | 0.46± 0.02 | 1.15± 0.06 |
| | B | 4 | 9.34 | 0.66± 0.03 | 1.90± 0.10 |
| | C | 4 | 11.13 | 0.62± 0.03 | 3.35± 0.17 |
| | D | 8 | 7.22 | 0.36± 0.02 | 1.50± 0.08 |
| | E | 8 | 9.34 | 0.76± 0.04 | 2.65± 0.13 |
| | F | 8 | 11.13 | 0.14± 0.01 | 3.20± 0.16 |
| | G | 10 | 7.22 | 0.67± 0.03 | 1.40± 0.07 |
| | H | 10 | 9.34 | 0.92± 0.05 | 2.60± 0.13 |
| | I | 10 | 11.13 | 1.62± 0.08 | 4.25± 0.21 |
| Control | LDPE 1 | 4 | 7.22 | 3.41± 0.17 | 9.34± 0.47 |
| | LDPE 2 | 8 | 9.34 | 4.14± 0.21 | 10.14± 0.51 |
| | LDPE 3 | 10 | 11.13 | 5.04± 0.25 | 12.37± 0.62 |

**Table 3.** Analysis of variance of the microbial loads influenced by pH and temperature conditions

| **Dependent Variable** | **Source of Variation** | **SS** | **df** | **MS** | **F-value** | **p-value** |
|---|---|---|---|---|---|---|
| Bacteria counts (cfu/g) | Corrected Model | 69.32 | 8 | 8.66 | 0.28 | $0.94^{n*}$ |
| | Intercept | 153.23 | 1 | 153.23 | 4.86 | $0.12^{n*}$ |
| | Temperature (T) | 0.68 | 2 | 0.34 | 0.01 | $0.99^{n*}$ |
| | pH (P) | 9.12 | 2 | 4.56 | 0.15 | $0.87^{n*}$ |
| | T× P | 56.37 | 4 | 14.09 | 0.45 | $0.71^{n*}$ |
| | Error | 94.56 | 3 | 31.52 | | |
| | Total | 405.52 | 12 | | | |
| | Corrected Total | 163.87 | 11 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Fungi counts (cfu/g) | Corrected Model | 14.48 | 8 | 1.81 | 0.34 | 0.90 n* |
| | Intercept | 16.38 | 1 | 16.38 | 3.01 | 0.18 n* |
| | Temperature (T) | 0.91 | 2 | 0.46 | 0.09 | 0.92 n* |
| | pH (P) | 0.32 | 2 | 0.16 | 0.03 | 0.97 n* |
| | T× P | 12.52 | 4 | 3.13 | 0.59 | 0.70 n* |
| | Error | 15.91 | 3 | 5.30 | | |
| | Total | 59.85 | 12 | | | |
| | Corrected Total | 30.39 | 11 | | | |

*Significant at $p< 0.05$

**Table 4.** Duncan Multiple range test for microbial loads

| | | Microbial loads ($\times 10^5$ cfu/g) | |
|---|---|---|---|
| | sample | Fungi count | Bacteria count |
| Film sample | A | $0.46\pm 0.02^{i}$ | $1.15\pm 0.06^{l}$ |
| | B | $0.66\pm 0.03^{g}$ | $1.90\pm 0.10^{i}$ |
| | C | $0.62\pm 0.03^{h}$ | $3.35\pm 0.17^{e}$ |
| | D | $0.36\pm 0.02^{j}$ | $1.50\pm 0.08^{j}$ |
| | E | $0.76\pm 0.04^{f}$ | $2.65\pm 0.13^{g}$ |
| | F | $0.14\pm 0.01^{k}$ | $3.20\pm 0.16^{f}$ |
| | G | $0.67\pm 0.03^{g}$ | $1.40\pm 0.07^{k}$ |
| | H | $0.92\pm 0.05^{e}$ | $2.60\pm 0.13^{h}$ |
| | I | $1.62\pm 0.08^{d}$ | $4.25\pm 0.21^{d}$ |
| Control | LDPE 1 | $3.41\pm 0.17^{c}$ | $9.34\pm 0.47^{c}$ |
| | LDPE 2 | $4.14\pm 0.21^{b}$ | $10.14\pm 0.51^{b}$ |
| | LDPE 3 | $5.04\pm 0.25^{a}$ | $12.37\pm 0.62^{a}$ |

*Sample with the same letter indicate no significant difference at $p< 0.05$.

## 4. Conclusions

A novel nanocomposite film was developed from the blend of starch and watermelon seed nanoparticles for locust beans packaging. The film was more permeable to carbon dioxide and hydrogen gases and less permeable to oxygen, methane, and nitrogen gases. A heterogeneous arrangement of the film matrix was seen which indicates inconsistencies in the particle size between 10.55 to 11.77nm. There was only a 4% weight degradation when the film was heated to 300$^{o}$C, which correspond to the threshold or glass transition temperature where it was stable thermally. There was also no significant difference ($p<0.05$) in the level of the microbial loads between the packaged samples, partly due to the presence of the nanoparticles in the matrix of the film, and partly due to the surface area between the molecules in the film mix.

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# LCMS STUDY OF THE INDIGENOUS FRUITS AND VEGETABLES OF INDIAN HIMALAYAN REGION

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**ABSTRACT**

Phenolics of *Prunus avium*, *Cydonia oblonga* & *Malva neglecta* and carotenoids of *Taraxacum officinale* of the selected indigenous fruits & vegetables of Indian Himalayan region (IHR) have been characterized and analysed for the first time. Phenolics & carotenoids were determined by LCMS/MS using a reverse phase C18 column. In *Prunus avium,* the phenolics like cyanidins, rutin, epicatechin & catechin were found. In *Cydonia oblonga*, the caffeoyl quinic acids were abundant. A total of 24 carotenoids were identified in *Taraxacum officinale*. The most important identified were (all-Z)-β-cryptoxanthin, antheraxanthin, (all-E & Z)-lutein, (all-E)-zeaxanthin and (all-Z)-violaxanthin. Hydrocarbon carotenoids found were (all-E)-β-carotene, (9Z)-β-carotene & (13Z)-β-carotene. In *Malva neglecta*, the major phenolics were quinic acid, chlorogenic acid, rutin, & gallic acid. This work will contribute to the development of scientific research about the bioaccessibity and bioavailabity of phenolics & carotenoids for better understanding of the phenolics and carotenoids impact on human health.

## 1.Introduction

The importance of diversity of fruits and vegetables in human diet to maintain good health is well established. Fruit and vegetable consumption is recommended in numerous food-based dietary guidelines and forms a key recommendation in many international statements related to healthy diets. There is enormous potential to better incorporate the wealth of diverse fruit and vegetable species into food systems. Indigenous fruits and vegetables are defined as locally-produced, socially and culturally accepted as local foods and eaten by previous generations or introduced for a very long time. They provide a variety of products like food, medicines, raw materials and are also an important source of renewable energy.

Chronic diseases like coronary heart diseases, cancers, immune dysfunction and diabetes mellitus are found less in people with increased fruit & vegetable consumption (Pandey et al., 2009). Fruits & vegetables being rich in health-promoting nutrients like vitamins, minerals, phenolics and flavonoids act as a primary food source. In addition to commercial fruits, edible wild fruits have drawn attention due to their exceptional antioxidant properties and increased polyphenol levels (Murillo et al., 2012; Siqueira et al., 2013). The Northern Himalayan tribes and rural residents rely heavily on wild edible fruits for their nutrition (Samant et al., 2001). For thousands of years, they have been used both as food and medicine (Gaur et al., 1999). Limited studies on wild edible fruits

& vegetables of Kashmir valley of North Himalayas have shown presence of abundant polyphenols and associated antioxidant, anti-microbial, anti-inflammatory and anti-proliferative activities (Badhani et al., 2015; Singh et al., 2015). *Prunus avium* & *Cydonia oblonga* as fruits and *Taraxacum officinale & Malva neglecta as vegetables* are the underutilized edible plants of Indian Himalayan region (IHR) selected for this study. In J &K, these plant species are abundantly found (Maikhuri et al., 2004).

One of the most liked temperate fruits is the sweet cherry (*Prunus avium*), which is well regarded by consumers and extensively researched by scientists for its flavour, colour, sweetness, nutritional and bioactive qualities. Sweet cherry fruits contain a variety of phenolics, including flavonoids (anthocyanins, flavan-3-ols, and flavonols) and phenolic acids (hydroxycinnamic derivatives), possessing antioxidant activity ((Ballistreri et al., 2013; Pacifico et al., 2014).

Quince fruit (*Cydonia oblonga*) is too acidic, astringent and tough to be consumed fresh. This fruit is readily accessible and a reasonably priced dietary source of phytoconstituents with positive health effects (Mir et al., 2016). Recent pharmacological studies have revealed that quince fruit has antioxidant, antibacterial, antiviral, anti-inflammatory, anti-ulcer, anticancer, and antihaemolytic properties, suggesting potential interest for pharmaceutical and nutraceutical uses. (Carvalho et al., 2010). Procyanidins are the other main constituents in quince fruits, and such compounds are known for their antioxidant, cardiac-vascular & cancer-related effects (Crozier et al., 2009). The large amount of phenolics and long use in traditional medicine of quince fruit prompted us to consider this natural product as a valuable source of phytoconstituents to be exploited in nutraceutical products (Patel et al., 2011).

*Taraxacum officinale* commonly known as dandelion, is a well-known herb that is consumed all over the world. Due to its wealth of nutrients, *Taraxacum* can also be utilised as a vegetable in addition to being a medicinal plant (Bajaj 1994). *Taraxacum* consumption has been shown to be protective against liver and lung damage in rats (Domitrovic et al., 2010; Liu et al., 2010). Numerous functional elements, including taraxacerin, triterpenoid, chlorogenic acid, saponin, and carotenoid, are responsible for this significant biological activity (Shi et al., 2008). Researchers have focused on *Taraxacum* species because of their antioxidant potential in addition to its analgesic, anti-inflammatory, anticarcinogenic, anti-allergic, anti-hyperglycemic and anti-inflammatory properties (Bajaj 1994). Furthermore, locals in the Northern Himalayas have consumed fresh *T. officinale* leaves as a vegetable diet. Additionally, because of its purported medical qualities, extracts are employed as flavouring agents in a variety of food products, such as alcoholic beverages, soft drinks, frozen dairy desserts, candies, baked goods and gelatins.

*Malva neglecta* is one of the most used herbal medicines. It is an edible plant wherein its boiled leaves are taken as wholesome vegetable. Leaves and roots of *M. neglecta* are used in traditional medicine for wound healing in several countries of the world (Ozudogru et al., 2011). The leaf possesses the ability to treat diabetes, cough, gynaecological diseases, and stomach aches. The herb is used to cure dermatitis, fractured bones, burns, and throat infections (Bushra et al., 2012).

Phenolic compounds constitute a large and heterogeneous class of compounds with a very wide distribution in taxa of higher plants. Despite this almost ubiquity, experimental evidence has demonstrated that each plant species is characterized by the presence of a limited number of compounds. Within each species, the nature of these compounds can vary from organ to organ but is constant enough toward several other factors. These facts have been used in recent years, in the characterization of several food products of plant origin by their phenolic profile. Factors contributing to the variability in phenolic distribution include cultivar and genetics, geographical origin, maturity, climate, position on tree and agricultural practices (Spanos et al., 1992).

The large amount of phenolics & carotenoids and long use in traditional medicine

of the selected minor fruits & vegetables prompted us to consider natural products as a valuable source of phytoconstituents to be exploited in nutraceutical products. The aim of the present work was to explore and characterize the polyphenolic & carotenoid composition of the selected indigenous fruits and vegetables by liquid chromatography–mass spectroscopy (LC-MS) analysis.

## 2. Materials & Methods

### 2.1. Chemicals and Reagents

MS grade formic acid, methanol, water & acetonitrile were purchased from Fisher Scientific (Mumbai, India). Double- deionised water from a Milli-Q-system from Millipore (Elix Technology, Bangalore India) was used. The standard compounds Quinic acid, Malic acid, tr-Aconitic acid, Gallic acid, Chlorogenic acid, Protocatechuic acid, Tannic acid, tr-Caffeic acid, Vanillin, P- Coumaric acid, Rosmarinic acid, Rutin, Hesperidin, Hyperoside, 4-OH-Benzoic acid, Salicylic acid, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Hesperidin, Luteolin, Kaempferol, Apigenin, Rhamnetin, Chyrsin, 3-O-Caffeoyl quinic acid, 4-O-Caffeoyl quinic acid, 5-O-Caffeoyl quinic acid, 3,5-di Caffeoyl quinic acid, Quercetin 3- galactoside, Kaempferol glycoside, Kaempferol 3-glycoside, Kaempferol 3-Rutinoside, Neochlorogenic acid, P-Coumaryl quinic acid, Cyanidin 3- glucoside, Cyanidin 3- rutinoside, Peonidin 3- glucoside, Pelargonidin 3- rutinoside, Peonidin 3-rutinoside, Catechin, Epicatechin, 9- Cis violaxanthin, Neochrome, All- Trans-neoxanthin, All-Trans-violaxanthin, 9-Cis-neoxanthin, Luteoxanthin, Cis-violaxanthin, Antheraxanthin, 9-Cis-violaxanthin, 13- Cis-lutein, All Trans lutein, All Trans zeaxanthin & 9 Cis- lutein, were purchased from Sigma Aldrich (St. Louis, MO, USA).

### 2.2. Plant collection and Extraction

The selected minor fruits and vegetables were collected from fields of the Pulwama district of J&K, India. Plants aerial part (leaves & fruits) were thoroughly washed to remove dust and superfluous material with distilled water and shade dried for two weeks. Then it was crushed into powder form by a mechanical grinder. The coarse powder (1 kg) was subjected to maceration with 1500 mL of 70% methanol for seven days in air tight container with occasional shake at room temperature. The macerate was passed through muslin cloth and then filtered through whatman filter paper no. 1. The filtrate was concentrated via rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) under reduced pressure at 35°C, which resulted in the formation 120 gm (12% yield) semisolid crude extract (Ullah et al., 2016; Zohra et al., 2019). The extract (100 mg) was re-dissolved in methanol (5 mL) and vortexed for 1 min. The final extracts were filtered with regenerated cellulose filters 0.2 µm, (Millipore, Bedford, MA, USA) to 2 mL HPLC vials for analysis.

### 2.3. Methodology

All the retention times and MS data were collected using the $C_{18}$ Accucore aQ column (100×2.1mm, Particle size 2.6µ, Part No. 17326-102130). For MS measurements, a positive/negative switching ion mode was used to obtain better tandem mass spectra and high resolution mass spectra. For all the compounds the high resolution mass data was in good agreement with the theoretical molecular formulas, all displaying a mass error of below 5 ppm thus confirming their elemental composition. In general, peak identities were consistent both within and between analyses. Fragment ions with intensities between 10% of the base peak were reported only when they were needed for comparison. The phenolics were positively identified by their typical UV-absorptions at 254, 280 and 320 nm.

### 2.4. LC Parameters

Qualitative analysis was conducted on a Quantum triple stage quadrupole (TSQ) mass spectrometer, equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector and an autosampler. An aliquot (10 µl) of each methanolic extract was chromatographed on a 100 × 2.1 mm, particle size 2.6 µm Accucore aQ (Part No. 17326-

102130) $C_{18}$ column which was heated to 30 °C. Analytes were separated using 0.1% formic acid + 5 mM ammonium formate in purified water (Mobile Phase A) and 0.1% formic acid + 5 mM ammonium formate in Methanol (Mobile Phase B) under a flow rate of 0.3 ml/min. The gradient employed was 0% B for 2 min, followed by an increase to 100% B over 18 min, and then hold for 1 min. Ions for mass spectrometry were generated using an electrospray source in either the positive or negative mode (depending on analyte ). MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were conducted.

### 2.5. MS Parameters

Mass Spectrometry (MS) analysis was performed on a Thermo Scientific TSQ Endura (TQH-E-1-0565) mass spectrometer with ESI source (Thermo Fisher Scientific, United States). The ionization interface was operated in both positive-ion (PI) electrospray mode for carotenoids and negative ion (NI) mode for other polyphenol compounds. The conditions were the same both in PI and NI electrospray modes. Source parameters were as follows: Sheath gas 30; Ion transfer tube temperature 200°C; vaporizer temperature 300 °C; auxiliary gas 10; sweep gas 1. Scan source parameters: positive ion spray voltage, 3500 V; negative spray voltage 2800 V. The following conditions were used for MS: scan range, 100-1000 (m/z); MS scan rate, 1.0 second; MS/MS scan rate, 4.0 seconds; and collision energy, 30 V. Dual source technology was applied for mass accuracy. ESI low concentration tuning mix was used to adjust the mass calibration of the instrument during analysis. Data was processed by Thermo LC Quan software.

## 3. Results and discussions

**Table 1.** $MS^n$ fragmentation of *Prunus avium* phenolics

| Peak No. | Compound | Molecular ion m/z [M-H] | $MS^n$ m/z (C.E) |
|---|---|---|---|
| 01 | Neochlorogenic acid | 354 | 191 |
| 02 | p- Coumaryl quinic acid | 337 | 163, 191 |
| 03 | Chlorogenic acid | 354 | 191(100), 179(60), 173(5), 135(50) |
| 04 | Cyanidin-3-O-glucoside | 449 | 287(100) |
| 05 | Cyanidin-3-O-rutinoside | 595 | 449(10), 287(100) |
| 06 | Peonidin-3-O-glucoside | 464 | 463(8), 301(100) |
| 08 | Peonidin-3-O-rutinoside | 609 | 463(8), 301(100) |
| 09 | Catechin | 289 | $MS^2$ → 245 (100), 205 (23), 179 (23); $MS^3$ → 203 (100), 227 (17), 189 (12), 161 (18) |
| 10 | Epicatechin | 289 | $MS^2$ → 245 (100), 205 (35), 179 (15); $MS^3$ → 203 (100), 227 (18), 189 (17), 161 (28) |
| 11 | Rutin | 609 | 301 |

C.E= Collision Energy for $ms^n$ Transition

**Table 2.** $MS^n$ fragmentation of *Cydonia oblonga* phenolics

| Peak No. | Compound | Molecular ion m/z [M-H] | $MS^n$ m/z (C.E) |
|---|---|---|---|
| 01 | 3-O-Caffeoylquinic acid | 354 | $MS^2$ → 191 (100); $MS^3$ → 127 (100), 173 (34), 85 (94) 109 (27); $MS^4$ → 109 (100) |
| 02 | 4-O-Caffeoylquinic acid | 354 | $MS^2$ → 191 (100); $MS^3$ → 173 (76), 127 (61), 85 (87), 93 (49); $MS^4$ → 111 (100) 3) |
| 03 | 5-O-Caffeoylquinic acid | 354 | $MS^2$ → 179 (100), 135 (19), 161 (2); $MS^3$ → 135 (100) |
| 04 | 3,5 Dicaffeoyl quinic acid | 516 | $MS^2$ → 245 (100), 205 (23), 179 (23); $MS^3$ → 203 (100), 227 (17), 189 (12), 161 (18) |
| 06 | Kaempferol 7-O-glucoside | 447 | $MS^2$ → 285 (55), 284 (100), 255 (27); $MS^3$ → 255(100), 267(20); $MS^4$ → 255 (100), 163 (30), 227 (34) |

C.E= Collision Energy for $ms^n$ Transition

**Table 3.** $MS^n$ Fragmentation of Carotenoids of *Taraxacum officinale*

| Peak No. | Compound Name | Parent ion m/z $[M+H]^+$ | Fragments $MS^n$ m/z |
|---|---|---|---|
| 01 | 9-or 9'-cis-Violaxanthin | 601.5 | 583 $[M+H-18]^+$, 565 $[M+H-36]^+$, 509 $[M+H-92]^+$, 491$[M+H-92-18]^+$ |
| 02 | Neochrome | 601.5 | 583 $[M+H-18]^+$, 399,421,477. |
| 03 | Trans Neoxanthin | 601.4 | 583.4$[M+H-18]^+$, 565 $[M+H-36]^+$, 547$[M+H-54]^+$, 521$[M+H-80]^+$ |
| 04 | Trans Violaxanthin | 601 | 583$[M+H-18]^+$, 565 $[M+H-36]^+$, 521$[M+H-80]^+$ |
| 05 | 9-or 9'-cis-Neoxanthin | 601 | 583 $[M+H-18]^+$, 565 $[M+H-36]^+$, 547$[M+H-54]^+$, 521$[M+H-80]^+$ |
| 06 | Luteoxanthin | 601 | 583 $[M+H-18]^+$ |
| 07 | Cis Violaxanthin | 601 | 583 $[M+H-18]^+$, 565 $[M+H-36]^+$ |
| 08 | Antheraxanthin | 585 | 567 $[M+H-18]^+$, 549 $[M+H-36]^+$, 505$[M+H-80]^+$ |
| 11 | 13, 13 – cis- Lutein | 569 | 551 $[M+H-18]^+$, 533 $[M+H-36]^+$ |
| 17 | β- Cryptoxanthin | 553 | 535 $[M+H-18]^+$, 461$[M+H-92]^+$ |
| 20 | 15, 15' cis- β-Carotene | 537 | 457 $[M+H-80]^+$, 413$[M+H-124]^+$, 123$[M+H-414]^+$, 177$[M+H-360]^+$, 137 $[M+H-400]^+$ |
| 21 | 9, 9' cis- β-Carotene | 537 | 457 $[M+H-80]^+$, 445$[M+H-92]^+$ |

| | | | |
|---|---|---|---|
| 23 | 13-or 13'-cis-β-Carotene | 537 | 457 $[M+H-80]^+$, 445$[M+H-92]^+$, 400 $[M+H-137]^+$, 269$[M+H-268]^+$, 177$[M+H-360]^+$, 137 $[M+H-400]^+$ |
| 24 | β-Carotene | 537 | 481 $[M+H-56]^+$, 445$[M+H-92]^+$ |

**Table 4.** $MS^n$ Fragmentation of phenolic compounds of methanol extracts of *Malva neglecta*

| **Peak. No.** | **Compound** | **Parent ion (m/z)** | **$MS^2$ (C.E)** |
|---|---|---|---|
| 01 | Quinic acid | 190.95 | 173, 127, 109, 85 (22),93 (22) |
| 02 | Malic acid | 133.05 | 115 (14),71 (17) |
| 04 | Gallic acid | 169.05 | 125 (14),79 (25) |
| 05 | Chlorogenic acid | 353 | 191 (17 |
| 07 | Tannic acid | 182.95 | 124 (22),78 (34) |
| 09 | Vanillin | 151.05 | 136 (17),92 (21) |
| 10 | p-Coumaric acid | 162.95 | 119 (15),93 (31) |
| 12 | Rutin | 609.1 | 300 (37), 271 (51), 301 (38) |
| 13 | Hesperidin | 611.1 | 303,465 |
| 15 | 4-OH Benzoic acid | 136.95 | 93,65 |
| 16 | Salicylic acid | 136.95 | 93,65,75 |
| 19 | Coumarin | 146.95 | 103,91,77 |
| 20 | Quercetin | 300.9 | 179,151,121 |
| 23 | Luteolin | 284.95 | 175,151,133 |
| 24 | Kaempferol | 284.95 | 217,133,151 |

C.E= Collision Energy for $ms^n$ Transition

The qualitative determination of secondary metabolites of the selected indigenous fruits (*Prunus avium & Cydonia oblonga*) and vegetables ( *Taraxacum officinale* & *Malva neglecta*) was performed by LCMS/MS and allowed identification of phytochemicals like organic acids, hydroxycinnamic and caffeic derivatives, catechin, procyanidins, and flavonols**.** Table 1, 2, 3 & 4 shows the identification data containing mass spectra of parent compound and fragments of *Prunus avium, Cydonia oblonga*, *Taraxacum officinale* ***&*** *Malva neglecta*.

### 3.1. *Prunus avium*

Figures 1 & 2 shows the HPLC chromatogram of standards mixture and methanol extract of *Prunus avium*. ESI–$MS^n$ mode revealed the presence of peak 1 with molecular ion peak at 354 m/z.

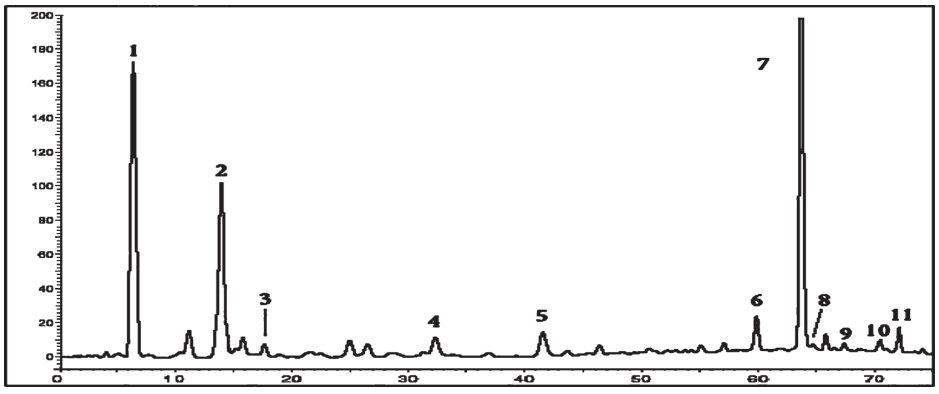

**Figure. 1.** HPLC profile of standards mixture of *Prunus avium*

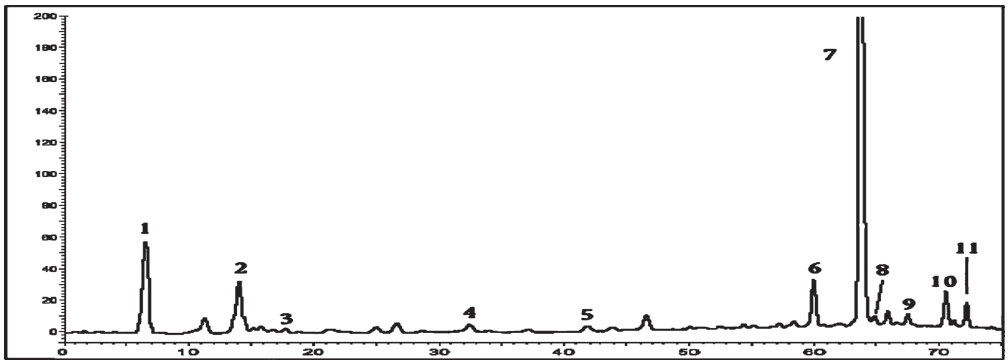


**Figure 2.** HPLC chromatograms of *Prunus avium* extract

The major fragments were at m/z 191. Based on molecular mass and fragmentation pattern, peak 1 was tentatively identified as Neochlorogenic acid. Peak 2 with molecular ion peak at 337 and m/z of its fragments as 163 & 191 was identified as p- Coumaryl quinic acid. Peak 3 having m/z 353 and fragment m/z as 191, 179, 173 & 135 was identified as Chlorogenic acid. Peak 4 with m/z 485 and m/z of its fragment as 287 identified as Cyanidin -3-glucoside. Peak 5 with m/z as 595 and fragments m/z as 449 & 287 identified as Cyanidin 3-rutinoside. Peak 6 with m/z as 463 and its fragment m/z 301 identified as Peonidin 3-glucoside. Peak 8 with m/z 609 with fragments m/z 463 & 301identified as Peonidin 3-rutinoside. Peak 9 & 10 with m/z 289 and its fragments m/z 245, 205, 179,203, 227, 189 & 161 was identified as Catechin & Epicatechin. Peak 11 with m/z 609 and its fragments m/z 301 identified as Rutin.

### 3.2. *Cydonia oblonga*

Figures 3 & 4 shows the HPLC chromatogram of standards mixture and methanol extract of *Cydonia oblonga*. ESI–$MS^n$ mode revealed the presence of peaks 1, 2 &3 as isomers with molecular ion peak at 354 m/z. The major fragments were at m/z 191. Based on molecular mass and fragmentation pattern, peaks 1, 2 & 3 were tentatively identified as 3-O- caffeoyl quinic acid, 4-O-caffeoyl quinic acid & 5-O-caffeoyl quinic acid. Peak 4 with molecular ion peak at 516 and m/z of its fragments is 163 & 191 was identified as 3, 5 dicaffeoyl quinic acid. Peak 6 with m/z 447 and its fragmentation with m/z 285, 255, 267 identified as Kaempferol 7-O-glucoside.

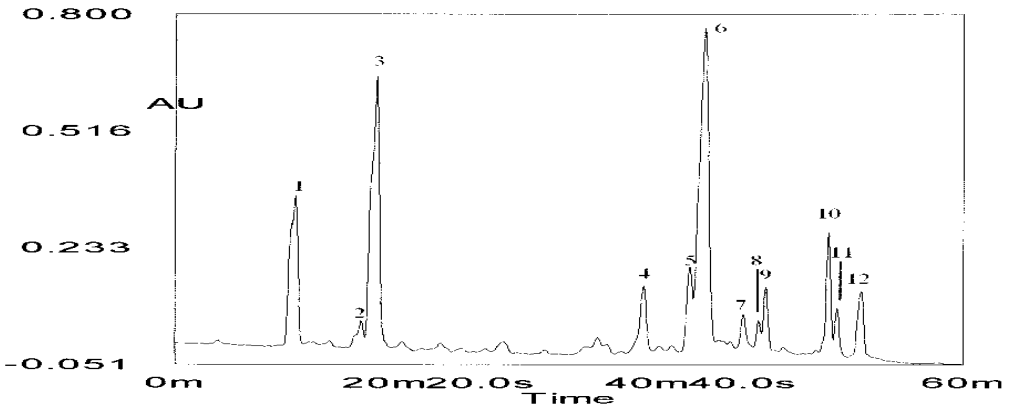


**Figure 3.** HPLC profile of a *Cydonia oblonga* standards mixture

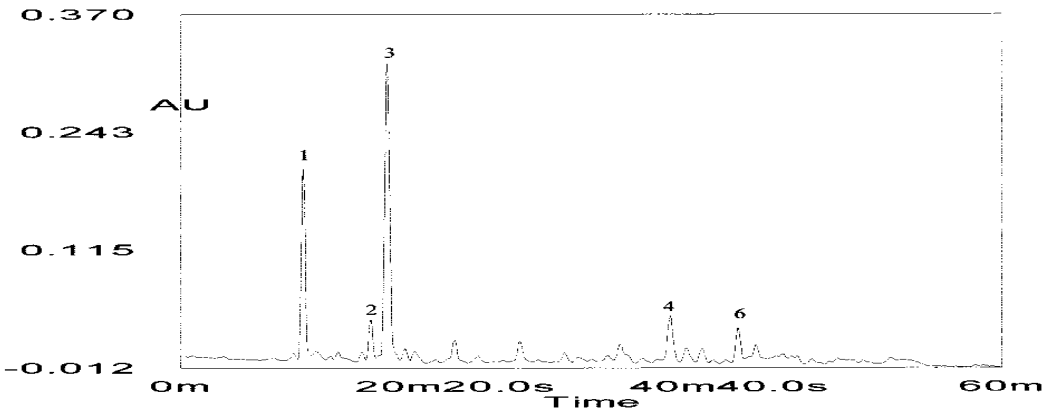


**Figure 4.** HPLC profile of *Cydonia oblonga* extract

### 3.3. Taraxacum officinale

Figures 5 & 6 shows the HPLC chromatogram of standards mixture and methanol extract of *Taraxacum officinale*. Peak 1 was identified as 9- or 9' –Cisviolaxanthin having $[M + H]^+$ at m/z of 601.5. The loss of one molecule of water leads to $[M + H-18]^+$ at m/z of 583 and loss of two water molecules to $[M + H -18-18]^+$ at m/z of 565. Loss of toluene molecule leads to $[M + H -92]^+$ at m/z of 509 and loss of toluene & water molecule to $[M + H -92-18]^+$ at m/z of 491. Peak 2 was identified as Neochrome due to hypsochromic shift of about 20 nm and m/z of protonated $[M+H]^+$ 601.5 with that reported by de Faria et al (2009). Both peaks 3 & 4 were identified as all Trans forms of neoxanthin and violaxanthin respectively based on epoxide test, absorption spectra and mass spectra characteristics. Peak 5 was identified as 9, 9' Cis- neoxanthin as a hypsochromic shift of about 6 nm and a weak absorption band at 326 nm occurred. Peak 6 with $[M + H]^+$ m/z 601 was identified as Luteoxanthin. This 5, 8- epoxy xanthophyll was characterized by the major product ion at m/z 583 $[M + H-18]^+$ and at m/z 221, resulting from the cleavage of $C_{10}$-$C_{11}$ bond in the polyene chain from the epoxy end group. The diagnostic ion at m/z 221 indicated the presence of an epoxy substituent in a b-ring with a hydroxyl group (Britton et al., 2004). The ion at m/z 221 corresponds to the oxo-ring fused to the 3-hydroxy-b-ring. Compound 7 exhibited $[M +H]^+$ at m/z 601 and $MS^2$ fragment at m/z 583 and was identified as Cis- violaxanthin. The mass spectrum displayed fragments at 583 $[M + H-18]^+$, 565 $[M + H-18-18]^+$ which correspond to the loss of one water molecule and two water molecules respectively. Peak 8 with protonated $[M + H]^+$ m/z at 601 and fragments at m/z 567 $[M+H -18]^+$ resulting from the loss of a water molecule and also a fragment at m/z 493 $[M + H-92]^+$ which correspond to the loss of toluene.

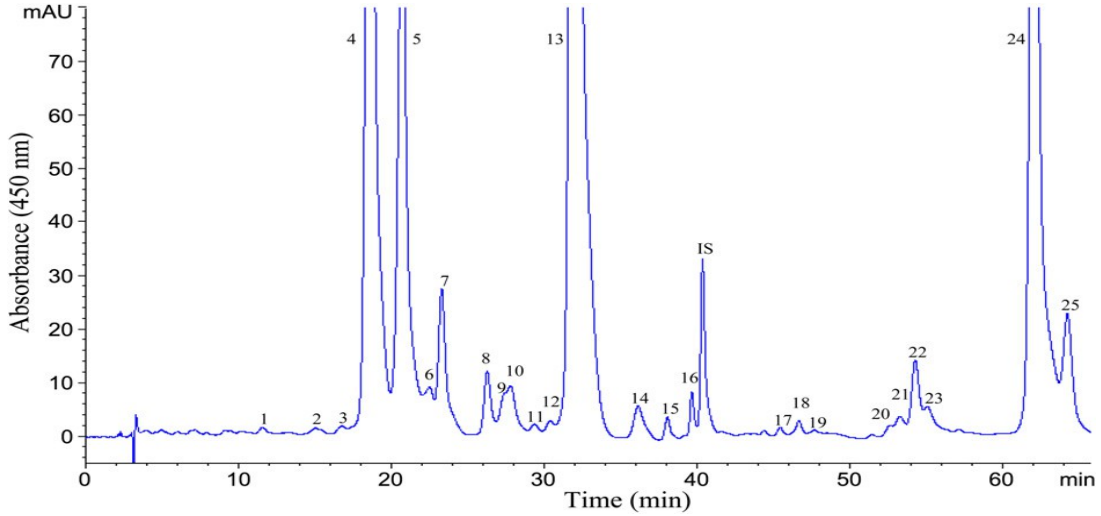


**Figure 5.** HPLC chromatogram of standards mixture of *T. officinale*

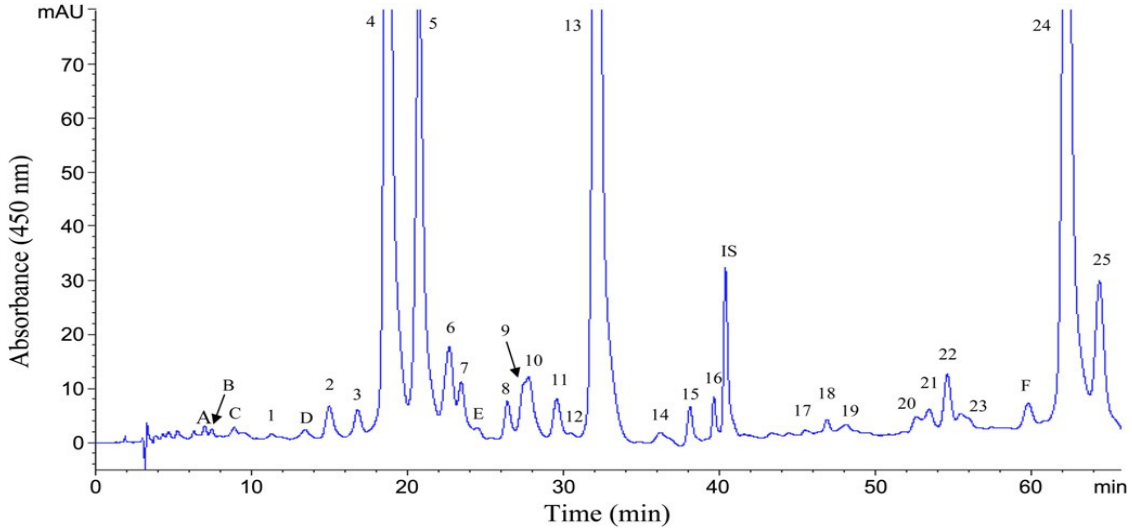


**Figure 6.** HPLC chromatogram of extract of *T. officinale*.
*Peak numbers with alphabetical letters (A-F) indicate additional compounds identified in extract fraction, while 1-25 denote the same standard compound.*

This fragmentation indicated the presence of extensive conjugation within the molecule. The ions at m/z 221 and 181 showed that the epoxide group was in a ring with a hydroxyl group. Peaks 17 and 24 were positively identified as all-Trans forms of β-cryptoxanthin and β-carotene, respectively. The mass spectrum of compound 11 identified as lutein (with one b-ring and one e-ring as end-groups), showed fragments at m/z 551 $[M + H-18]^+$, corresponding to the loss of a water molecule (Crupi et al., 2010). Owing to the presence of a double bond allylic to the hydroxyl group, the fragment at m/z 551 is more stable than the protonated molecule (Rivera et al., 2012).The $MS^2$ spectrum included the fragments $[M + H-18-18]^+$ at m/z 533, $[M + H-18-56]^+$ at m/z 495 and $[M + H-18- 92]^+$ at m/z 459, in agreement with previous studies (Ren et al., 2008). MS analysis of compound 24 showed molecular ion $[M + H]^+$ at m/z 537 and fragment ions $MS^2$ (m/z 481, 457, 445, 400, 269, 177, 137), and was referred as β-carotene (Dequires et al., 2010). In extracts, the additional peaks A-F were identified as Auroxanthin, 13- cis-neoxanthin, Violaxanthin and 9-cis-β-carotene.

### 3.4. *Malva neglecta*

Figures 7 & 8 shows the HPLC chromatogram of standards mixture and methanol extract of *Malva neglecta*. ESI–$MS^n$

mode revealed the presence of peak 1 with molecular ion peak $[M+H]^+$ at m/z 191. The major fragments were at m/z 173, 127,109, 85 & 93. The fragment peak with m/z of 173 occurs due to loss of one molecule of water $[M + H - 18]^+$. Based on molecular mass and fragmentation pattern peak 1 was tentatively identified as Quinic acid. Peak 2 with molecular ion peak at 133 and m/z of its fragments is 115 & 71. Peak 4 having m/z 169 and fragments m/z as 125 & 79 was identified as Gallic acid. Peak 5 with m/z 353 and m/z of its fragment as 191 identified as Chlorogenic acid. Peak 7 with m/z of 183 and fragmentation m/z as 124 & 78 identified as Tannic acid. Peak 9 with m/z as 151 and its fragments m/z as 136 & 92 identified as vanillin. Peak 10 with m/z 163 with fragments m/z 119 & 93 identified as p- coumaric acid. Peak 12 with m/z 609 and its fragments m/z 300, 271 & 201 was identified as Rutin. Peak 13 m/z 611 and its fragmentation with m/z 303 & 465 identified as Hesperidin. Peaks 15 & 16 were isomers with m/z 137 identified as 4- OH – benzoic acid and salicylic acid having m/z of fragments as 93, 65 & 75. Peak 19 having m/z 147 and its fragments m/z 103, 91 & 77 identified as Coumarin. Peak 20 with m/z 301 and fragments m/z 179,151 & 121 identified as quercetin. Peaks 23 & 24 with m/z 285 having fragments m/z 175, 151 & 121 was identified as luteolin for peak 23. Peak 24 with fragments m/z 217, 133 & 151 was identified as Kaemferol.

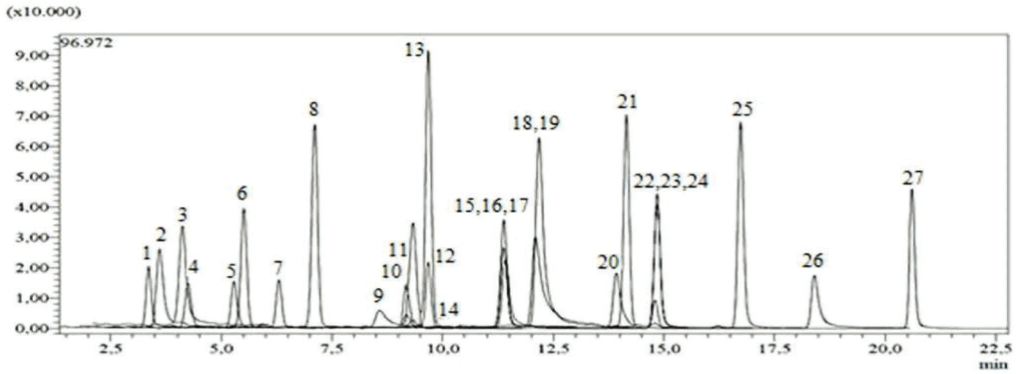


**Figure 7.** HPLC chromatogram of standards mixture of *Malva neglecta*

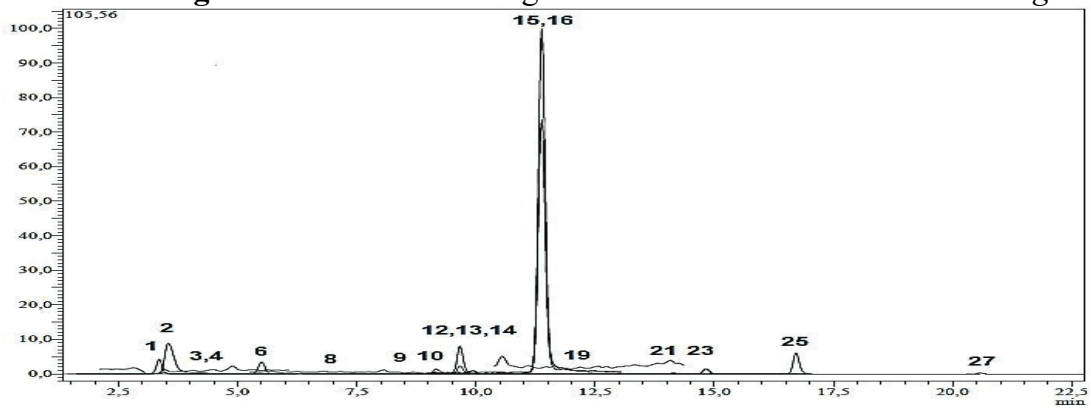


**Figure 8.** HPLC profile of *Malva neglecta* methanol extract

## 4.Conclusions

The results from our study, with identified phenolics & carotenoids, together with the generalization of literature data, allowed us to update the knowledge on the phytochemical constituents of *Prunus avium, Cydonia oblonga, Taraxacum officinale* & *Malva neglecta*. Apart of the well-known and largely explored beta-carotene, they contain carotenoids (e.g. lutein, luteoxanthin, antheraxanthin, zeaxanthin) considered as high-value functional products with extensive applications in human affairs.

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**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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**Ethical Approval**

This study did not involve any animal or human testing.

**Data Availability**

No data was used for the research described in the article.

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# DEVELOPMENT AND CHARACTERIZATION OF MILK FERMENTED WITH VIILI ADDED OF CURCUMA LONGA

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**ABSTRACT**

The promotion of health through proper nutrition is a growing concern in daily lives of many people, so the demand for functional foods is increasing. This study aimed to develop a fermented milk with natural dye by Turmeric fermented by mixed culture called Viili, that contains fungus, yeasts and lacto bacteria. The Turmeric was added as a dye and also for its functional and antioxidant properties. Six fermented milk formulations were prepared: Fm (pure fermented milk), Tm (fermented milk added turmeric - 0.6% w/v); Sm (fermented milk added sugar 10% w/v); STm (fermented milk added turmeric - 0.6% w/v and sugar 10% w/v); Scm (fermented milk added salt 1.3% w/v); ScTm (fermented milk added salt 3% w/v and turmeric). The samples were stored for 30 days and subjected to analysis in the 1st, 15th and 30th day of storage. All formulations had *Lactococcus lactis* counts above $10^8$ CFU / mL. Samples without the addition of turmeric in the are lighter than samples that have added turmeric to the formulation. The pH of the samples showed a significant reduction with variation in acidity. Formulations added turmeric presented curcuminoids unchanged in all samples until the end of storage. Samples without the addition of turmeric showed lower amounts of total phenolics. The viscosity varied between the samples. The general sensorial acceptance of the product using the hedonic scale reached greater acceptance in the product with the addition of salt. When with the addition of sugar, there was a reduction in acceptance.

## 1.Introduction

The concept of functional foods associates to several products the ability to provide physiological benefits and contribute to the health of consumers. The market for products with functional claims has grown significantly, stimulating research and development of new ingredients and products.

Viili is a very viscous fermented milk traditional to the Nordic countries, most commonly found in Finland. Traditionally, it is made from non-homogenized milk, which results in the formation of a cream layer on the surface of the milk (Leporanta, 2003) The fermentation is carried out by a mesophilic culture, which contains *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar diacetylactis and *Leuconostoc mesenteroides* subsp. *cremoris*, along with the fungus *Geotrichum candidum*, which cover the surface of the product (Tamime and Marshall, 1997; Vasilkevic and Shah, 2008). In addition, viili also contains yeasts *Kluyveromyces marxianus* and *Pichia fermentans*, all microorganisms form a unique symbiotic system (López et al., 2010). *Lactococcus lactis* subsp. *cremoris* produces exopolysaccharides

(EPS), thus conferring characteristic intense viscosity.

Several benefits are possible from such microorganisms, including control of intestinal infections, control of cholesterol levels, positive influences on the immune system, improved utilization of lactose in people who do not digest it well, and anticarcinogenic action. This group of bacteria and yeasts that contribute to the regulation of the composition of the intestinal microbiota and offer the possibility of influencing the development of the mucosa and systemic immunity are called probiotics (Gilliland, 2001). Lactic acid bacteria (LAB), of some genera are proven probiotics, can ensure intestinal homeostasis as well as interact with epithelial cells as well as immune cells associated with the gut to induce activation of the immune system (Galdeano et al., 2010). Most administered probiotic cells exert health benefits through adhesion to intestinal cells. However, it has been recommended that oral administration of probiotics be continuous, as cells are constantly eliminated from the digestive tract through feces and host physiological changes and/or administration of antimicrobial agents such as antibiotics (Fung et al., 2011).

Exopolysaccharides (EPS) are long-chain polysaccharides consisting of repetitive units of sugars or sugar derivatives. They are present on the surface of many bacteria, including some lactic acid bacteria (LAB) and may be attached to the bacterial surface forming a capsule, weakly attached, or may be fully secreted into the environment (Ramchandran & Shah, 2010). In general, EPS-producing bacteria exhibit good adhesion properties that may be of interest for transient colonization of the gut. EPS can interact with host cells, and thus are of biological, biotechnological or medical interest (Lopez et al., 2012). In addition EPS can present a selective advantage for probiotic bacteria to survive adverse conditions in the gastrointestinal tract after food ingestion (Salazar et al., 2011; Oerlemans et al., 2021).

EPS-producing LABs have gained considerable attention in the industry, they have traditionally been used for the manufacture of fermented dairy products due to their ability to confer desirable sensory attributes such as increased viscosity, consistency and improved stability and texture. EPS produced by lactic acid bacteria are a natural alternative as a replacement for commercial additives of plant or animal origin, and the use of EPS can result in a safe, natural and healthy final product with improved texture and stability, which can have an important impact on the development of new products (Derriche et al., 2021).

Some studies with EPS, isolated from lactic acid bacteria, showed that they were able to neutralize the effect of bacterial toxins and enteropathogens, thus conferring a potential benefit to the host (Werning et al., 2022; Thorakkatu et al., 2022). Other benefits attributed to EPS are antitumor and immunostimulant activity and prebiotic effect (Cázares-Vásquez et al., 2021; Prete et al., 2021). In addition, studies associate the consumption of EPS with cholesterol level reduction, in addition to antioxidant, anti-inflammatory, anti-cancer activity and promotion of natural immunity (Bengoa et al., 2021; Jurásková et al., 2022).

Turmeric is also known as saffron or golden ginger, it is a plant with the scientific name *Curcuma longa L*., from the Zingiberaceae family, native to South and Southwest Asia and extensively cultivated in India, China, Taiwan, Japan, Burma, Indonesia, and the African continent (Jyotirmayee & Mahalik, 2022). The crop was introduced in Brazil in the 1980s, the plant is easy to cultivate and has the advantage of not requiring special cultural treatments, developing well in various tropical conditions (Tanwar et al., 2022). Figure 1 shows images of the rhizome and turmeric powder. It is used as a dye in food and beverages, as a condiment, as a flavoring agent, and as medicine (Pelissari et al., 2022). As for its sensory characteristics, turmeric has a weakly aromatic odor, reminiscent of ginger, a pungent and slightly bitter taste (ANVISA, 2010). Interest in turmeric has increased significantly in recent years. This is due to the fact that it is a natural product and has color characteristics similar to those of tartrazine, synthetic yellow dye widely used in

the food and pharmaceutical industry, which can cause adverse reactions to man (Somasundaram et al., 2002).

Besides being known for its coloring and flavoring properties, it is well known and exploited by traditional Asian medicine as an anti-inflammatory, anti-arthritic, bile function regulator and cholesterol level reducer, carminative, antispasmodic, antioxidant, anti-diarrheal, and diuretic (Abd El-Hack et al., 2021; Jyotirmayee & Mahalik, 2022). Through studies developed using extracts of turmeric rhizome, antioxidant, antimicrobial, anti-inflammatory and anticancer activities were identified (Shi et al., 2021; Lee et al., 2003). In addition to antioxidant effects, several benefits have been attributed to turmeric, including anticarcinogenic activity. Fuloria et al. (2022) showed that the addition of turmeric ethanolic extract at concentrations of 0.5 to 1%, in the diet of mice, significantly inhibited tumor multiplicity, tumor burden, and tumor incidence, when the administered at early tumor stage. Curcumin, a dietary polyphenol found in turmeric, has been shown in studies to have an anti-adipogenic function. Curcumin inhibits the synthesis of fatty acids, suppressing the accumulation of lipids. Through its interaction with diverse signal transduction pathways, curcumin can reverse insulin resistance, hyperglycemia, and other inflammatory symptoms associated with obesity and metabolic diseases (Boaz, 2011).

Due to the increasing expansion of dairy products in the functional food segment, the objective of this work was to develop different formulations of a fermented milk, named viili, with exopolysaccharides (EPS) and containing probiotic Lactococcus lactis, added of a natural dye, an ingredient that presents functional and antioxidant property. To characterize and analyze the viability of microorganisms and constituent compounds of the formulations, over 30 days of storage at 4°C, in order to enable health benefit claims with the ingestion of the products. To also perform a sensory analysis to verify the acceptance of the fermented milks by potential consumers.

## 2. Materials and methods

### 2.1. Materials

#### *2.1.1. Viili Culture*

To promote milk fermentation, a culture of Mesophilic viili powder contained *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. biovar diacetylactis, *Leuconostoc citrovorum*, *Kluyveromyces marxianus* and *Geotrichum candidum*.

The culture was reactivated by three successive fermentations in skimmed milk (Molico, Néstle) reconstituted at 13% (w/v) and frozen (-18 ºC) until use with 20% glycerol (v/v) in 5mL portions (inoculum).

#### *2.1.2. Curcuma longa*

The turmeric used in the preparation of the formulations was acquired in the local market, in the form of powder, of the same brand (Kirin) and same batch.


**Figure 1**. Images of turmeric in rhizome and powder form (Himesh et al., 2011)

## 2.2. Methods

### *2.2.1. Fermented milk*

Powdered milk was used for the production of fermented milk. skimmed, reconstituted at 13% (w/v) in distilled water. The milk was heated (95°C), in a water bath with thermostat, for 5 minutes, cooled to 20°C. 4%(v/v) of the mesophilic starter culture inoculum added.

The fermentation was carried out in 1L glass flasks at 20°C for 24h. Six different formulations were produced, being them Fm - natural fermented milk without any addition, Tm – milk fermented with the addition of turmeric, Sm – fermented milk with the addition of sucrose, STm – fermented milk with addition of sucrose and turmeric, ScM – milk fermented with addition of sodium chloride, ScTm– fermented milk with addition sodium chloride and turmeric. After the fermentation, the products were refrigerated to 4°C for up to 30 days. The amount of turmeric powder was determined according to the maximum concentration of turmeric, as a dye, allowed by Brazilian legislation in fermented milk (BRASIL, 2000).

### *2.2.2. Lactic Acid Bacteria Count*

The quantification of lactic acid bacteria was performed in MRS agar (Man Rogosa and Sharp, pH 6.5 ± 0.2), with addition of cycloheximide (200 $mg.L^{-1}$), to inhibit fungus. The plates were incubated at 30°C for 72 hours, in anaerobiosis. The count was in $CFU.mL^{-1}$ of the milk fermented (Irigoyen et al, 2005).

### *2.2.3. Lactococcus lactis count*

The count of Lactococcus lactis was performed using the medium M17 added with cycloheximide (200$mg.L^{-1}$) to inhibit fungi (pH 7.2 ± 0.2) with incubation at 30°C under anaerobic conditions for 48 hours, at methodology was performed according to Irigoyen (2005).

### *2.2.4. Total Fungi count*

The fungi count was performed in PDA medium added with acid 10% tartaric acid (pH 3.5 ± 0.2), with incubation temperature of 25°C, in aerobic conditions, for 120 hours. The result was expressed in $CFU.mL^{-1}$ of the fermented milk (BRASIL, 2003).

### *2.2.5. Color*

Color measurements were made with digital colorimeter (Konica Minolta Sensing, Inc., Tokyo, Japan) in CIELab system (L*, a* and b*) with illuminant D65.

### *2.2.6. Viscosity*

The viscosity of the samples was determined using Brookfield digital viscometer, with spindle 4, speed of 12 rpm, in 600mL of a sample kept under refrigeration (Hassan et al., 2022).

### *2.2.7. Curcuminoids*

Curcuminoids present in turmeric powder and samples added turmeric were determined qualitatively by analysis of Thin Layer Chromatography. As support for the stationary phase, it was A silica plate was used to apply and run the samples. the phase mobile consisted of chloroform, ethyl alcohol and glacial acetic acid, mixed in the ratio of 95:5:0.5. The chromatogram obtained was examined under light ultraviolet at a wavelength of 365nm (ANVISA, 2010).

### *2.2.8. Total phenolic content*

The extracts for the analyzes were obtained with 1 gram of the sample lyophilized and addition of 80% ethanol in the proportion 1:10, with stirring for 20 min. The mixture was centrifuged at 3500 rpm, following the supernatant to concentration on a rotary evaporator at 70°C to 10 mL. The solution was then stored at -22°C until used. The analysis followed the methodology described by Adom and Liu (2002).

The determination of phenolic compounds was performed using 2.5 mL of Folin-Ciocalteau reagent (10%), 2.0 mL of sodium carbonate 7.5% and 0.5 mL of sample extracts. The phenolic compounds were determined with reading in spectrophotometer at 760 nm. The quantification was performed by the standard gallic acid curve and the results were expressed in mg equivalents of gallic acid/100g on a dry basis (Swain and Hills, 1959).

### *2.2.9. Antioxidant activity*

The antioxidant capability of the extracts with respect to the ABTS+• free radical (2,2-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid], Sigma Aldrich Chemie, Steinheim,

Germany) was determined using the method described by Sanchez-Gonzales et al. (2005). The absorbance was read at 730 nm in a UV-vis spectrophotometer (model Libra S22, Biochrom, Cambridge, UK). The quantification was based on a standard curve of Trolox (100–2000 mM), and the results were expressed in TEAC as µmol Trolox/g sample on a dry basis. The antioxidant activity by scavenging activity of DPPH• (2,2-diphenyl- 1-picrylhydrazyl, Sigma Aldrich Chemie, Steinheim, Germany) was performed according to Brand-Williams et al. (1995), with absorbance was read at 517 nm in a UV-vis spectrophotometer (model Libra S22, Biochrom, Cambridge, UK). The quantification of the extracts was performed using a standard curve of Trolox (100–1000 mM, Sigma Aldrich Chemie, Steinheim, Germany), and the results were expressed in Trolox equivalent antioxidant capacity (TEAC) as µmol Trolox/g sample on a dry basis.

***2.2.10 pH, titratable acidity and Centesimal***

Acidity by titration with 0.1M NaOH solution, expressing the results in g of lactic acid/100 g fresh weight (IAL, 2008). pH measurements were made with digital potentiometer (Hanna, HI 223) and the centesimal composition of the fermented milk samples was determined through analysis of lipids, ash, moisture, protein and carbohydrates per difference (AOAC, 2006).

***2.2.11. Exopolysaccharides (EPS)***

EPS quantification was performed following Schiavão-Souza et al. (2007), with modifications. The procedure consisted of weighing 10g of the samples in 50mL centrifuge tubes, added with 0.25 mL of acid 80% trichloroacetic acid, the samples were stirred under refrigeration (4°C) for 30 minutes. Afterwards, they were centrifuged at 3000 rpm for 10 minutes at 4°C. The EPS were precipitated by addition of cold ethanol in the supernatants obtained. The EPS were separated through a new centrifugation under the same conditions as above.

Total carbohydrates were determined with the precipitate obtained diluted in 2mL of distilled water. 1mL of the diluted precipitate was added to 1mL of 5% phenol plus 5 mL of concentrated sulfuric acid. The tubes were left to rest for 10 minutes and then warmed up in a water bath at 30ºC for 20 minutes. After this procedure, reading in spectrophotometer at 490nm. The total carbohydrates were determined from the glucose calibration curve at concentrations of 10 to 100 µg/mL.

***2.2.12. Sensory analysis***

The project was submitted and approved by the Ethics Committee in Research Involving Human Beings (Process No. 117.625) of State University of Londrina.

A sensory test was carried out for each product, with the addition of sucrose with the participation of 99 potential consumers and added sodium chloride with 74 potential consumers to evaluate product attributes. The judges were instructed o indicate how much they liked or disliked the products in relation to the attributes of color, aroma, texture, flavor and overall acceptance, through a structured nine-point hedonic scale ranging from “I disliked extremely (1)” to “Like Extremely (9)” (Stone and Sidel, 2004).

After, the judges were also asked to indicate their intention to purchase the product, using a seven-point scale ranging from “Certainly not would buy (1)” to “Certainly would buy (7)” (Stone and Sidel, 2004).

***2.2.13. Statistical Analysis***

The physical, chemical and microbiological analyzes followed the completely randomized design. The analysis was carried out in triplicate and the results were submitted to the Analysis of Variance (ANOVA) and Tukey's test, for comparison of means at the 5% level of significance. The experimental designs for sensory evaluation were randomized block design, where the treatments were the formulations and the blocks the potential consumers. The results were submitted to ANOVA and Tukey's test.

## 3. Results and discussions

### 3.1. Content of curcuminoids

The qualitative analysis during the 1st, 15th and 30th day was performed in fermented milk samples added with 0.6% (w/v) of turmeric and kept under refrigeration at 4°C.

The 3 main curcuminoids present in yellow turmeric and that contribute to its functional properties are: curcumin, dimethoxycurcumin and bisdemethoxy curcumin (Reddy et al., 2019). For comparisons, the curcuminoids present in white curcuma were also analyzed qualitatively by TLC.

From a thin layer chromatogram read under ultraviolet light at a wavelength of 365nm, it was possible to observe and compare the bands of the components in yellow turmeric powder and those present in the samples. In Figure 2 it can be verified for turmeric powder in three distinct bands.

When examined under ultraviolet light (365 nm), turmeric powder presented in the middle part of the silica plate, a green spot fluorescent, which corresponding to demethoxycurcumin, corresponding in figure to point 2. In the upper third, the stain referring to the curcumin, point 3, also fluorescent green and in the lower third, the stain with the same color as the previous ones referring to the bisdemethoxycurcumin, point 1. The fluorescent bands that appear in the figure before point 1, are the sample application sites (ANVISA, 2010).

After reading the plate under UV light, the Retention Factor calculations (Rf) were performed at each of the points to certify that each point actually belonged to the above mentioned compounds. From the descriptions of identification and calculation of Rf for curcuminoids provided by the Pharmacopoeia Brazilian (ANVISA, 2010), the presence of the 3 main curcuminoids in yellow turmeric used for milk formulations fermented. The analysis also allowed to observe that the 3 compounds did not degraded or underwent changes over the 30 days of storage. In Figure 3 one can observe differences in the bands between the white and yellow turmeric.

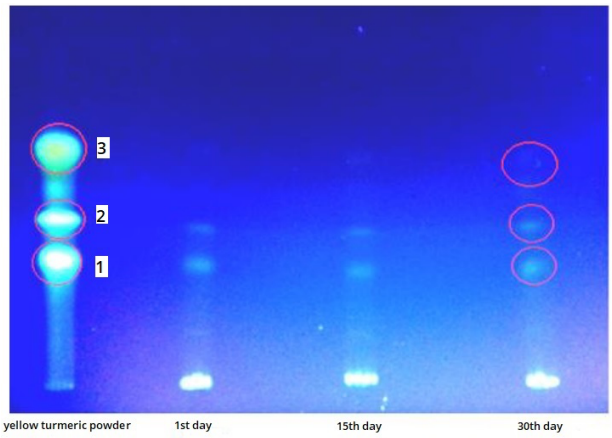


**Figure 2.** Curcuminoids present in yellow turmeric powder and fermented milk samples with turmeric added during the 30 days of storage. Bisdemethoxycurcumin (1); demethoxycurcumin (2); curcumin (3).

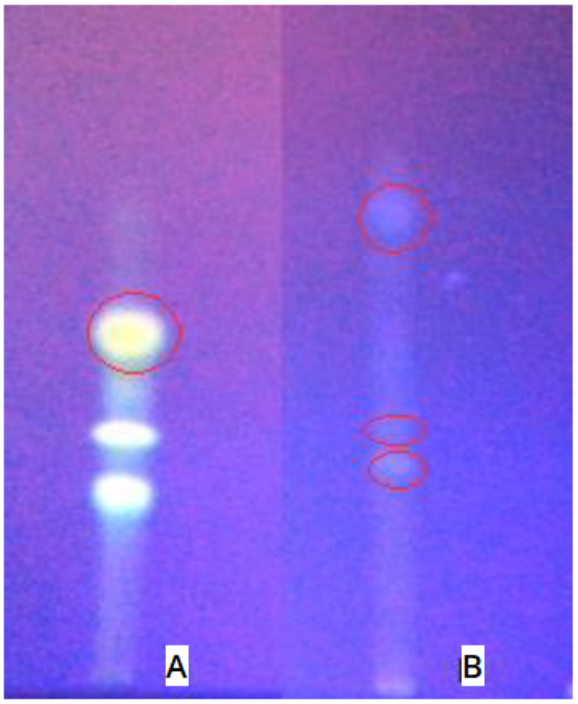


**Figure 3.** Curcuminoids present in yellow turmeric powder (A) and white turmeric (B).

### 3.2. Lactic Acid Bacteria and *Lactococcus lactis* Counts

The table shows the results during the 1st, 15th and 30th day of storage for total lactic bacteria count and *Lactococcus lactis* in the 6 tested formulations. All formulations showed results above 8 Log CFU.mL$^{-1}$ during the 30 days of storage, maintaining high counts, being considered a source of probiotics.

The analysis for fungi was also performed in this work, although *Geotrichum candidum* was described as a of the constituent microorganisms in the culture, none of the treatments identified the presence of the fungus, which in traditional viili grows on the surface of the product and forms a velvety covering, but there is also a type of viili without the fungus (LEPORANTA, 2003). The absence of this microorganism was probably due to the form of fermentation, since pasteurized milk and inoculated with the mixed culture of viili was fermented in 1L closed flasks, which resulted on a reduced contact surface and oxygen limitation, essential for the fungal development.

**Table 1.** Counts of Lactic Bacteria and *Lactococcus lactis* during 30 days of storage.

| Samples** | Time (days) | Lactic Bacteria* (Log CFU.mL$^{-1}$) | *Lactococcus lactis** (Log CFU.mL$^{-1}$) |
|---|---|---|---|
| **Fm** | 1 | 9.18±0.10$^{a}$ | 9.32±0.01$^{a}$ |
| | 15 | 9.08±0.03$^{a}$ | 9.00±0.11$^{abc}$ |
| | 30 | 9.04±0.01$^{ab}$ | 9.11±0.02$^{abcd}$ |
| **Tm** | 1 | 9.30±0.14$^{a}$ | 9.30±0.17$^{a}$ |
| | 15 | 8.70±0.01$^{b}$ | 9.18±0.01$^{abc}$ |
| | 30 | 8.30±0.02$^{c}$ | 8.72±0.01$^{e}$ |
| **Sm** | 1 | 9.08±0.01$^{a}$ | 9.23±0.02$^{a}$ |
| | 15 | 8.80±0.23$^{bc}$ | 9.20±0.01$^{ab}$ |
| | 30 | 8.30±0.11$^{c}$ | 8.96±0.04$^{de}$ |
| **STm** | 1 | 9.28±0.01$^{a}$ | 9.30±0.13$^{a}$ |
| | 15 | 9.00±0.01$^{ab}$ | 9.00±0.02$^{bcd}$ |
| | 30 | 9.00±0.10$^{ab}$ | 9.30±0.01$^{a}$ |
| **Scm** | 1 | 9.15±0.05$^{a}$ | 9.11±0.01$^{a}$ |
| | 15 | 8.70±0.04$^{b}$ | 9.18±0.01$^{abc}$ |
| | 30 | 8.41±0.02$^{bc}$ | 8.87±0.16$^{e}$ |
| **ScTm** | 1 | 9.15±0.02$^{a}$ | 9.30±0.02$^{a}$ |
| | 15 | 9.60±0.00$^{a}$ | 9.00±0.00$^{abc}$ |
| | 30 | 8.48±0.00$^{bc}$ | 8.85±0.07$^{d}$ |

*Means in the same column accompanied by equal lowercase letters do not differ from each other at the level of $p \leq 0.05$. Lactococcus lactis counts in M17 pH 7.2 ± 0.2 at 30°C for 48 hours, total lactic acid bacteria in MRS pH 6.5 ± 0.2 at 30°C for 72h, both with addition of cycloheximide (200mg L$^{-1}$) with counts under anaerobic conditions.

** Fm (pure fermented milk), Tm (fermented milk added turmeric - 0.6% w/v); Sm (fermented milk added sugar 10% w/v); STm (fermented milk added turmeric - 0.6% w/v and sugar 10% w/v); Scm (fermented milk added salt 1.3% w/v); ScTm (fermented milk added salt 3% w/v and turmeric).

After fermentation, on the first day of storage all samples showed counts of total lactic acid bacteria and Lactococcus lactis close to $10^9$ CFU.mL$^{-1}$, however over the 30 days of storage this value was reduced in Tm, Sm, Scm and ScTm samples.

The Fm and STm formulations maintained the counts at 9 log CFU.mL$^{-1}$, until the last day of storage. Maintaining the original number of microorganisms, in samples of pure viili, added only with turmeric and added sugar and turmeric, it probably happened because these formulations presented more favorable conditions for the maintenance of bacteria. According to Donkor et al. (2007) the presence of inhibitors, such as sodium chloride, sucrose, hydrogen peroxide, metabolites, nutrients and buffers, are factors that affect the survival of the microorganism throughout from storage.

In a study carried out by Fadaei, Mortazavi & Pourahmad, (2012), the growth average of *Lactococcus lactis* in fermented milk with Viili culture was 8.39 log CFU.mL$^{-1}$ 5 days after fermentation. As reported, the count of

microorganisms decreased significantly until the 15th day of storage, which did not happen in the present study.

### 3.3. Centesimal composition and Color

The centesimal composition of the fermented milks was stable during the storage at 4°C for the same formulation, showing no significant difference at a 5% significance level over the 30 days.

The percentage of moisture between the different formulations showed a significant difference ranging between 85.00 and 90.16%, with the product without any additive showed the highest humidity, and the one added of turmeric and sugar to less. With the values presented, it can be seen that at as other components are added to the fermented milk its moisture decreases, due to the greater amount of total solids that these present.

On average, the products presented the following compositions for Moisture, Ash, Proteins, Lipids and Carbohydrates respectively. Fm (90.08%, 0.81%, 3.14%, 0.04%, 5.92%). Tm (89.90%, 0.85%, 3.71%, 0.04%, 5.48%). Sm (85.59%, 0.70%, 3.35%, 0.02%, 10.61%). STm (85.33%, 0.80%, 3.67%, 0.04%, 12.64%). Scm (87.10%, 1.93%, 3.31%, 0.01%, 7.63%). ScTm (85.25%, 2.15%, 3.80%, 0.05%, 9.31%). As expected, the added turmeric samples presented the highest value of proteins and those with addition of sucrose, the highest carbohydrate content. Among the parameters, the formulations were similar in most aspects analyzed. With the variation of additives, the variations were concentrated in moisture and carbohydrates on a larger scale.

Table 2 shows the results of the different formulations of the fermented milk with and without added turmeric for color parameters in the CIELAB system (L*, a* and b*). The samples without the addition of turmeric in the formulation presented, on average, values for the parameter L* of 79.10; 80.92 and 81.50 respectively for samples Fm, Sm and Scm, these samples being clearer when compared to the Tm samples, STm and ScTm that had addition of turmeric in its formulation having an average for L* values of 75.52; 77.57 and 77.90 respectively. According to Rein and Heinonen (2004), the increase in pigments causes a decrease in luminosity, a fact that can be observed when comparing the L* values for the formulations that had the addition of turmeric, with a reduction in the values for the parameter L*, thus being darker.

Regarding parameters a* (red (+) and (-) green) and b* (yellow (+) and (-) blue), it is observed that all formulations showed negative values for the parameter a* and positive for b*, that is, all formulations have yellowish-green coloration, with differences in their intensities. To the samples without added turmeric (Fm, Sm and Scm) the values of a* and b* are lower than those of the formulations with the addition of turmeric (Tm, STm and ScTm), having on average presented values respectively for a* and b* of -3.21 and 6.36 in formulations without turmeric and -8.85 and 39.81 for formulations containing turmeric. These differences are the result of the presence of turmeric, which has curcumin (CC) and two other methoxy compounds (DMC and BDMC), being these the compounds that give to turmeric the yellow color. According to Yan et al., (2021), curcumin has a green-yellow in acidic conditions, which is possible to observe in the samples containing turmeric, which showed lower values for a* and higher to b*.

Almeida et al., (2018) evaluated in his work the color parameters of the three different curcuminoids. In this work it is possible verify that each curcuminoid contributes specifically to the parameters L*, a* and b*, that is, although it is not possible to differentiate in the present work of the curcuminoid fractions, it is possible to conclude that is possible that color presented by the formulations containing turmeric is not due exclusively to its addition, but also to the concentration of each fraction of curcuminoids, thus existing difference during storage due to possible variations of these fractions. According to the authors, curcumin presents for the parameters L* (72.84), a* (16.84) and b* (110.06). For DMC the L* (72.15), a* (1.96) and b* (82.73). For BDMC the L* (81.54), a* (-4.72) and b* (49.44).

**Table 2.** Color parameters for the formulations during 30 days of storage.

| Samples** | Time (days) | L* | a* | b* |
|---|---|---|---|---|
| **Fm** | 1 | 80.76±0.00$^{b}$ | -3.16±0.00$^{bc}$ | 6.40±0.04$^{i}$ |
| | 15 | 78.40±0.22$^{d}$ | -3.20±0.04$^{bc}$ | 7.35±0.02$^{ij}$ |
| | 30 | 78.14±0.24$^{d}$ | -3.59±0.01$^{d}$ | 7.08±0.53$^{jk}$ |
| **Tm** | 1 | 76.22±0.06$^{e}$ | -7.32±0.00$^{ef}$ | 34.30±0.42$^{g}$ |
| | 15 | 74.80±0.54$^{g}$ | -8.27±0.05$^{ef}$ | 45.87±2.32$^{a}$ |
| | 30 | 75.55±0.51$^{g}$ | -10.09±0.21$^{e}$ | 39.73±0.04$^{e}$ |
| **Sm** | 1 | 79.43±0.43$^{c}$ | -3.08±0.00$^{b}$ | 6.20±0.02$^{l}$ |
| | 15 | 81.62±0.39$^{b}$ | -3.28±0.00$^{c}$ | 5.13±0.03$^{m}$ |
| | 30 | 81.72±0.11$^{b}$ | -2.91±0.04$^{a}$ | 6.05±0.21$^{l}$ |
| **STm** | 1 | 76.71±0.29$^{e}$ | -8.82±0.05$^{h}$ | 38.17±0.04$^{f}$ |
| | 15 | 77.78±0.08$^{d}$ | -9.51±0.02$^{j}$ | 41.09±0.06$^{c}$ |
| | 30 | 78.23±0.11$^{d}$ | -9.38±0.03$^{j}$ | 40.52±0.04$^{d}$ |
| **Scm** | 1 | 81.64±0.35$^{b}$ | -3.21±0.00$^{bc}$ | 7.59±0.08$^{i}$ |
| | 15 | 79.66±0.19$^{c}$ | -3.26±0.01$^{bc}$ | 4.72±0.04$^{m}$ |
| | 30 | 83.22±0.19$^{a}$ | -3.27±0.02$^{bc}$ | 6.80±0.31$^{k}$ |
| **ScTm** | 1 | 78.77±0.45$^{d}$ | -8.45±0.05$^{fg}$ | 37.31±0.07$^{g}$ |
| | 15 | 76.56±0.27$^{e}$ | -9.20±0.02$^{g}$ | 39.69±0.17$^{e}$ |
| | 30 | 78.34±0.45$^{d}$ | -8.62±0.06$^{i}$ | 41.68±0.01$^{b}$ |

* Means in the same column accompanied by equal lowercase letters do not differ from each other at $p \leq 0.05$. Parameters L* black (0) – white (100), a* red (+) – green (-), b* yellow (+) blue (-).

** Fm (pure fermented milk), Tm (fermented milk added turmeric - 0.6% w/v); Sm (fermented milk added sugar 10% w/v); STm (fermented milk added turmeric - 0.6% w/v and sugar 10% w/v); Scm (fermented milk added salt 1.3% w/v); ScTm (fermented milk added salt 3% w/v and turmeric).

### 3.4. pH and acidity

After fermentation, viili has a pH of 4.43 and an acidity content of 0.9% (Kalkan & Balpetek, 2022). The values found for the pH of the samples on the first day of storage ranged from 4.41 for the fermented milk with no addition, to 4.58 for the sample with added salt (Figure 4). Due to the accumulation of lactic acid during storage, after 30 days, the values obtained for the pH of the samples showed a significant reduction.

In the work of Thamer and Penna (2006), the pH of dairy beverages probiotics, fermented with skimmed milk powder, varied between 4.72 and 4.83. Casarotti et al. (2014), found values between 4.38 and 4.13 when analyzing milk fermented with probiotic culture over 28 days of storage.

The products presented an acidity variation between 0.90 and 1.4% of lactic acid during storage. The values found are within those established by current legislation, which determines the value minimum of 0.6 and maximum of 1.5 g of lactic acid/100mL of fermented milk (Brasil, 2000). The increase in acidity and decrease in pH value can be justified by the accumulation of lactic acid, because even after fermentation, under cold storage, fermenting bacteria hydrolyze lactose (Ugidos-Rodríguez, Matallana-González & Sánchez-Mata., 2018). In samples with turmeric, even if added in a small amount, 0.6%, there was a slight increase in acidity.

The acidity found by Vélez-Ruiz (2019) in yogurts with probiotic characteristics ranged from 0.45 to 0.63 g.100mL-1. Celik & Temiz, (2022) found values in yogurts that varied between 0.80 and 0.93 g.100mL-1, while Hakimi, Zahraee, & Rohani (2018), when analyzing yogurts from commercial brands obtained acidity values of 0.90 to 0.95 g of lactic acid in 100mL.

### 3.5. Phenolic Compounds and Antioxidant Capacity

The phenolic compounds present in samples are presented in Figure 5. The addition of turmeric powder promoted a significant increase in total phenolics, even in small quantity (0.6%). For the total phenolic compounds, the samples without the addition of turmeric: Fm, Sm and Scm, ranged from 66.15 to 92.39 mg gallic acid equivalents. $100g^{-1}$. Samples with added turmeric powder ranged from 90.79 to 130.88 mg gallic acid equivalents.$100g^{-1}$. With the increase in the levels of phenolic compounds, there was also an increase in the antioxidant capacity of the samples, as shown in Figure 5, in the DPPH and ABTS tests. Naturally, due to the greater amount of phenolics, there was a greater availability for antioxidant action.

According to the literature, Curcuma longa has a high content of total phenolic compounds, the turmeric rhizome contains from 4 to 8 mg.100g-1 of curcuminoid pigments. In some cases, the concentration of curcuminoid pigments can reach up to 11.80 mg.100g-1. These variations can be due to several factors such as different varieties or

cultivars, period of bulb development and cultural practices (Mathai, 1976; Nguyen et al., 2021).

Phenolic compounds are also present in the formulations of viili without the addition of turmeric. The occurrence of phenolic compounds in milk and dairy products can be a consequence of several factors, such as example, the consumption of certain forage crops by livestock, the protein

catabolism by bacteria, contamination with sanitizing agents, process-induced incorporation or its deliberate addition (O'Connell & Fox, 2001).

### 3.6. Exopolissaccharides (EPS)

Table 3 contain the results obtained for glucose and an estimation of viili exopolysaccharide sugar fractions and total EPS in the samples evaluated during 30 days of storage.

In this study were found for the 6 formulations of viili analyzed, different values referring to the amount of EPS produced. In between the samples without added sugar the lowest amounts of EPS were found in Fm and Tm, with 75.71 and 74.48 mg/L and the highest for Scm and ScTm with 90.46 and 93.19mg/L respectively. For Nguyen et al., (2020), for some strains of EPS-producing lactic acid bacteria, such as those of Lactococcus, through variations in physiological conditions it can be increase EPS biosynthesis.

Viili is characteristic for having a sticky texture and high viscosity due to exopolysaccharides (EPS) produced by Lactococcus lactis ssp. cremoris (Gotoh et al., 2021). The yield of EPS produced by different LAB can usually vary between 50-2700mg/L (Yusra et al., 2022). The EPS of this fermented milk is described as being a pentasaccharide composed of galactose, glucose, rhamnose in a similar proportion 02:02:01 (Kumar et al., 2022). Through this proportion, the amount of EPS present in the viili can be estimated by analysis of total carbohydrates, using glucose as standard.

Studies indicate that glucose was more efficient than fructose, lactose or galactose as a carbon source for growth and biosynthesis of EPS to Lactobacillus fermentum F6 (Zhang et. al, 2011). Other works also found similar results using glucose as a source of carbon for the production of EPS by many bacteria (Kumar et al. 2019). Mıdık et al., (2020) concluded that Lactococcus delbrueckii subsp.bulgaricus NCFB 2772, produced three times more EPS with glucose as the source of sugar. Gotoh et al., (2021) found that Lactococcus lactis subsp. cremoris NIZO B40, produced about nine times more EPS with glucose as a sugar source under acidifying conditions.

Yang et al., (1999), found a variation between 164 and 263 mg/L of EPS produced by strains of Lactococcus lactis ssp. cremoris grown in skimmed milk.

### 3.7. Viscosity

The values obtained for the viscosity analysis, performed with Brookfield viscometer, were between 810.70 and 923.76 centipoise (Table 4). The viscosity of fermented milk is largely due to by the presence of EPS. According to Damodaran, Parkin, Fennema (2010), soluble polymers such as proteins also promote increased viscosity, being greater the higher the protein concentration.

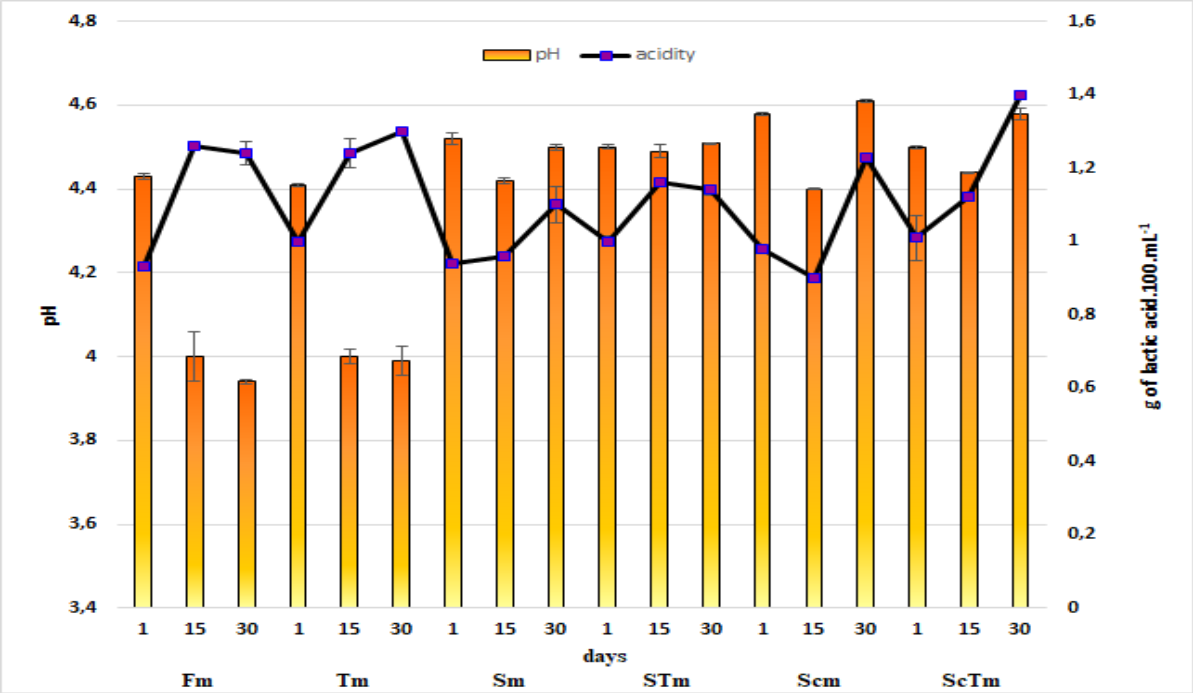


**Figure 4**. pH and acidity during 30 days of storage

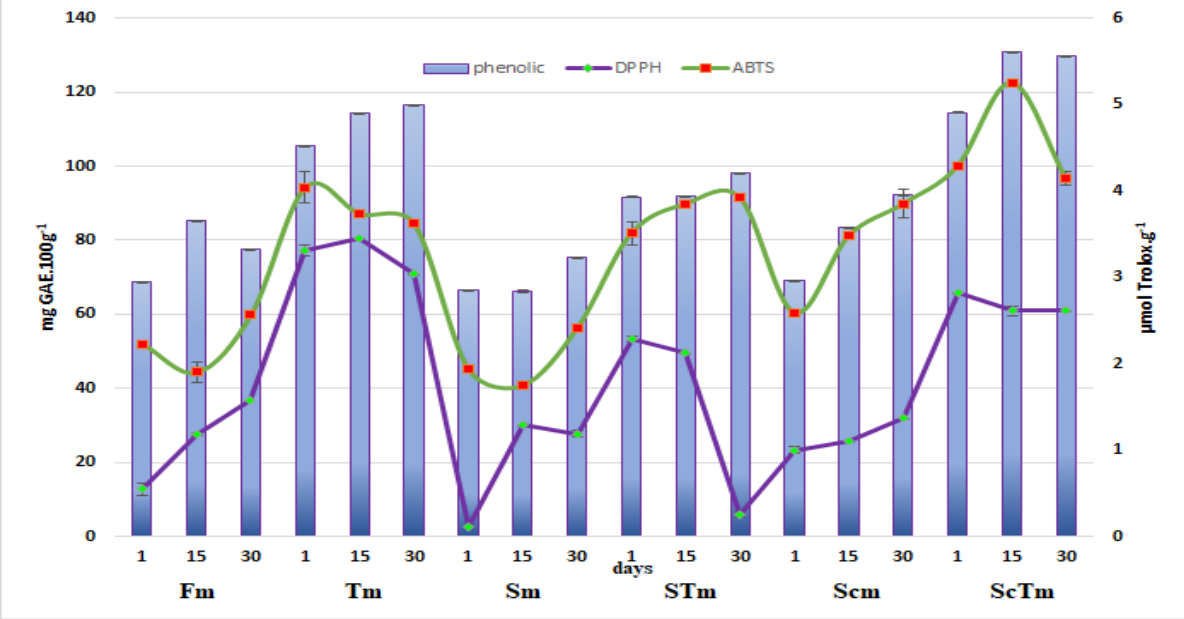


**Figure 5**. pH and acidity during 30 days of storage

EPS is produced by lactic acid bacteria gradually during acidification and during changing environmental conditions, the interactions between polysaccharides and proteins will continue (Yang et al., 2021). The increase in viscosity during the storage it is attributed to the interactions that occur between the EPS and the protein over time, as can be seen in Table 4.

The viili formulations that showed the highest viscosity were Sm and STm. The viscosity of samples without added sugar ranged from 845.11 for ScTm to 896.76 cP for Fm.

**Table 3.** Analysis of glucose (phenol-Sulfuric) of the formulations and estimation of the values of EPS fractions of viili and total EPS during 30 days of storage.

| ** Sample | Time (days) | Phenol-sulfuric glucose (mg/L)* | mean | EPS fractions | | | Total EPS mg/L |
|---|---|---|---|---|---|---|---|
| | | | | Glucose( 2) | Galactose (2) | Rhamnose (1) | |
| **Fm** | 1 | 32.27±0.037$^{def}$ | 30.28 | 30.28 | 30.28 | 15.14 | 75.71 |
| | 15 | 28.29±0.007$^{f}$ | | | | | |
| | 30 | 30.28±0.033$^{def}$ | | | | | |
| **Tm** | 1 | 30.25±0.017$^{def}$ | 29.79 | 29.79 | 29.79 | 14.90 | 74.48 |
| | 15 | 30.99±0.004$^{def}$ | | | | | |
| | 30 | 28.14±0.00$^{4f}$ | | | | | |
| **Sm** | 1 | 61.00±0.002$^{g}$ | 67.68 | 67.68 | 67.68 | 33.84 | 168.21 |
| | 15 | 63.46±0.006$^{g}$ | | | | | |
| | 30 | 78.59±0.014$^{ab}$ | | | | | |
| **STm** | 1 | 65.63±0.004$^{bc}$ | 75.29 | 75.29 | 75.29 | 37.65 | 188.24 |
| | 15 | 81.17±0.107$^{a}$ | | | | | |
| | 30 | 78.77±0.008$^{ab}$ | | | | | |
| **Scm** | 1 | 41.44±0.077$^{def}$ | 36.18 | 36.18 | 36.18 | 18.09 | 90.46 |
| | 15 | 29.08±0.018$^{def}$ | | | | | |
| | 30 | 38.03±0.022$^{def}$ | | | | | |
| **ScTm** | 1 | 43.56±0.083$^{de}$ | 37.27 | 37.27 | 37.27 | 18.64 | 93.13 |
| | 15 | 37.28±0.021$^{def}$ | | | | | |
| | 30 | 44.40±0,045$^{d}$ | | | | | |

* Means in the same column accompanied by equal lowercase letters do not differ from each other at $p \leq 0.05$.

** Fm (pure fermented milk), Tm (fermented milk added turmeric - 0.6% w/v); Sm (fermented milk added sugar 10% w/v); STm (fermented milk added turmeric - 0.6% w/v and sugar 10% w/v); Scm (fermented milk added salt 1.3% w/v); ScTm (fermented milk added salt 3% w/v and turmeric).

### 3.8. Sensory analysis

The sensorial analysis of the fermented milks was carried out by evaluating the acceptance of the samples through the 9-point hedonic scale. For the product added with sucrose, presented in the form of fermented milk, the test had the participation of 99 volunteers where 53.5% were women aged 17 to 50 and 46.5% men aged 17 to 35 years. The test with the added product of chloride of sodium, presented in the form of salad dressing, had the participation of 74 volunteers, 63.5% women aged 17 to 50 and 36.5% men aged 17 to 35 years.

Of the total number of participants, 76% stated that they liked and consumed fermented milk, 54% reported the same in relation to the sauce for salad and 89% said they liked and consumed probiotic products.

In the acceptance test, the attributes appearance, aroma, flavor, texture and overall acceptance of four milk formulations fermented (Sm, STm, Scm, ScTm).

For products with added sucrose, presented in the form of fermented milk, there was greater

acceptance of the attributes for formulation Sm, without the turmeric (Table 5). Products presented in the form of salad dressing, with addition of sodium chloride, had a greater acceptance than those with addition of sucrose, with no significant difference between the two formulations with added salt, Scm and ScTm (Table 5).

The results obtained for acceptability, approval, indifference and rejection referring to each of the 4 formulations are found in Figure 6A. The Acceptability between formulations ranged from 4.47 to 7.13, with that the formulations with sugar (Sm 5.32 and STm 4.47) presented lower significant acceptability compared to those with salt (Scm and ScTm).

There was difference significant for the acceptability among the added samples of sucrose, with and without the addition of turmeric. The sample Sm, with the addition of sucrose and without turmeric was the most accepted compared to STm. Among the samples with salt, which had very close average acceptability values, there was no difference significant. The standard deviation between acceptability values was quite high, mainly for the first two formulations (Sm and STm), this shows the divergence in the values provided by each taster.

**Table 4**. Viscosity of formulations after 30 days of storage, expressed in centipoise

| Sample ** | Viscosity (cP) | | |
|---|---|---|---|
| | 1* | 15* | 30* |
| **Fm** | 808.04±10.50$^{f}$ | 865.83±14.89$^{bcd}$ | 896.76±11.47$^{abc}$ |
| **Tm** | 866.91±20.71$^{bcd}$ | 887.56±9.91$^{bcd}$ | 893.07±17.28$^{abc}$ |
| **Sm** | 889.96±17.29$^{abc}$ | 906.66±11.55$^{ab}$ | 920.53±7.33$^{a}$ |
| **STm** | 891.44±17.96$^{abc}$ | 915.29±15.68$^{a}$ | 923.76±6.43$^{a}$ |
| **Scm** | 810.70±2.04$^{f}$ | 827.16±19.01$^{ef}$ | 856.17±16.81$^{cde}$ |
| **ScTm** | 812.03±8.68$^{f}$ | 814.56±9.15d$^{ef}$ | 845.11±20.78$^{def}$ |

* Days. Means in the same column accompanied by equal lowercase letters do not differ from each other at $p \leq 0.05$.

** Fm (pure fermented milk), Tm (fermented milk added turmeric - 0.6% w/v); Sm (fermented milk added sugar 10% w/v); STm (fermented milk added turmeric - 0.6% w/v and sugar 10% w/v); Scm (fermented milk added salt 1.3% w/v); ScTm (fermented milk added salt 3% w/v and turmeric).

**Table 5**. Acceptance of sensory attributes of fermented milk with viili culture*

| Sample** | Appearance | Aroma | Flavor | Texture | Global Acceptance |
|---|---|---|---|---|---|
| **Sm** | 6.8±1.8$^{a}$ | 5.3±1.9$^{a}$ | 5.5±2.2$^{a}$ | 5.2±2.3$^{a}$ | 5.3±2.1$^{a}$ |
| **STm** | 5.6±2.3$^{b}$ | 4.7±2.1$^{b}$ | 5.4±2.2$^{a}$ | 4.1±2.4$^{b}$ | 4.5±2.2$^{b}$ |
| **Scm** | 7.6±1.6$^{a}$ | 7.2±1.5$^{a}$ | 7.0±1.6a | 7.2±1.8$^{a}$ | 7.2±1.7$^{a}$ |
| **ScTm** | 7.7±1.1$^{a}$ | 7.2±1.3$^{a}$ | 7.0±1.6$^{a}$ | 7.2±1.8$^{a}$ | 7.2±1.4$^{a}$ |

** Sm (fermented milk added sugar 10% w/v); STm (fermented milk added turmeric - 0.6% w/v and sugar 10% w/v); Scm (fermented milk added salt 1.3% w/v); ScTm (fermented milk added salt 3% w/v and turmeric).

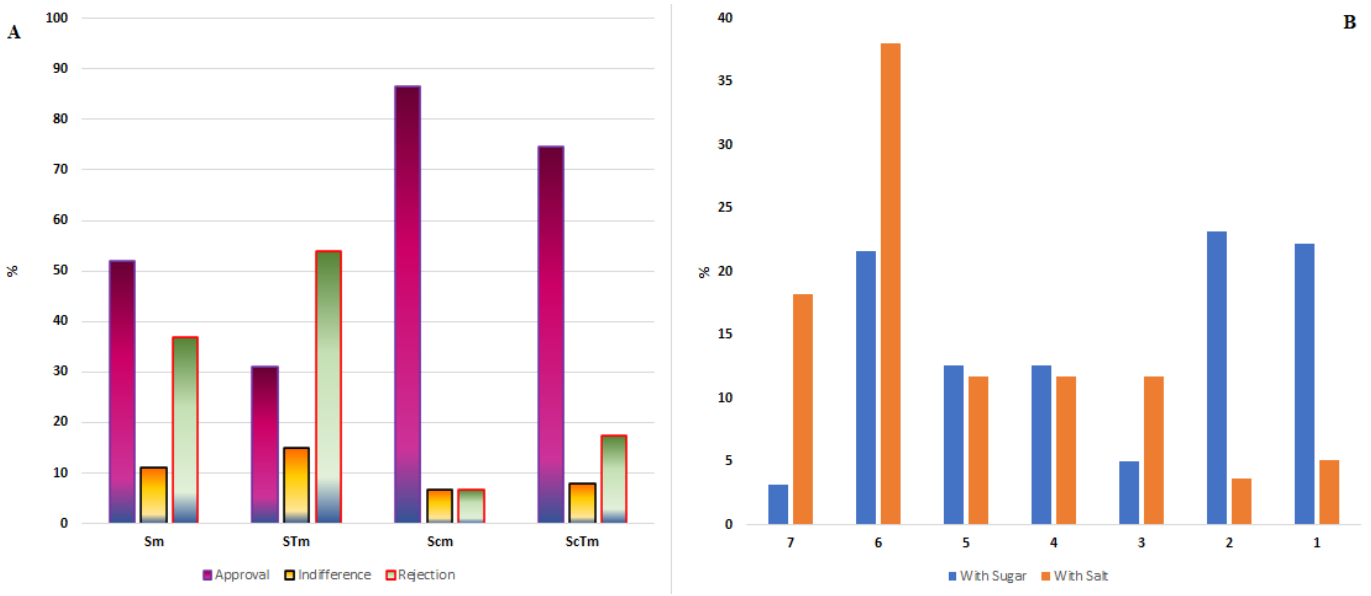


**Figure 6**. **A:** Percentage of approval, indifference and rejection of products (Approval, percentage of grades from 6 to 9), (Indifference, percentage of grades 5) and (Rejection, percentage of scores from 1 to 4). **B:** Tasters' purchase intention for milk fermented with sugar or salt. (7 – Would certainly buy), (6 – would possibly buy), (5 – maybe buy), (4 – Maybe buy it, maybe not), (3 – Maybe not by), (2 – I might not buy) and (1 – I certainly would't buy).

The approval, indifference and rejection percentages allow observe the perception of the panelists in relation to each formulation (Figure 6A). The sample with added sugar only was approved by 52% of the panelists who gave scores between 6 (I liked it slightly) and 9 (I liked it extremely), 11% if were indifferent to it with a score of 5 (I neither liked nor disliked it) and 37% of the judges rejected the formulation As, with the attribution of grades from 1 (dislike extremely) to 4 (dislike slightly).

For the formulation with added sugar and turmeric (STm) an approval was 31%, indifference 15% and rejection 54%.

The product with addition of salt, served in the form of salad dressing was the one with the highest acceptability 7,13 and also the highest approval rating 86.6%. The formulation with salt and turmeric had high approval by judges 74.7% and low levels of indifference and rejection, 8% and 17.3%, respectively.

For the acceptability of probiotic low-fat yogurt, Karaca et al., (2019) obtained values between 4.4 and 7.6. In similar work, Boukria et al., (2020), found values for the acceptability of fermented skimmed milk per mixed culture between 6.18 and 6.65. The viscous fermented milks typical of the Nordic countries are very widespread and appreciated in northern Europe. Finland and Denmark present the highest consumption of the Nordic group with an average of 41 kg per person year, with an average consumption of 100 g per day/person (Fondén et al., 2006; Ganina & Krasnova, 2021). Nonetheless in Brazil, viili is not known, and its sensory characteristics, such as high viscosity and acidity, are a novelty for the palate of its potential consumers.

Figure 6B contains the percentages obtained with the application of the purchase intention test, the two products developed, fermented milk (with sugar) and salad dressing (with salt) were evaluated.

## 4. Conclusions

The addition of turmeric, salt or sugar to milk fermented with the mixed culture viili, did not cause a decrease in microbial viability, being

thus, the claim of a probiotic product can be maintained for up to 30 days refrigerated storage.

The addition of turmeric to the viili fermented product contributed to the increase in the content of phenolic compounds in the product, causing an increase in antioxidant activity

The product viili added with turmeric and salt can be used as a functional salad dressing, as in addition to maintaining the aforementioned benefits, it was well accepted by potential consumers.

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