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# QUALITY CHARACTERISTICS OF MUFFINS PREPARED FROM REPLACEMENT OF WHEAT WITH BARLEY: NUTRITIONAL, ANTI-OXIDATIVE AND MICROBIAL POTENTIAL

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**ABSTRACT**

The objective of present study was to investigate the sensorial, nutritional and microbial value and acceptance of muffins prepared from barley flour as partial replacement of wheat flour. The barley flour was incorporated in the proportion of 100:0, 95:5, 90:10, 85:15, 80:20 and 75:25% of wheat flour for the formulation of muffins and found that muffins containing barley flour were nutritionally superior. The results revealed that with increase in incorporation of barley flour, a positive increase in protein, total phenolic compounds and scavenging activity toward $ABTS^{+}$ and DPPH of muffins was observed. However, baking led to a reduction in phenolic and antioxidant properties. The muffins were also found microbiologically safe for human consumption. This study suggested that partial replacement of wheat flour with barley flours rich in nutritional and bioactive compounds, diversify the utilization of barley flour in various bakery products.

## 1. Introduction

Barley (*Hordeum vulgare* L.) is an ancient and one of the most widely cultivated cereal grain possess functional components (β-glucan, B-complex vitamins, tocopherols and phenolic compounds) which provides associated with human health (Punia & Sandhu, 2015). Being rich in dietry fibres and bioactive compounds barley is still an underutilized cereal and used only in brewing industry and as animal feed. Wheat is a dominant portion of a standard diet and basic ingredients of bakery products. In bakery industries, before product formulation, wheat is processed into refined wheat flour. The refined wheat flour have poor quality protein deficient in essential amino acids. Therefore it is recommended that refined wheat flour may be fortified with essential nutrients to meet the need of humans. As reported by Baba et al. (2016) barley possess nutty flavour, better chewiness, good consistency and have potential to reduce blood glucose level and cholesterol.

Therefore, this may be an interesting opportunity to incorporate barley into bakery products to improve their nutritional behaviour.

Barley flour incorporated with wheat flour has been previously incorporated in the production of rusks (Punia et al., 2020); cakes (Yaqoob et al., 2018), chocolate chips cookies (Frost et al., 2011) and bread (Holtekjolen et al., 2008) have been studied previously. The previously reported studies have shown that addition of barley flour into nutritionally poor cereal is a successful attempt of improving their nutritional and antioxidant behaviour. There is a growing interest in developing novel bakery products supplemented with natural antioxidants.

Among breakfast products, muffin ranks 3rd and attract a broad range of consumers (Rosales-Soto et al., 2012) and consumed worldwide at all economic levels due to different varieties, ready-to-eat nature and reasonable cost. The supply of barley functional compounds through muffins

may be an effective way to supply the bioactive compounds. To the best of our knowledge, studies regarding the incorporation of barley in muffins appear to be limited. This prompted us to investigate the effect of incorporation of barley at level of 5,10, 15, 20, and 25 % on the nutritional, antioxidant, microbial and sensorial properties of muffins.

## 2. Materials and methods

### 2.1. Materials

Wheat and barley cultivar were procured from local market of Sirsa, Haryana for preparing muffins. Grains were milled into fine powder using grinder for further evaluation. Wheat flour (WF) was incorporated with barley flour (BF) and the blends were reported as WF (100% wheat flour), BF-5%+WF-95%, BF-10%+WF-90%, BF-15%+WF-85%, BF 20%+WF-80%, and BF-25%+WF-75%, respectively.

### 2.2. Proximate Composition

WF and WF-BF blends were tested for their moisture, ash, fat, and protein contents by employing the standards methods of analysis (AOAC, 1990). The carbohydrate content was calculated by difference.

### 2.3. Muffin formulation and preparation

The muffins were prepared following the method as described by Nicol (1995). Wheat flour (WF) /Wheat flour-barley flour (WF-BF) blends (100g), butter (50g), sugar (50g), eggs (50g), milk (50g), baking powder (3.3g) and salt (0.4) were used as ingredients for muffins. Firstly, sugar, eggs, milk and butter were blended using a mixer and then baking powder, wheat flour and salt were added and mixed properly to produce uniform batter. Further, batter poured into muffin mould and baked at 200 $^{o}$C for 25 min.

### 2.4. Pasting properties of blends

Pasting properties of flours were studied using Modular Compact Rheometer (MCR 52, Austria). Parameters recorded were pasting temperature, peak viscosity, trough viscosity, final viscosity, breakdown viscosity and setback viscosity. All the measurements were replicated thrice.

### 2.5. Total phenolic content (TPC) of wheat flour- barley flour (WF-BF) blends and muffins

TPC of flours from different wheat cultivars were determined by following the Folin-Ciocalteau method as described by Gao et al. (2002). Gallic acid was used as the standard, and results are expressed as µg gallic acid equivalents (GAE)/g of flour.

### 2.6. Total flavonoids content (TFC) of wheat flour- barley flour (WF-BF) blends and muffins

TFC was determined by following the method described by Jia et al. (1998). Catechin was used as standard and the results were reported as µg catechin equivalents (CE)/g of sample.

### 2.7. Antioxidant activity (AOA) of wheat flour- barley flour (WF-BF) blends and muffins

AOA was measured using a modified version of the method described by Brand-Williams et al. (1995). Methanol was used as a blank, and antioxidant activity (AOA) was calculated as percent discoloration.

% AOA= (1- (A of sample$_{t=0}$ / A of control$_{t=30}$) x100

### 2.8. Metal chelating activity (MCA) of wheat flour- barley flour (WF-BF) blends and muffins

MCA of wheat extract was measured by following the method described by Dinis et al. (1994). The chelating activity of the extract for $Fe^{2+}$was calculated as follows:

Iron ($Fe^{2+}$) chelating activity (%) = {1-( Absorbance of sample /Absorbance of control)}x 100.

### 2.9. $ABTS^+$ scavenging capacity of wheat flour- barley flour (WF-BF) blends and muffins

$ABTS^+$ scavenging activity was measured by following the method described by Re et al. (1999). A standard curve was prepared by using different concentrations of vitamin C similar to DPPH assay. $ABTS^+$ scavenging property was expressed as vitamin C in μmol/g of wheat.

### 2.10. Textural parameters of wheat flour-barley flour (WF-BF) muffins

For textural properties, TA-$XT_2$ texture analyzer (stable micro systems, haslemeres, England) were used. From the force time curves of the texture profile analysis (TPA), hardness, firmness, chewiness, cohisiveness and springiness were calculated.

### 2.11. Sensory evaluation of wheat flour-barley flour (WF-BF) muffins

The muffins prepared were evaluated by a semi-trained sensory panel member who assessed the muffins for various sensory attributes such as color, flavor, texture, and overall acceptability, using a 9-point hedonic rating scale ranging from like extremely (9) to dislike extremely (1). Sensory evaluation was done by panel of 30 judges in the age group of 20–35 years, comprising postgraduate students, research scholars, and faculty members of the department.

### 2.12. Microbiological analysis of wheat flour-barley flour (WF-BF) muffins

Muffins were studied for total plate count (TPC) and mould count (MC) as the method described by de-Almeida Marques et al. (2016). A 10-fold dilution was prepared by homogenizing the sample in sterile saline and serially diluted in the same diluents. 1 ml of these dilutions was poured on agar plates for TPC and MC respectively. Plates were then incubated at 37 °C for 24 h and 24 °C for 48 h for determining TPC and MC respectively, prior to counting. The results were expressed as log of colony forming units per gram (log cfu/g) of sample.

### 2.13 Statistical analysis

The data reported in all the tables are expressed as mean ± standard deviation of three independent replications. Analysis of variance (ANOVA) was used to determine significant variations among the samples. When an effect was found to have significant effect by the ANOVA, Turkey's Multiple Comparison Test was used to determine which levels of the effect were significantly different at $P < 0.05$.

## 3. Results and discussions

### 3.1 Proximate composition and Physical and functional properties of WF-BF flour blends

The proximate composition of blends prepared from wheat flour (WF) and barley flour (BF) blends are presented in Table 1a. Incorporation with BF had a significant effect on proximate composition of flour blends. A significant ($p<0.05$) increase was observed in moisture content of flour blends was observed. Barley has been reported to have higher moisture content than wheat that has been associated to greater fiber content of barley (Haruna et al., 2011). For the WF, ash, protein and fat content of 1.83, 12.23 and 2.46 % was observed. A progressive increase in ash, protein and fat, contents of muffin with the increase in WF-BF blends was observed. Blending with BF significantly, ($P<0.05$) increased the protein content of WF-BF blends from 12.56 to 14.23 %. The increase in these nutritional compounds may be attributed to the presence of greater of these compounds in BF than WF. As reported by Yaqoob et al. (2018), an increase in moisture, ash, protein and fat content was observed for cake formulated from wheat-barley blends. Replacement of WF with BF significantly decreased the carbohydrate content to 72.55 to 68.22 %.

Bulk density of WF-WB flour blends decreased with the increase in level of BF from 5 to 25% (Table 1b). The wheat flour showed the highest value of bulk density. Functional properties of WF-BF flour blends increased with the increase in level of BF from 5 to 25%. Incorporation of BF to WF significantly ($p< 0.05$) increased both WAC and OAC (Table 1b) and was found the

highest for WOF 25%. This increase in WAC and OAC may be attributed to the increase in the β-glucan level (Bhatty, 1986). Foaming capacity (FC) for WF was observed 32.2%. As the incorporation of BF was increased, FC was also observed and the values between 32.9 to 35.2 %. For WF and WF-BF flour blends, the emulsion activity was found in the range between 33.3 to 38.5%.

**Table 1a.** Proximate composition of WF-BF blends

| Blends | Moisture (%) | Crude protein(%) | Crude fat (%) | Ash (%) | Carbohydrates (%) |
|---|---|---|---|---|---|
| WF | $9.55^{a}$±0.03 | $12.23^{a}$±0.05 | $2.46^{a}$±0.02 | $1.83^{a}$±0.05 | $72.55^{f}$±0.06 |
| BF-5%+WF-95% | $10.11^{b}$±0.02 | $12.56^{b}$±0.03 | $2.69^{b}$±0.02 | $1.99^{b}$±0.03 | $71.89^{e}$±0.05 |
| BF10%+WF-90% | $10.36^{c}$±0.01 | $12.98^{c}$±0.05 | $2.94^{c}$±0.03 | $2.11^{c}$±0.03 | $70.21^{d}$±0.03 |
| BF-15%+WF-85% | $10.57^{d}$±0.02 | $13.11^{d}$±0.04 | $3.35^{d}$±0.01 | $2.56^{d}$±0.02 | $70.37^{c}$±0.04 |
| BF-20%+WF-80% | $10.78^{e}$±0.01 | $13.90^{e}$±0.02 | $3.68^{e}$±0.01 | $3.07^{e}$±0.02 | $69.03^{b}$±0.05 |
| BF25%+WF-75% | $10.89^{f}$±0.01 | $14.23^{f}$±0.04 | $3.99^{f}$±0.02 | $3.44^{f}$±0.03 | $68.22^{a}$±0.03 |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

**Table 1b**. Functional properties of WF-BF blends

| Sample | Bulk density (g/ml) | WAC (%) | OAC (%) | FC (%) | EA (%) |
|---|---|---|---|---|---|
| WF | $0.534^{f}$±0.02 | $135^{a}$±0.01 | $107^{a}$±0.02 | $32.2^{a}$±0.02 | $33.3^{a}$±0.01 |
| BF-5%+WF-95% | $0.523^{e}$±0.01 | $145^{b}$±0.02 | $123^{b}$±0.01 | $32.9^{b}$±0.01 | $35.23^{b}$±0.01 |
| BF10%+WF-90% | $0.499^{d}$±0.01 | $177^{c}$±0.02 | $134^{c}$±0.03 | $33.6^{c}$±0.02 | $36.2^{c}$±0.02 |
| BF-15%+WF-85% | $0.467^{c}$±0.03 | $197^{d}$±0.01 | $156^{d}$±0.01 | $34.7^{d}$±0.01 | $37.4^{d}$±0.01 |
| BF-20%+WF-80% | $0.411^{b}$±0.01 | $201^{e}$±0.03 | $177^{e}$±0.01 | $35.1^{e}$±0.01 | $38.1^{e}$±0.01 |
| BF25%+WF-75% | $0.356^{a}$±0.01 | $205^{f}$±0.01 | $194^{f}$±0.02 | $35.2^{f}$±0.03 | $38.5^{f}$±0.01 |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

### 3.2. Pasting properties of WF-WB flour blends

Pasting properties of WF and WB flour blends are summarized in Table 2. Viscosity (PV and FV) of WF was observed to be 945 cP and 1329 cP, respectively whereas incorporation of 5 to 25% BF in WF increased PV and FV and the range observed was from 998 to 1198 cP and 1357 to 1599 cP, respectively. Sullivan et al. (2011) reported that BF has higher PV as compared to WF and incorporation of BF into WF significantly ($p<0.05$) increased the pasting properties. SV also increased as the incorporation of BF increased in WB blends and the values ranged from 539 to 707 cP. As proportion of BF in WF increased, a progressive increase in PV, FV and BV was observed, which may be due to the soluble fibers in the BF.

Pasting temperature (PT) of wheat flour was 84.6°C and at increased level of incorporation of BF no significant increase was observed.

**Table 2.** Pasting properties of WF-BF blends

| Blends | Peak viscosity (cP) | Trough Viscosity (cP) | Breakdown Viscosity (cP) | Final Viscosity (cP) | Setback Viscosity (cP) | Pasting Tempera-ture |
|---|---|---|---|---|---|---|
| WF | $945^{a}\pm11$ | $788^{a}\pm31$ | $157^{a}\pm14$ | $1329^{a}\pm13$ | $539^{a}\pm22$ | $84.6^{a}\pm24$ |
| BF-5%+WF-95% | $998^{b}\pm15$ | $799^{b}\pm10$ | $199^{b}\pm21$ | $1357^{a}\pm11$ | $560^{b}\pm31$ | $84.3^{a}\pm19$ |
| BF10%+WF-90% | $1035^{c}\pm21$ | $823^{c}\pm12$ | $212^{bc}\pm25$ | $1418^{b}\pm15$ | $595^{c}\pm33$ | $83.5^{b}\pm17$ |
| BF-15%+WF-85% | $1111^{d}\pm26$ | $825^{d}\pm9$ | $286^{c}\pm28$ | $1497^{c}\pm17$ | $672^{c}\pm31$ | $83.3^{b}\pm25$ |
| BF-20%+WF-80% | $1123^{e}\pm18$ | $829^{e}\pm25$ | $294^{d}\pm33$ | $1523^{d}\pm21$ | $703^{d}\pm18$ | $82.4^{b}\pm31$ |
| BF25%+WF-75% | $1198^{f}\pm22$ | $892^{f}\pm27$ | $306^{e}\pm19$ | $1599^{e}\pm25$ | $707^{e}\pm15$ | $82.1^{b}\pm26$ |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

### 3.3. Textural parameters of WF-BF blends muffins

The textural parameters of the muffins were significantly altered by the replacement of wheat with barley flour (Table 3). The hardness of control WF muffins was 8.46 and as the incorporation of BF increased, an increase hardness was observed. Blending of WF with different proportions of BF, a significant ($P \leq 0.05$) increase in cohesiveness with values between 0.27 to 0.30 was observed when compared with WF muffin (0.25). A significant decrease in springiness values from 31.03 to 28.14 in comparison to WF- muffin (31.22) was observed with replacement of WF with BF.

For WF-muffin, chewiness was found to be 37.11 and the values increased from 38.11 to 45.66 as the BF incorporated upto 25%.Yaqoob et al. (2018) observed an increase in springiness and decrease in chewiness in barley incorporated cake. Replacement of WF with BF also increased the firmness of muffins with values between to 6.31 to 7.33 in comparison to WF muffins (6.01). Texture attributes of muffins made with WF was reported to improved with incorporation of BF. Consequently, WF-BF blends may be considered a functional source for quality improvement of wheat muffins by incorporating BF.

**Table 3**. Texture attributes of WF-BF blends muffins

| Blends | Hardness (N) | Cohisive-ness | Springiness (mm) | Chewiness (Nmm) | Adhesive-ness (Nmm) | Firmness (N) |
|---|---|---|---|---|---|---|
| WF | $8.46^{a}\pm0.02$ | $0.25^{a}\pm0.01$ | $31.22^{a}\pm0.02$ | $37.11^{a}\pm0.02$ | $1.19^{a}\pm0.01$ | $6.01^{a}\pm0.02$ |
| BF-5%+WF-95% | $8.63^{b}\pm0.03$ | $0.27^{b}\pm0.02$ | $31.03^{b}\pm0.02$ | $38.11^{b}\pm0.03$ | $1.35^{b}\pm0.01$ | $6.31^{b}\pm0.03$ |
| BF10%+WF-90% | $8.91^{c}\pm0.01$ | $0.29^{c}\pm0.03$ | $30.87^{c}\pm0.03$ | $38.44^{c}\pm0.02$ | $1.48^{c}\pm0.03$ | $6.62^{c}\pm0.02$ |
| BF-15%+WF-85% | $9.11^{d}\pm0.01$ | $0.29^{d}\pm0.03$ | $30.43^{d}\pm0.01$ | $42.11^{d}\pm0.01$ | $1.56^{d}\pm0.02$ | $6.87^{d}\pm0.01$ |
| BF-20%+WF-80% | $9.26^{e}\pm0.01$ | $0.29^{e}\pm0.02$ | $29.11^{e}\pm0.01$ | $43.67^{e}\pm0.03$ | $1.77^{e}\pm0.02$ | $7.06^{e}\pm0.01$ |
| BF25%+WF-75% | $9.41^{f}\pm0.03$ | $0.30^{f}\pm0.03$ | $28.14^{f}\pm0.02$ | $45.66^{f}\pm0.02$ | $2.01^{f}\pm0.03$ | $7.33^{f}\pm0.03$ |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

### 3.4. Total Phenolics and flavonoids of WF-BF blends and muffins

Phenolic compounds have become the essentiality of food products for their remarkable antioxidant activities and ability to scavenge free radicals to prevent free radicals chain reactions (Gangopadhyay et al., 2016).The total phenolic content (TPC) of WF and BF was

1134 and 2699 µg(GAE)/g. Sandhu et al. (2016) reported TPC in wheat in the range between 974 and 1399 mgGAE/g. Incorporation of BF flour to WF led to a significant ($p < 0.05$) increase in TPC by 5.7, 8.8, 24.4, 42.2 and 61% when 5, 10, 15, 20 and 25% of WF was replaced with BF (Table 4a). After baking significant decrease in TPC was observed for muffins made from WF and flour blends. In case of muffin made from WF, TPC decreased by 9.8% whereas for flour blends the decrease was from 4.3 to 17.95. WOF-10 % had the largest decrease in TPC upon baking. Holtekjolen et al. (2008) reported similar decrease in TPC for bread made by incorporation of barley flour to wheat flour. Leenhardt et al. (2006) reported heat produced during baking may be reason of reduction of phenolic content. Molecular structure of phenolic compounds changes as a result of heating which leads to either reduced chemical reactivity or decreases their extractability due to certain degree of polymerization. TFC of WF was observed 121 µg CE/g and replacement of WF by BF at levels of 5, 10%, 15%, 20% and 25% exhibited TFC of 152, 197, 219, 335 and 398µg CE/g, respectively (table 5). Baking, however led to led to a significant ($p < 0.05$) decrease in TFC. Barley is a rich source of flavonoids as compared to wheat therefore, increasing its proportion in WF increased the TFC significantly ($p<0.05$). Baking led to a significant ($p<0.05$) decrease in TFC. The muffins prepared by WF exhibited TFC of 89 µg CE/g whereas those prepared by incorporating 5, 10, 15, 20 and 25% BF to WF showed TFC values of 132, 145, 166, 211 and 234µg CE/g, respectively. Holtekjolen et al., (2008) and Angioloni and Collar (2011) also observed a reduction in flavonoids content of breads during thermal processing, As reported by Xu and Chang (2008), flavonoids are heat sensible and during processing of food products, flavonoids are started to degrade, however, the extent of degradation of flavonoids depends upon the duration and processing conditions.

**Table 4a.** Total phenolic content and total flavonoids content of WF-BF blends and muffins

| Sample | TPC (µgGAE/g) | | TFC (µgCE/g) | |
|---|---|---|---|---|
| | Before baking | After baking | Before baking | After baking |
| WF | $1134^{a}\pm23$ | $743^{a}\pm25$ | $121^{a}\pm20$ | $89^{a}\pm24$ |
| BF-5%+WF-95% | $1199^{b}{}_{\uparrow5.7}\pm32$ | $887^{b}\pm19$ | $152^{b}{}_{\uparrow25.6}\pm16$ | $132^{b}\pm31$ |
| BF10%+WF-90% | $1234^{c}{}_{\uparrow8.8}\pm15$ | $801^{c}\pm20$ | $197^{c}{}_{\uparrow62.8}\pm12$ | $145^{c}\pm33$ |
| BF-15%+WF-85% | $1411^{d}{}_{\uparrow24.4}\pm25$ | $887^{d}\pm34$ | $219^{d}{}_{\uparrow80.9}\pm24$ | $166^{d}\pm 17$ |
| BF-20%+WF-80% | $1613^{e}{}_{\uparrow42.2}\pm11$ | $934^{e}\pm37$ | $335^{e}{}_{\uparrow193}\pm36$ | $211^{e}\pm 29$ |
| BF25%+WF-75% | $1826^{f}{}_{\uparrow61}\pm26$ | $1122^{f}\pm19$ | $398^{f}{}_{\uparrow228}\pm15$ | $234^{f}\pm31$ |
| Barley | $2699^{g}\pm17$ | - | $1568^{g}\pm26$ | - |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

### 3.5. Antioxidant potential of WF-BF blends and muffins

Various antioxidant assays are currently being used by the researchers to detect the antioxidant potential in natural extracts. Owing to the complex nature of bioactive compounds in natural extracts, single assay is not enough to quantify the antioxidant potential. In the present study DPPH, ABTS, and metal chelating activity assays were used to assess the

antioxidant activity in the extracts. The results obtained from antioxidant analysis of different extracts revealed that BF incorporated muffins significantly ($p$<0.05) improve the antioxidant potential (Table 4b). For WF muffins, DPPH antioxidant activity and Metal chelating activity was observed to be 10.3 and 22.4%, respectively. Incorporation of BF proportion in WF increased the antioxidant activity in terms of DPPH and $ABTS^{+\cdot}$ $ABTS^{+}$ scavenging activity of WF was 7.22%. Increasing the level of BF in the WB blends progressively increased the scavenging activity from 8.11 to 11.78 µmol/g. The increase in antioxidant activities may be due to the higher scavenging activity of barley as compared to wheat. Baking resulted in a decrease in AOA for wheat and WF-BF muffins. Antioxidative compounds are very heat sensitive and reported a reduction in TPC as well as loss of AOA in products during the heat treatment.

**Table 4b.** Antioxidant potential of WF-BF blends and muffins

| Blends | DPPH (%) | | $ABTS^{+}$ (µmol/g) | | Metal chelating activity(%) | |
|---|---|---|---|---|---|---|
| | Before baking | After baking | Before baking | After baking | Before baking | After baking |
| WF | $10.3^{a}$ ±0.03 | $6.7^{a}$±0.03 | $7.22^{a}$±0.01 | $5.11^{a}$±0.01 | $22.4^{a}$±0.02 | $17.4^{a}$±0.03 |
| BF-5%+WF-95% | $12.9^{b}$±0.02 | $8.4^{b}$±0.01 | $8.11^{b}$±0.03 | $5.23^{b}$±0.03 | $23.9^{b}$±0.01 | $17.6^{b}$±0.03 |
| BF10%+WF-90% | $14.9^{c}$±0.01 | $9.7^{c}$±0.02 | $8.75^{c}$±0.02 | $6.03^{c}$±0.01 | $26.8^{c}$±0.02 | $18.1^{c}$±0.01 |
| BF-15%+WF-85% | $17.4^{d}$±0.01 | $12.5^{d}$±0.01 | $9.95^{d}$±0.01 | $6.46^{d}$±0.01 | $32.3^{d}$±0.02 | $18.4^{d}$±0.02 |
| BF-20%+WF-80% | $19.3^{e}$±0.02 | $14.6^{e}$±0.03 | $11.21^{e}$±0.01 | $7.13^{e}$±0.03 | $36.6^{e}$±0.01 | $19.3^{e}$±0.01 |
| BF25%+WF-75% | $21.4^{f}$±0.03 | $16.4^{f}$±0.02 | $11.78^{f}$±0.02 | $7.28^{f}$±0.02 | $39.2^{f}$±0.03 | $20.2^{f}$±0.03 |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly (p < 0.05)

### 3.6. Sensorial attributes of muffins

The sensorial scores reported by the test panel by following 9 point hedonic scale are shown in Table 5. Statistically significant (p<0.05) variations were observed for organoleptic quality (color, flavour, aroma, taste and overall acceptability) of muffin by semitrained panel of judges. The sensory results showed that the chewing properties, taste, aroma and overall acceptability of muffin were best for muffin made from only from wheat flour (WF). Increasing levels of barley flour (BF) slightly decreased the sensorial scores. The results showed that BF supplementation at different levels (5, 10, 15, 20 and 25%) into formulation had considerable effects on the muffins quality. Upto 15% incorporation of BF into WF did not affect sensory attributes of muffin considerably, after that color of muffins became more darker. Gumminess increased for muffins made by BF incorporation to WF. Incorporation more than 20% resulted in low score and the muffin were not acceptable. Incorporation of BF to WF at levels of 25% decreased the sensory scores drastically and muffin were unacceptable due to their irregular shape, dilution of gluten, gummy mouthfeel and barley characteristic flavor/aroma.

According to sensory analysis, overall acceptance of muffin were found the best for control sample. Muffin prepared with BF addition to WF were liked moderately and like slightly by panelists. Although as the incorporation of BF in WF for making muffin

has increased, bioactive potential and antioxidants, but addition upto 15% BF in flour gave satisfactory sensorial results in terms of overall acceptability.

**Table 5.** Sensory analysis of WF-BF blends muffins

| Blends | Color | flavor | Taste | Aroma | Overall acceptability |
|---|---|---|---|---|---|
| WF | $9.1^{f}\pm0.02$ | $8.4^{f}\pm0.02$ | $9.1^{f}\pm0.03$ | $9.2^{c}\pm0.03$ | $9^{f}\pm0.03$ |
| BF-5%+WF-95% | $8.7^{e}\pm0.02$ | $8.1^{e}\pm0.02$ | $8.8^{e}\pm0.01$ | $8.8^{c}\pm0.02$ | $8.7^{e}\pm0.02$ |
| BF10%+WF-90% | $8.3^{d}\pm0.02$ | $7.7^{d}\pm0.01$ | $8.6^{d}\pm0.01$ | $8.5^{c}\pm0.02$ | $8.2^{d}\pm0.02$ |
| BF-15%+WF-85% | $7.8^{c}\pm0.02$ | $7.3^{c}\pm0.02$ | $8.3^{c}\pm0.02$ | $8.3^{c}\pm0.01$ | $8.1^{c}\pm0.02$ |
| BF-20%+WF-80% | $6.8^{b}\pm0.03$ | $6.4^{b}\pm0.01$ | $7.2^{b}\pm0.01$ | $7.3^{b}\pm0.02$ | $6.3^{b}\pm0.01$ |
| BF25%+WF-75% | $6.1^{a}\pm0.01$ | $5.9^{a}\pm0.01$ | $6.1^{a}\pm0.01$ | $6.3^{a}\pm0.02$ | $5.9^{a}\pm0.02$ |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

### 3.7. Microbiological analysis of WF-BF blends muffin

Regarding the shelf life of bakery products, microbiological spoilage is a major limiting factor. Spoilage from microorganism by bacteria, yeast and moulds is the concern in high moisture products which causes manufacturer's financial loss and a cause of threat to consumer's health. Improper handling in packaging, storage conditions, sanitary practices may be the possible reasons of such loses. Result of microbiological analysis of the samples is shown in Table 6. Total plate count reflects the conditions in which the food is manufactured, and stored, this count may be used as a tool to check the keeping quality of the finished product. An increased in incorporation of BF in WF, did not show a significant ($p<0.05$) increase in total plate count and mould count of muffin till $10^{th}$ day .The microbial content of all WF-BF blends muffins suggested that barley incorporated muffins are microbiologically safe for human consumption.

**Table 6.** Microbial analysis of WF-BF blends muffins

| | TPC (log10cfu) | | | | Mold (log10cfu) | | | |
|---|---|---|---|---|---|---|---|---|
| Blends | 0 day | 5th day | 10th day | 15th day | 0 day | 5th day | 10th day | 15th day |
| WF | - | $0.632^{a}\pm0.05$ | $1.432^{a}\pm0.03$ | $3.454^{a}\pm0.02$ | - | $0.211^{a}\pm0.03$ | $1.032^{a}\pm0.02$ | $3.001^{a}\pm0.05$ |
| BF-5%+WF-95% | - | $0.624^{b}\pm0.03$ | $1.445^{b}\pm0.02$ | $3.789^{b}\pm0.02$ | - | $0.221^{b}\pm0.02$ | $1.078^{b}\pm0.05$ | $3.022^{b}\pm0.03$ |
| BF10%+WF-90% | - | $0.597^{c}\pm0.02$ | $1.478^{c}\pm0.05$ | $3.997^{c}\pm0.02$ | - | $0.256^{c}\pm0.03$ | $1.103^{c}\pm0.03$ | $3.156^{c}\pm0.03$ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| BF-15%+WF-85% | - | $0.571^{d}$ ±0.02 | $1.501^{d}$± 0.03 | $4.235^{d}$± 0.03 | - | $0.288^{d}$± 0.02 | $1.143^{d}$± 0.01 | $3.212^{d}$± 0.02 |
| BF-20%+WF-80% | - | $0.521^{e}$± 0.02 | $1.536^{e}$± 0.05 | $4.457^{e}$± 0.02 | - | $0.312^{e}$± 0.05 | $1.167^{e}$± 0.02 | $3.245^{e}$± 0.02 |
| BF25%+WF-75% | - | $0.593^{f}$± 0.03 | $1.578^{f}$± 0.03 | $4.657^{f}$± 0.03 | - | $0.324^{f}$± 0.02 | $1.898^{f}$± 0.02 | $3.567^{f}$± 0.05 |

Mean ±SD- mean and standard deviation of triplicate analysis

Values followed by the same superscript within the column do not differed significantly ($p < 0.05$)

## 4.Conclusions

The nutritional, sensorial and antioxidant properties of muffins were enhanced by progressive enhancement of barley flour in wheat flour. Results showed that substituting WF with BF at level upto 15% produced muffins with better properties almost similar to the control muffins. Baking tests showed that BF addition with <15% significantly impaired the color, taste, aroma and overall acceptability of muffins. Therefore, muffins made from WOF-15% was found to be acceptable in terms of sensory, nutritionally and microbiologically. From health point of view, development of such functional bakery products would be beneficial to improve the nutritional status of consumer.

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## ETHICAL STATEMENTS

Conflict of interest: The authors declare that they do not have any conflict of interest.

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# STATE-OF-THE ART IN AGRICULTURE DIGITAL MANAGEMENT ROMANIA CASE

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**ABSTRACT**

Agriculture, animal and plants management are nowadays subject of profound transformations aimed at sustainable development.

The article show up performance of digital agricultural management by implementing appropriate software and hardware elements in areas subject to desertification and beyond. Digitalization and Tele_ transmission both on s&m farms and on large plantations used to forecast and eliminate disasters caused by pests, dryness and nutrient compatibility are already implemented but are still topics of discussions and reluctant on the part of Romanian farmers.

A survey was launched throughout Romania and it was disseminated by National Rural Development Network (NRDN) to find out the weaknesses and threats but also the opportunities and strengths that would determine the implementation of digital methods and infrastructure in Romanian agricultural management, in order to keep pace with the imposed one by the economic, demographic and climatological conditions.

## 1. Introduction

Agriculture and allied businesses are critical to the economy's long-term development and prosperity. The key difficulties for agricultural production are decision making, crop selection, and supporting systems for enhanced crop output. (Pallathadka, et al., 2021)

But, agricultural habits are changing today and knowledge have to be updated as quick as smart technologies forces the entrance in all fields of activity and even change our life itself. But knowledge that does not change behaviors is useless.

At a time when digitalization, as well as globalization, population ageing and climate changes are elements shaping humankind future, an unseen competition is undergoing between human and machine, between Human Intelligence and Artificial Intelligence. It is, in fact, a kind of race between the verb "have", with all its meanings and the verb "exist".

For the purpose of this study, I look at the extent to which modernization means tackling climate change, desertification and even avoiding a widespread agricultural crisis.

What I find is that Smart Technologies aligning with dedicated software allow a technological revolution on Agriculture (Figure 1). Jobs are reinvented, even at a much faster rate than the ability to train and create new other specializations. In this frame the future jobs in the agriculture, but not only, are and will be reshape, so that humans have to be mates with Artificial Intelligence (AI), Machine Learning (ML), Internet of Things (IoT), Drones, machines which are and will be programmed for this. (Channe, et al., 2015).

This reality push agricultural management activities to require nowadays major transforming and to implement "Processes

Remote Control (PRC) and broadly to use Big Data, as well as GPS, ZigBee technology, GPRS network and Internet to monitor and management the fresh agricultural products and the logistics process." (J. Li et al., 2010)

In the same trend the use of "Wireless Sensor Network (WSN) with IP network in Precission Agriculture (PA) would be an added advantage to existing solutions of PA system." (Z. Suryady, et al., 2011)

Another face of digitalization in agriculture is the introduction of radio-frequency identification (RFID) tags in supply chains which "engenders the need for incorporating and utilizing the additional generated data. It is generally assumed that these data, once generated, are complete and rife with necessary information for making decisions." (Yu-Ju, et al., 2011).

Machine Learning, is another stunning digital tool: as long as it accumulates data and experience, it "identifies the challenges in agriculture". ML is in fact a smart software implemented on matched devices, and it is the one that monitors and alerts regarding the necessity or opportunity of the operations such as treating the plantations or harvesting.

The implementation of smart software solutions in agriculture and suitable hardware also, helps increasing the yield of the farm by "making the best use of the resources that the farmers have at hand" and by rising the "level of quality and health of the crops".(Chandhini, et al., 2016).

Thus, technical performance that mankind has gotten, allows the dedicated software and hardware to ensure the management of agricultural operations for multiple plots and lands.

This article done an overview of the most significant and the newest tracks regarding Digitalization and Trends & Technologies used in Agriculture, thus being helpful for informing and rising awareness as much as many stakeholders possible. The main goal of the study actually, is to reveal the attitude of the Romanian farm managers regarding smart technologies implementation and what are their fears and expectations.

## 2. Materials and methods

The main, up-to-date and relevant scientific literature, reports, notes and statistics was studied in order to be known the state-of-art, as well the latest modern techniques, devices and tools used nowdays in agriculture An analysis was made, in order to highlight the strengths and weaknesses, as well as the opportunities and threats regarding smart technology and its implementation in Romanian agriculture. In this sense, a survey was launched and, thanks to the National Network for Rural Development (NRDN), it was disseminated throughout Romania.

Approaching the problem in a descriptive and easy to understand way, the results of the analysis and the conclusion should substantiate the future economic and agricultural strategies for maintaining the step in Romanian agriculture with the most efficient, effective and green solutions and technologies.

**Figure 1.** Digital farm management
Source photo: https://industrywired.com/new-age-of-agriculture-state-of-digital-farming-in-2020/

## 3.Results and discussions

- What is now the state-of art and what is to come?
- What are Strengths and Opportunities of Full Automation of the Farm & Plantation?
- What does Precision Agriculture Applications mean and what departments benefit from digitalized farm?

These are the questions.

In answer to them, is very representative and synthetically showed by Chandhini. K* et al. ,in the International Journal of Innovative Technology and Research, throughout the ”Flow Chart Agricultural Production System” (Figure 2) how the floor farms, are empowered both with Clouds solutions, drone and satellites transmission and Smart ERP platforms& GIS solutions.

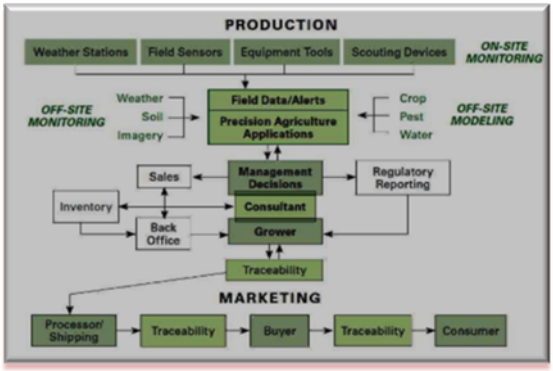



**Figure 2.** Flow Chart Agricultural Production System

*Source; Chandhini. K., et al., (2016).*

Thus, the functions generated by digital tools kit for ensuring the implementation of precision agriculture with very great accuracy are:

- monitoring crops
- ensuring financial management
- planning, executing and monitoring of agricultural works
- allocating the necessary resources, forecasting treatments for being applied just-in-time
- real-time alerts, inspections and observations
- devices management
- supply, stocks, sales evidence
- reports for farms, plots, works and activities. (Figure 3)

Agri Digital Management is accomplished based on global scenarios and a system based on AI (https://emerj.com/ai-sector-overviews/ai-agriculture-present-applications-impact/) and Machine Learning. According with description made by software developers, the software for improved agriculture performance incorporated a GIS mapping and geolocation engine so that performance is achieved as the mapped plots and lands are managed by the machine ”according to its own type of plantation soil, treatments and operations that the respective culture requires”.

It is noteworthy that Machine Learning (ML), is able to learn as it accumulates data and experience. Machine learning (ML) is fast becoming a powerful tool for increasing agricultural production, for instance, ML predicts weather and yield through satellite images. (Fan, et al., 2020)

The machine is able to send alerts identifying the optimal time for harvesting, spraying or treating plantations. All needed information are collected from the sensors of IoT devices installed in weather stations, on the land, in ”electrical tanks”, to correlate and monitor activities and operations, for launching alerts in real time and indicating treatments and operations that the respective culture requires”.

System based on AI and ML allow the identification of plant diseases and application of solutions just-in-time.

Due to conception, AI and ML has the ability to connect to multiple weather stations for providing real-time weather information.

All above mentioned features are affordable, being available even for mobile devices for farmers around the world and very pervasiveness. (https://emerj.com/ai-sector-overviews/ai-agriculture-present-applications-impact/)


**Figure 3.** High Tech Agriculture Management

All functions and capabilities mentioned above are STRENGTHS related with digital tools kit, ensuring the implementation of precision agriculture with very great accuracy.

Predictive Analytics for Process Automation and Mechanization (PAM), for innovative approach towards Automation &Technological Development (ATD), for New Business Models (NBM) indicates the rise of global agricultural production with 69% between 2010 and 2050. Technological innovation in farming is coming to support world's population increase at 9.7 billion by 2050.
(http://publications.tno.nl/publication/34636797/vmt66M/TNO-2020-R11009.pdf).

Drone Fly, a leading DJI Enterprise, FLIR infrared UAV, estimates that drones can spray fertilizer 40 to 60 times faster than doing so by hand.
Business Insider Intelligence projects there to be around 12 M agricultural sensors installed globally by 2023 (https://www.dronefly.com/).

Already tractors has begun connecting to the Internet and was created a method to display data about farmers' crop yields, even on phones and an undergoing project is about self-driving tractors.

Meanwhile, when smart machines will be spread the field, agricultural farm managers would be free up to perform other tasks with more efficiency.

Statistics demonstrated that the management provided by the AI and ML has made it feasible to produce more plants than ever before, according with vHT-agri system develop by Maharashtra State Agricultural with ”the same or a lower input of raw materials” (Satoshi N., et al., 2018).
As AI, ML, IoT are integrated into conventional agriculture, income from the agri sectors will go up.

The outcome of using digital technology is that the input ratio fall accordingly. The composition ratio of conventional input materials such as fertilizer and agricultural chemicals decrease. This is OPPORTUNITY agreeing with the green deal policies.

High Tech Agri Management based on Artificial Intelligence and Machine Learning allows easily planning, monitoring and carrying on analyses regarding all the activities of the farm.

Belated plant protection affects 20-40% of the global yield every year. Artificial Intelligence and Machine Learning for High Tech Management being equipped with alarm algorithms for detecting harmful insects and diseases can prevent for the risk of harmful insects or diseases.
Land cultivation, planting, crop protection, fertilization, irrigation, harvesting and all other activities are managed through a friendly interface. The HT interface allow to ”track the quantities used at the entrance, the costs and the working hours for each activity”(Agrivi booklet).

### 3.1. Romania agriculture and high technology- case study

At the beginning of 2021, a questionnaire was launched on the Romanian market, disseminated through the National Rural Development Network, for this article purpose with the aim of identifying the conditionalities and interest regarding digital technologies in farm management, by Development Regions.

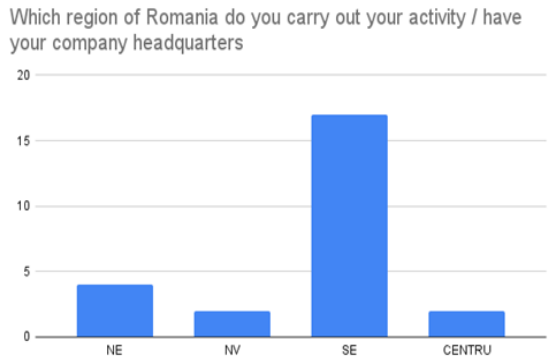



**Figure 4.** Q1_applied questionnaires: Which region of Romania do you carry out your activity / have your company headquarters.

The number of respondents was relatively small, this aspect being a subject for another analysis.

Those who were interested in answering the questions were mostly from the SE region: (Tabel 1)

**Table 1** Distribution of answers by Romanian regions

| REGION | NW | CENTRER | SE | SW OLTENIA | SUD-MUNTENIA | NE |
|---|---|---|---|---|---|---|
| **Respon-dents %** | 3.8 % | 7,7 % | 76.9 % | 7.7 % | 0% | 3,8 % |

The next two questions sought to identify the legal form of organization in order to have an image on the degree of coagulation of the Romanian agricultural properties. It is already proven that small properties can only ensure a subsistence production, the return obtained being insufficient for digital management implementation and smart devices controlled activities.

Because 64% of owners land are not organized as societies, reveal a weak force representing the interests of farmers which are spread in small entities without juridical personality (Figure 6).

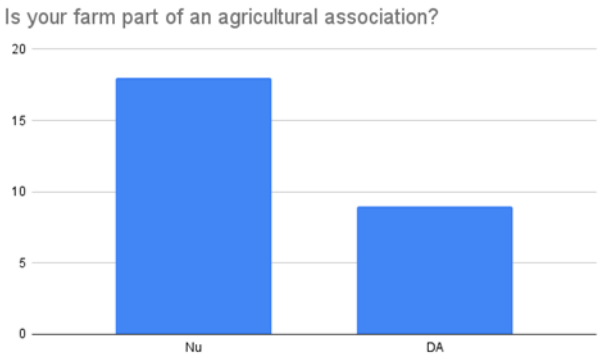



**Figure 5.** Q2_applied questionnaire: Is your farm part of an agricultural association?

In a majority of (28%) of the total respondents, farmers are owners of SMEs, as a legal form organization and (4%) are Agriculture societies and 4% are Research centers.(Figure 6)

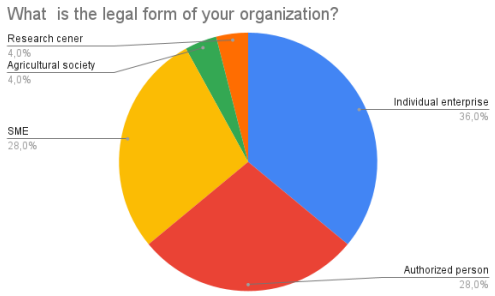
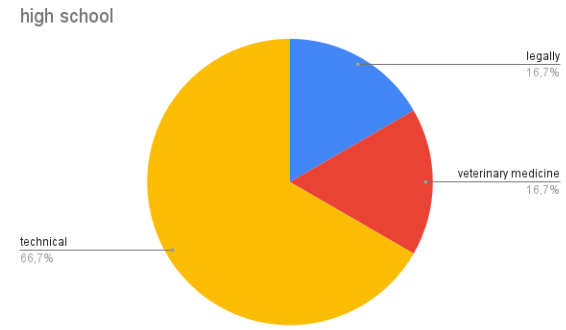



**Figure 6.** Q3_applied questionnaire: What is the legal form of organization ?

I also wanted to know the degree of training and the initial field of education of the interviewed farm managers. The dominance of the share of managers with a technical profile, as managers of farms and plantations (67,7% HS, 33,3% Univ Degree, 37,8% MD, ) it is explained, in my opinion, by the industrial sector dereliction after 1989. So that, engineers, almost regardless of their specialization, invested time, training, finances in inherited or leased land. (Figure 7)

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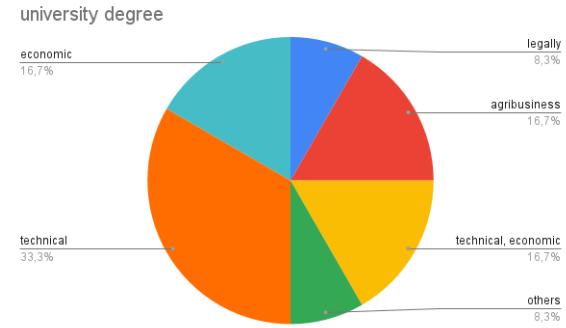


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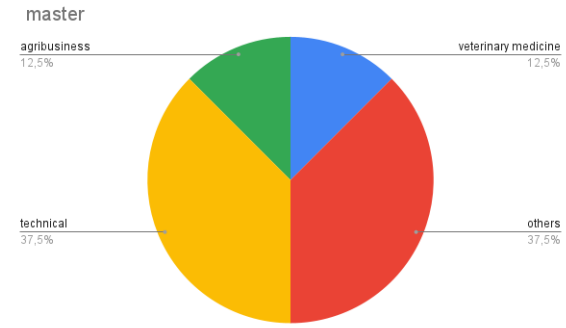



C

**Figure 7.(A,B,C)** Q4_applied questionnaire: What is the qualification level of the company manager?

As reveals the chart bellow, 66,7% of legal entities, with economic activity in agriculture are micro-enterprises, with up to 9 workers, being not known the value of other resources like financial, material and the surface of the land. These are followed by societies (25,9%) with 10-50 employees (Figure 8)

The most significant problems the managers confront with, appear in the graph represented in Figure 9: (19,2%) are too old workers, (23.1%) have no proper skills and almost (50%) have a kind of seasonal activity and are not very involved in the workplace.

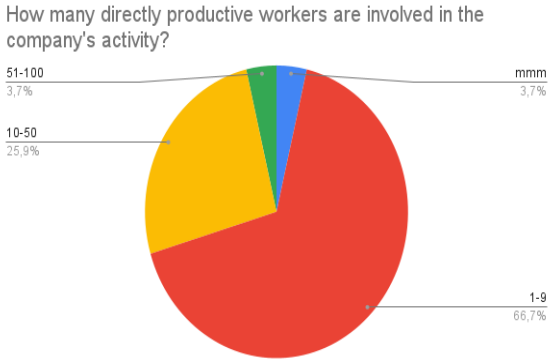



**Figure 8.** Q6_applied questionnaire: How many directly productive workers are involved in the company's activity

The state of the art regarding the lack of efficiency and / or the rarity of a motivated and well-trained workforce should be a trigger for farm manager, to move on for implementing new technologies in agriculture.

Unfortunately, this aspect will have the consequence of removing workers from some jobs, this implying their retraining and mastering the skills regarding new technologies, where it is possible and for whom it is possible. Not all current agricultural workers can acquire the skills and knowledge thus to become a mate with digital machines.

Additionally, surveyed managers think more than half of the number of workers create troubles, cause damage, even theft and are difficult to retain. Maybe, all before, because small wages and earnings?

Last but not least are poorly trained. (Figure 9)

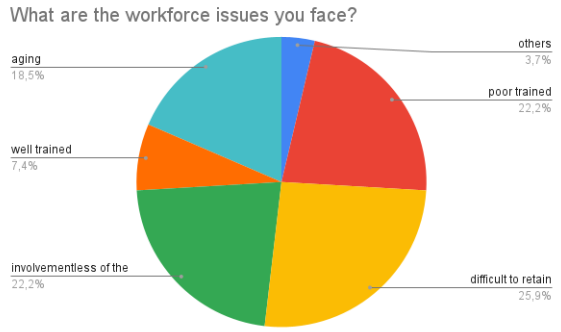



**Figure 9.** Q7_applied questionnaire: What are the workforce issues you face?

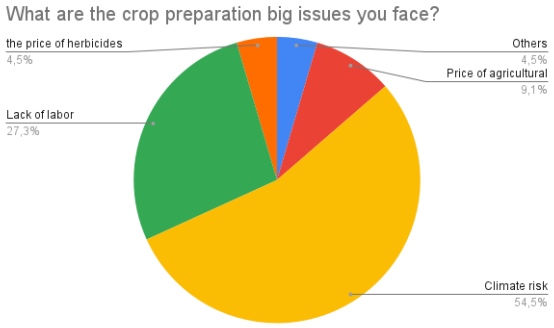



**Figure 10.** Q8_applied questionnaire: What are the crop preparation big issues you face?

Not only the labor force raises concerns for Romanian agricultural farms managers.

It seems that the major fear, (54.5%), is the climate risk followed by lack of labor and skilled workers (27.3%), the price of agri works 9.1% and the price of seeds and herbicides, (4 %) (Figure 10).

So what should be done, now in the 21st century, when precision agriculture is already an answer? Let's see further what are the weaknesses and threats but also the opportunities and strengths of Romanian farmers.

According to the analyzed answers, histograms reveals (Figure 11) there are many managers (Green bar) who know and have documented themselves about new technologies (IoT, Clouds, SaaS, Drone). But only few managers implement digital technology and do precision agriculture in Romania (Mauve bar).

There are only very few who are not interested regarding this issue (Blue bar). Could it be a cause for concern? Probably yes, considering that there is a predictable and predicted food crisis added to a global competition and Romanian agriculture would not stay aside.

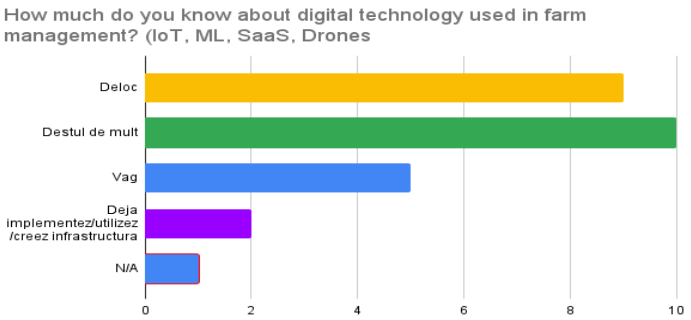



**Figure 11.** Q9_applied questionnaire: How much do you know about digital technology used in farm management?

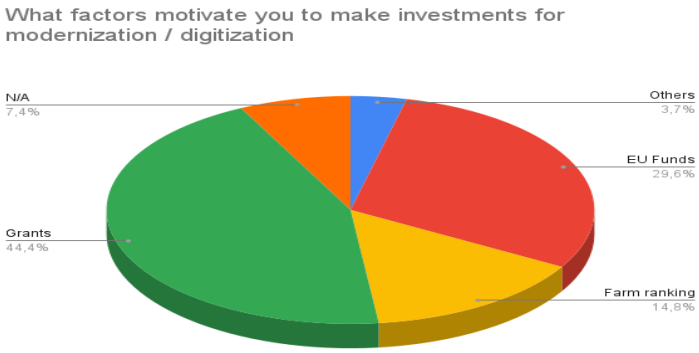



**Figure 12.** Q10_applied questionnaire: What factors motivate you to make investments for modernization / digitization

Going forward (Figure 12) hope seems to be reborn. All managers are much and very much interested in implementing digital agriculture, being highly motivated by competition for Grants (44,4%), EU funds (29,6%) and by the aim of bringing the own farm to a leading competitive position (14,8%). Asking for possible causes as major weakness on moving to digital agriculture (Figure 13 A,B), most managers (40,7%) responded as being "less true" the lack of access to documentation. Regarding the "fear of theft of the digital infrastructure" (55.5%) responded as being "very true" and "true". Fear of failure followed by the impossibility of reimbursement of the loan was another significant weakness regarding the digital agriculture.

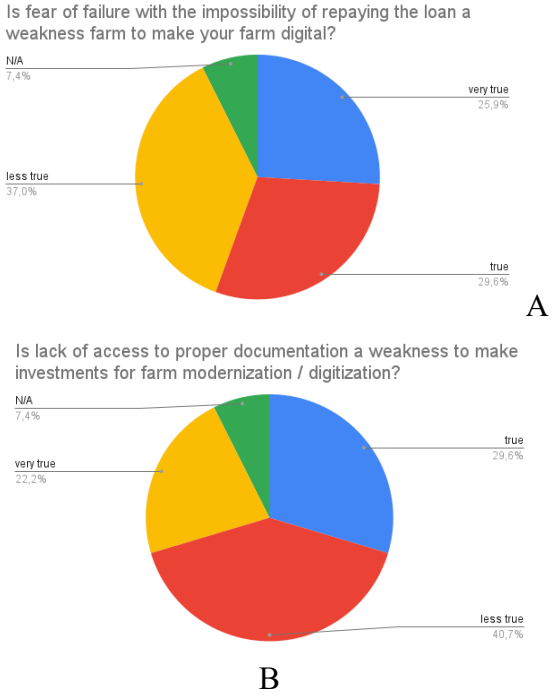
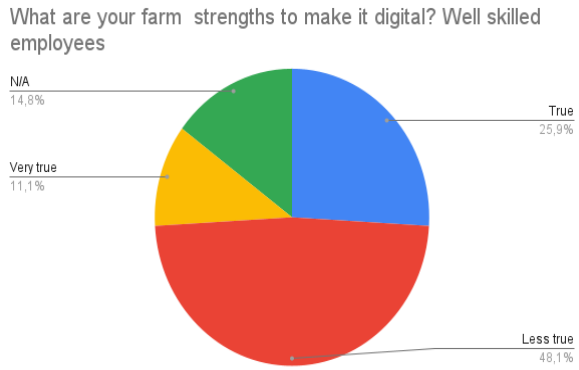



**Figure 13.**(A,B) Q11_applied questionnaire: What are the farm weakness to make investments for modernization / digitization?

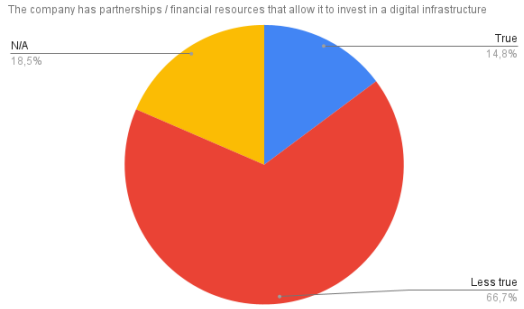
The strengths of the company identified by the interviewed managers were ranked as follows: with a majority share was the option

The analyzed issues exposed by survey (Figure 14 A,B,C) were as follows:

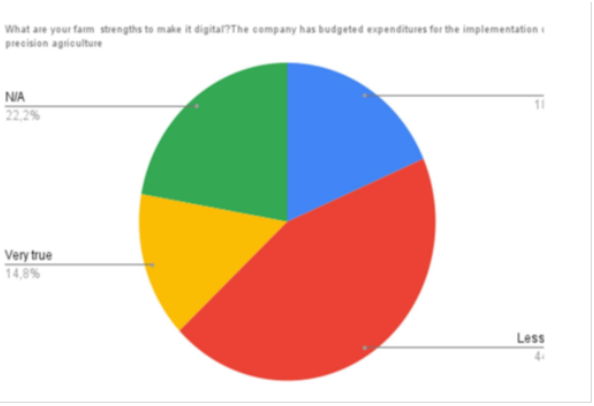
- The company has well-skilled employees, 48,1%"less true" ;
- the company provides motivating salaries to employees, so that they get involved with enthusiasm.
- The company has partnerships / financial resources that allow it to invest in a digital infrastructure : 66,7%"less true";
- The company has budgeted / is willing to budget expenditures for the implementation of precision agriculture: 66,7%"less true".




A




B




C

**Figure 14** (A,B,C). Q12_applied questionnaire: What are your farm strengths to make it digital?

Regarding threats, the respondents consider the biggest one the prices charged for digital technology implementation, services and very high rents for SaaS.

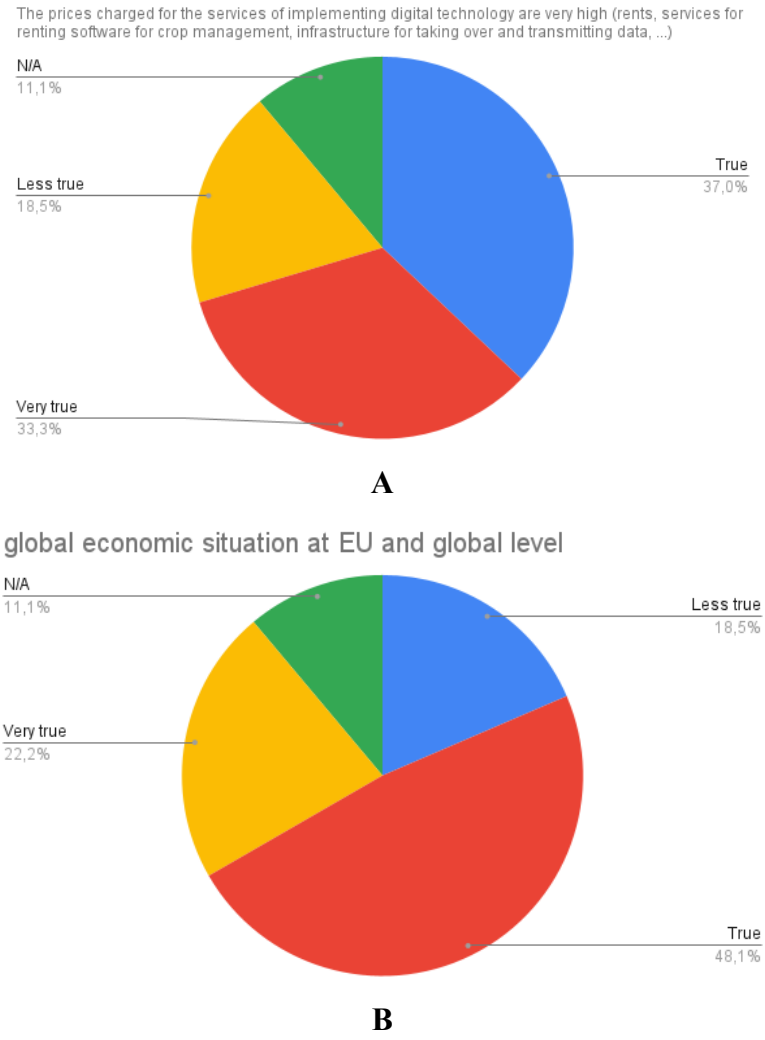



**Figure 15.** (A,B). Q13_applied questionnaire: What are your farm threats to make investments for modernization / digitization?

for crop management, infrastructure for taking over and transmitting data: ”true” and ”very true” represents (70,1%), (Figure 15A).

The general economic situation involving the impossibility of recovering expenses on a volatile global market, populated by customers having low purchasing power was ranking as follows: ”true” and ”very true” represents 70,3% (Figure 15B).

Asked about the company's opportunities in terms of digitalization, managers consider the interest of importers for Romanian agricultural products, given for export is (48.1%) "true" (Figure 16 A) and European policies on this matter are (44.4%)"very true" (Figure 16 B).

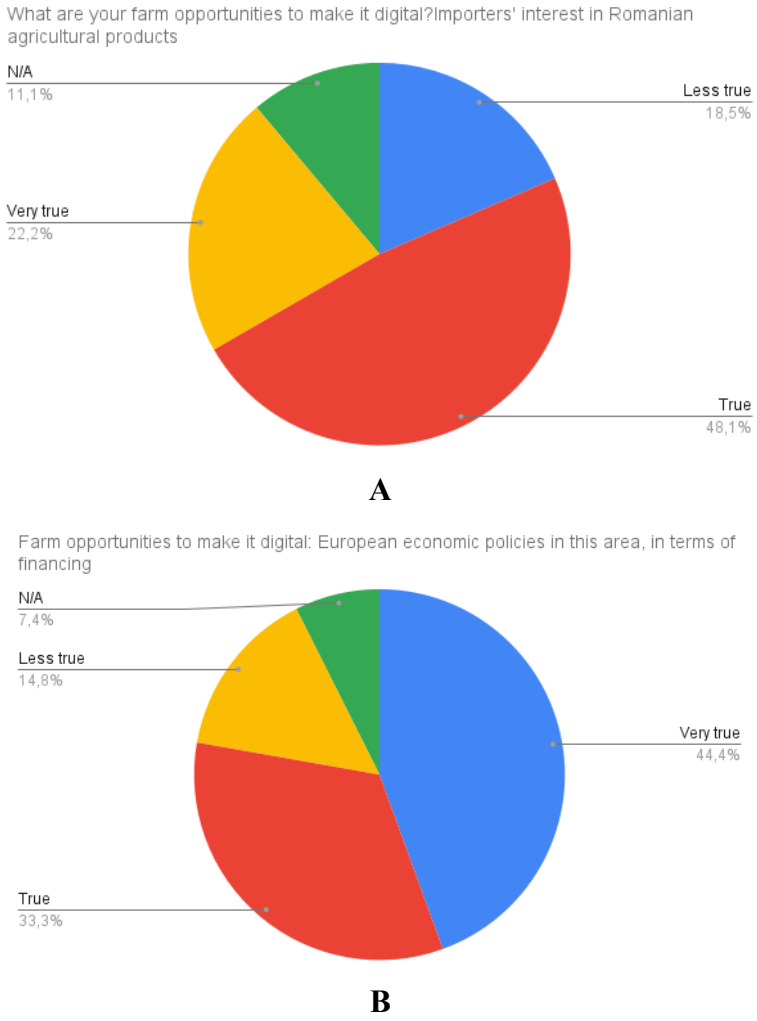



**Figure 16. (A,B)** Q14_applied questionnaire: What are your farm opportunities to make investments for modernization /digitization?

Was noticed an interest of managers for implementing digital technology. They estimated investments to be realized as follows:

Regarding infrastructure and sensors for collecting data on temperature, humidity and NPK, (33,3%) consider time for implementation over 1 year **(**Figure 17A).

Regarding drone (s) (33,3%) answered that time for implementation is 1-3 years and (22,2%) consider 3-5 % **(**Figure 17B).

Regarding dedicated software, SaaS and pest monitoring systems, 29,6% estimated 1-3 years and others 22,2% consider over 10 years will be necessary for implementation **(**Figure 17C).

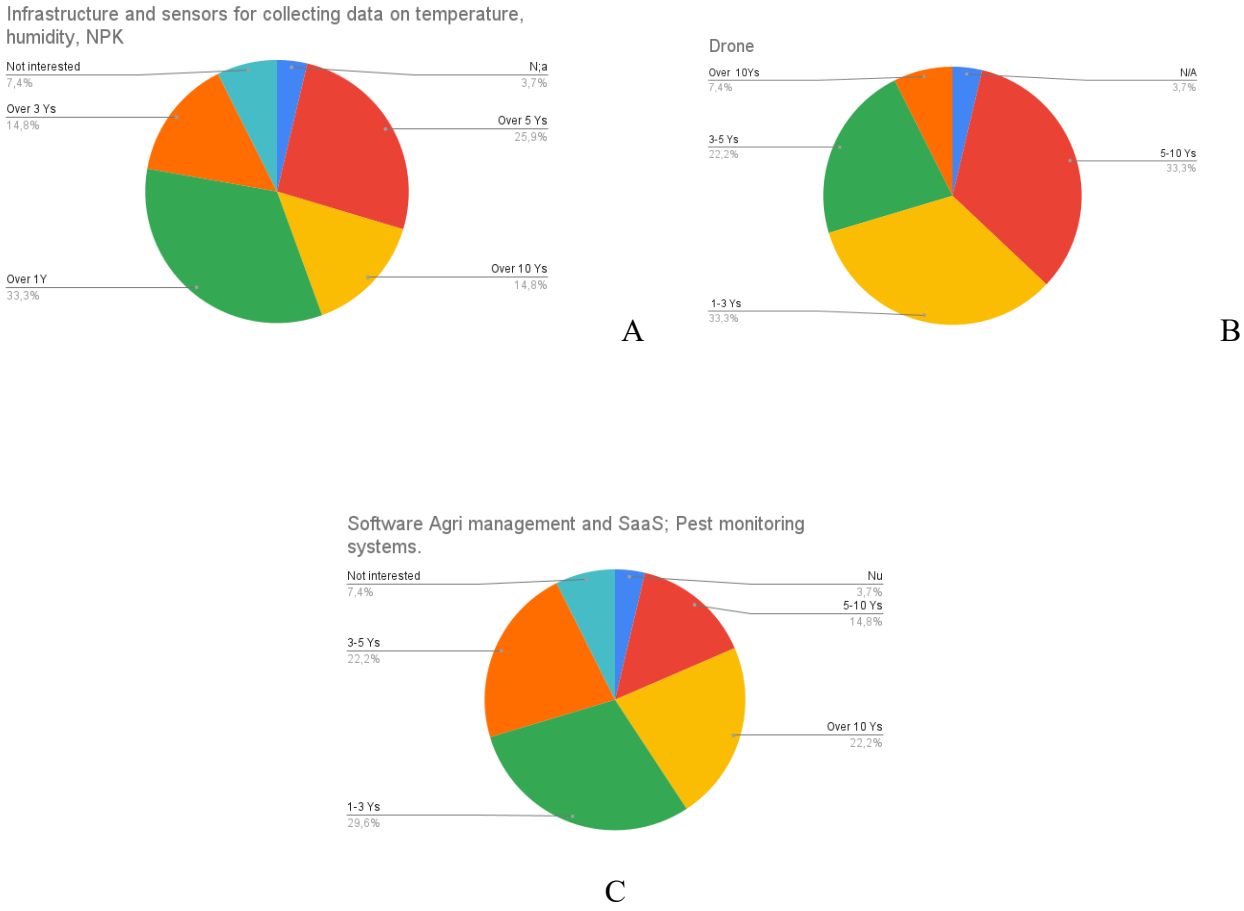



**Figure 17**.(A,B,C) Q15_applied questionnaire: What are the digital equipment your farm will be equipped with?

## 4.Conclusions

The agricultural progress has been a crucial factor in worldwide social and economic change. (Salunke, et al., 2015)

Romanian farmers are open and aware regarding the added value of farm management through the implementation of digital technologies. However, the high costs of the digital infrastructure, as well as the lack of financing resources, maybe also the blockages created in the economic policies by the current sanitary problem, all of these make the Romanian farms to be under digitized. The result of the analysis should not take us by surprise, because most of the answers came from small and very small companies, which still cannot afford to implement digitalization in farm management in order to be efficient, in a competitive way, and to keep pace with the great transformations in agriculture.

On the other hand, the large farms are already applied top technologies.

A well-known example is Agricost Insula Mare a Brailei, the largest farm in Romania, but also a very important one in Europe, which has 80 tractors with Trimble automatic guidance. Crop development monitoring is done at the Agricost with up to 29 weather stations which are and will be integrated in the coming time to monitor air temperature and humidity, precipitation, wind speed, humidity and soil temperature, to receive alerts on the possible occurrence of diseases in agricultural crops.

This means that while some farmers will be able to expand their operations, others will be severely challenged in their efforts to compete

in the sector and meet the stringent food quality and safety standards required by processors and retailers.
(https://www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050_Global_Agriculture.pdf, pp 4), accede on 05112021.

Crop management on difficult meteorological conditions as well as just-in-time treatments against diseases and pests, and transmissions with drones of field information, and AI faster added decisions for reducing and simplifying field work, applying the appropriate treatments only when necessary and in the required quantities, obtaining rich crops and healthy agricultural products throughout agricultural chain, are aspects with robust interest for farmers.

Application of digitalization for agriculture still remains "a formidable task, since integration of diverse domains for online monitoring of agricultural supply chain and management of complex agro ecosystems require concerted and collaborative efforts in a structured manner." (Patil, et al., 2012).

All above have to be envisage by the Romanian forward thinking educational and agricultural policies makers, for creating robust support for sustainable Romanian agricultural.

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# SIMULATION OF POLY-(HYDROXYBUTYRATE) FROM METHANE IN VERTICAL LOOP BIOREACTOR

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*Vertical loop bioreactor.*

**ABSTRACT**

Bio-plastics are eco-friendly biopolymer finding tremendous application in food and pharmaceutical industries. Bio-plastics have suitable physicochemical, mechanical properties, and does not cause any type of hazardous pollution upon disposal but have high production cost. This can be minimized by screening potential bio-polymers producing strains, selecting inexpensive raw material, simulation and optimization of cultivation condition. In this study, simulation of bacterial production of poly-β-hydroxybutyrate from methane in vertical loop fermentor was carried out by Comsol 5.2 software in 3-dimensional mode. Mass transfer in the process of bacterial growth was investigated via the feed of methane substrate. The graphs of cell density and growth confirmed the results of the simulation according to time. Meshing and independence analysis of the mesh carried out. The initial concentration of microorganism was 0.001 g/L than in the optimal condition and different duration of time was reached 50% of methane and 50% of gas in the reactor that was the highest value of growth microorganism. The results of the simulation were confirmed to experimental results with less than 5% error.

## 1.Introduction

One of the most important poly-hydroxy-alkanoates is poly-hydroxybutyrate (PHB), which is formed as intracellular granules in different microstructures. Three unique characteristics of PHB are thermoplasticity, water resistance and biodegradability Lee (1996); Booma, et al. (1994); Golzar, et al.

(2020); Malmir, et al. (2020). However, their world wide application is still limited due to the high production cost. PHB total cost depends on some parameters e.g. the substrate, microorganism, cultivation system, and down-stream processing. The utilization of cheap substrates Khosravi-Darani and Bucci (2015); Darani, et al. (2006); Khosravi-Darani, et al. (2019); Ghoddosi, et al. (2019); Mokhtari-Hosseini, et al. (2009); Mokhtari-Hosseini, et al. (2009); Bozorg, et al. (2015); Koller, et al. (2017); Khosravi-Darani, et al. (2013), model-ing Shahhosseini, et al. (2003), suitable bioreactor and design of experiments Khosravi Darani, et al. (2003); Vasheghani Farahani, et al. (2004), as well as recovery method Khosravi-Darani and Vasheghani-Farahani (2005); Khosravi Darani, et al. (2003); Vasheghani Farahani, et al. (2004). These factors would not only decrease the production cost of PHB but also help in increasing productivity Khanna and Srivastava (2005).

One way to increase PHB production from natural gas is to use new bacterial species in bio-fermenters with appropriate hydrodynamics Khanna and Srivastava (2005); Rashidi, et al. (2020); Reddy, et al. (2003). Among the carbon sources available, methane is the most suitable substrate for PHB production, which is both readily available and inexpensive. It is also necessary to design a proper fermenter for fermentation with sufficient efficiency. For biomass production from methane, the fermenter must be designed to allow adequate mass transfer from air to liquid flow, $CO_2$ removal, mixing and oxygen supply Shah, et al. (2008); Okan, et al. (2019); S.R. Mofradnia, et al. (2018); Mofradnia, et al. (2019); Rahnama, et al. (2012); Moradi, et al. (2019). Mixing with blades is not suitable for biomass production in large fermenters due to high-energy consumption. In addition, high energy is needed for running these kind of fermenters. Also, it is possible that a contaminating micro-particle enters the fermenter during the blade's design and installation Rahnama, et al. (2012); Zhang, et al. (2008); Moradi, et al. (2019); Khosravi Darani, et al. (2018); Rezapour, et al. (2019). In a loop fermenter, mixing and aeration performs well through the circulation of the medium. These fermenters perform best in heat transfer, mass transfer, aeration and substrate mixing and require local sampling and analysis. These fermenters are very high. In addition to these benefits, the energy required to transfer each kg of oxygen is low Yazdian, et al. (2009); Wendlandt, et al. (2005); Memari, et al. (2020).

According to the above, in this study, the simulation of a vertical loop bioreactor, a type of airlift bioreactor, to produce PHB was investigated by COMSOL software.

## 2. Materials and methods

### 2.1. Simulation

Loop bioreactor simulation was performed by COMSOL Multi-physics simulation software as a set of simulations that can solve the differential equations of nonlinear systems by partial derivative with finite element method in 1, 2 and 3D spaces. Also in this software, the problem can be presented I the form of a mathematical formula (in the form of equations) and a physical one (selection of the physical model, such as the transfer of dilute components in solution) Soheil Rezazadeh Mofradnia, et al. (2018); Mofradnia, et al. (2019); Multiphysics (2012).

For the present simulation, the dilute component transfer module is used. In order to define the desired module, the properties and constants available in the COMSOL software are used for the simulation module. COMSOL software has a library of inform-ation on material properties and constants. However, if the property of the material in question is not present in the software library,

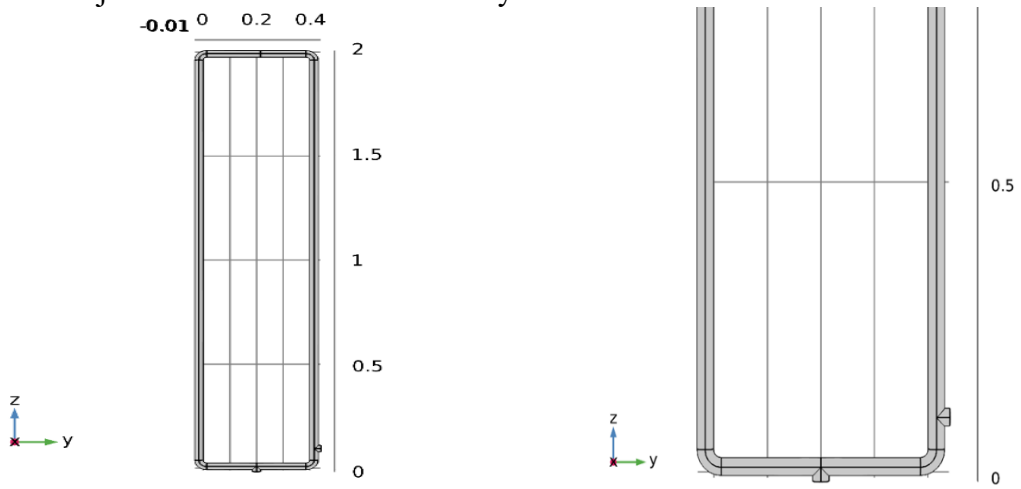
its value can be directly defined in the module used as a parameter Mousavi, et al. (2010). The dilute compon-ent transfer module is used for both the liquid and gas phases. As in the gas phase, only the mass transfer from the gas bulb to the gas- liquid boundary occurs, and in the liquid phase, dissolved gases are transferred to the liquid bulk and the biomass production reaction takes place in the liquid phase. Since the momentum equilibrium is not in the bioreactor, the velocity of the liquid phase and the gas phase in the equations are considered constant. Average velocity of the gas phase and the liquid phase in the bioreact-or were 0.035 and 0.35 m/s.

## 2.2. The geometry of the bioreactor

The bioreactor's dimensions including length, height, diameters as well as diameter of gas and biomass injection port were 0.45, 2.00, 0.03 and 0.03 m, respectively. Figure 1 shows the overall scheme of the loop bioreac-tor (Figure 1a) and the ports of gas and biomass injection are more accurately specified (Figure 1b). As shown in table 1, for the bioreactor walls the non-slip boundary condition means zero velocity and fixed boundary of the system. Also the condition of no flux, i.e. n. Ni=0, means that none of the system's components are going out of the system. The gas output condition is also given by n. Di∇Ci =0, which means the components of the system are leaving through the fluid. In the initial condition of this model, the concentration of component in the liquid and gas phases at t=0 are considered to be 0. Initial and boundary conditions are presented in table 1,

## 2.3. Meshing

Meshing for the present 3D geometry is done through finite number of elements. The meshing is considered as triangular elements. In figure 2a, an overview of the meshed bioreactor is shown. Also in figure 2b, this meshing is partially displayed at the injection site.

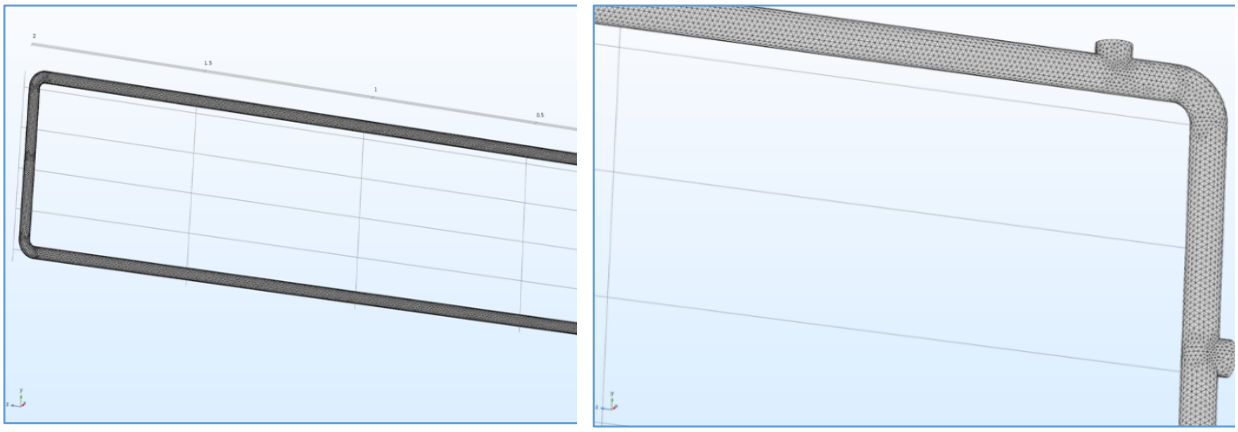



(**a**) (**b**)

**Figure 1.** The overall scheme of the bioreactor (a) and location of gas and biomass injection (b).

**Table 1. The boundary conditions used in the simulation.**

| Boundary | Value | Explanations |
|---|---|---|
| Gas injection | $CH_4$=20.1 mol/$m^3$<br>$O_2$=4.221 mol/$m^3$ | Dynamically (time dependent) |
| Biomass injection | Cbio=0.0088 mol/$m^3$ | Zero moment injection |
| Walls | No flux condition through the wall | |
| Gas outlet | Outflow condition | |



(**a**) (**b**)

**Figure 2.** An overview of the meshed bioreactor with triangular elements (a), Close view of the bioreactor meshed by gas and biomass injection sites (b).

## 3.Results and discussions

In this study, the performance of Hirsutas methylocystis for the production of PHB was simulated. According to the equations and information, simulating the bioreactor in the COMSOL software, the mean change of the concentration of the components in the liquid phase in different ratios of methane to air, 0.2/0.8 (methane to air), 0.5/0.5 (methane to air) and 0.8/0.2 (methane to air) were calculated by time.

### 3.1. Changes in the concentration of methane and oxygen substrates over time

As can be seen in figure 3 (a and b), the concentration of both components initially increases, because the gas mass reaches liquid-gas contact surface. In the study of Aghamiri et al. (2012), the kinetics of protozoan production of natural gas by Methylomonas microstructure in the gas phase with an equal volume of air and natural gas was studied experimentally and by mathematical modeling Wu, et al. (2012). Different models of Mund, Moser, Tseir, Iba, Andrews and Novak have been used to describe the kinetics of cell growth. Methane and Oxygen as growth restrictive substrates, were considered in the concentration range of 2 to 10 mg/l were used to establish the phases of inactivity and death using a time-dependent death coefficient. The results of this study showed that mathematical models can express well the cell mass production's dynamic in the growth till death phases. The kinetic parameters of each model were extracted by it's comparison with the

experimental data. The results also showed that Mund and Moser models more accurately describe cell mass production than other models in this study.

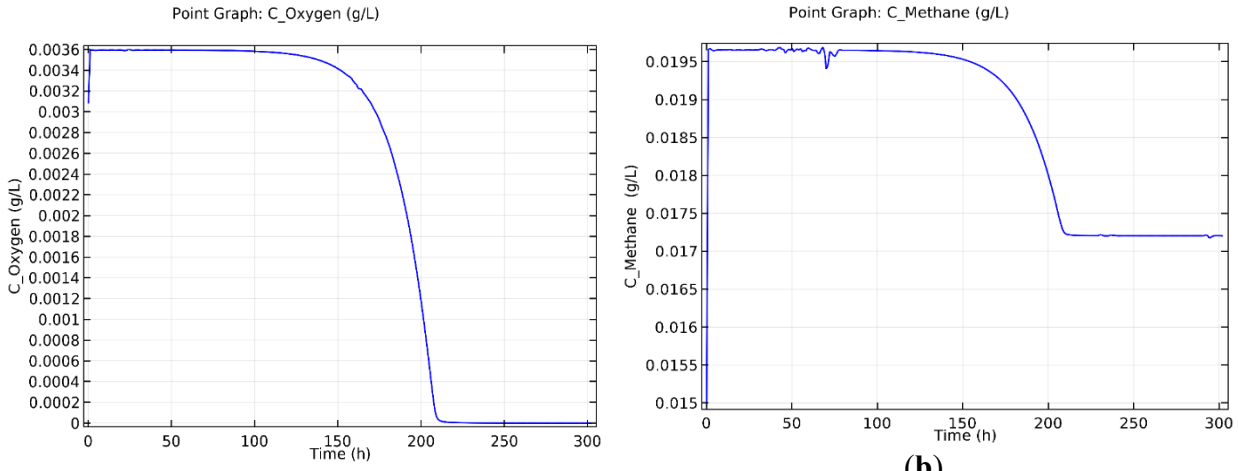
It also showed that the amount of methane and dissolved oxygen in the cell culture medium decreased with increasing cell mass production rate. In other words, when cell mass growth is low, the amount of methane and oxygen in reach of the cell is high and as cell growth increases, the amount of methane and dissolved oxygen consumption increases Yazdian, et al. (2010).

Figure 4 (a & b) show the increase in methane and oxygen concentration in the gas-liquid interface more precisely. According to the figures, it can be seen that after the injection of methane and air into the bioreactor, their concentration stabilized after 1.5 h, indicating that the two components reach saturation. Then, the reaction of these two components with biomass begins over time and their concentration decreases (Figure 4 c & d). This process is the same for all of the methane/ air ratios.

### 3.2. Changes in methane and oxygen substrate's concentrations over time

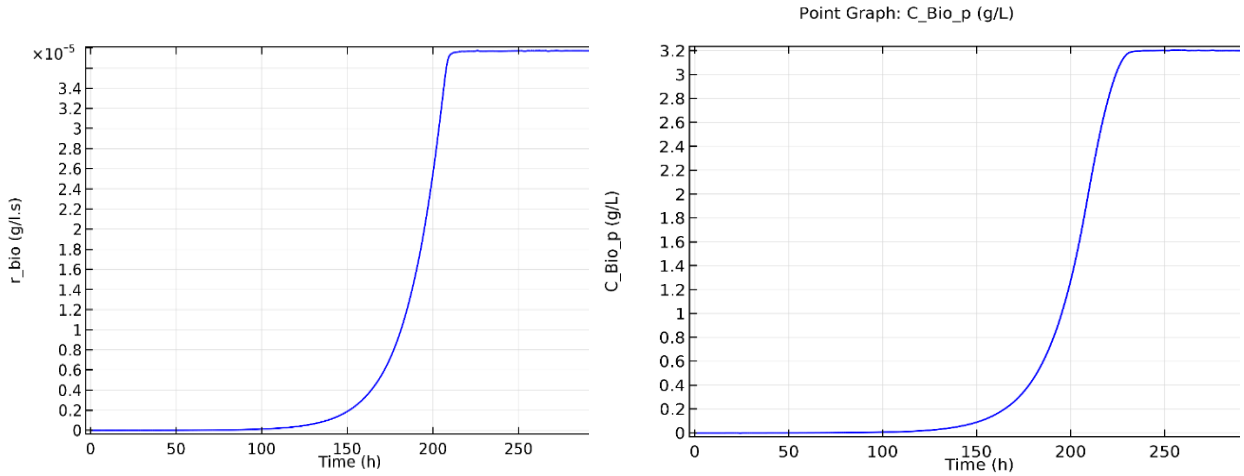
Figure 4 shows the change in biomass growth rate and the biomass concentration in the ratio of 50% methane and 50% air. According to the biomass growth diagram in Fig. 4, initially the mass concentration increases and then with the constant growth rate, reaches it's maximum over time, these findings are in good agreement with the obtained results by Bagde, et al. (2014).

According to the Fig 4., the biomass growth rate is initially controlled by the concentration of methane and air. Given the constant μ dependence on the concentration of these two components, the growth rate of the mass is fixed after some time (Eq. 3.4). In other words, μ reaches it's maximum value and becomes independent of the concentration of the components.




(**a**) (**b**)

**Figure 3.** Profile of $O_2$ (a) and $CH_4$ concentration over time (b).




**(c)** **(d)**

**Figure 4.** Changes in oxygen (a) and methan (b) concentration as well as biomass growth rate (c) and biomass concentration (d) over time.

Over time, the biomass growth rate becomes dependent on the mass concentration. This has led to another increase in the biomass growth rate, with the concentration of air and methane eventually falling. As the result, the μ also decreases and the biomass growth rate remains constant over time.

In 2012, mathematical modeling of loop bioreactor to produce protozoan protein from methane by aerobic microstructure of Methylomonas species was simulated by Bagde, et al. (2014). The mass equilibrium equation was written for each part separately and according to the available assumptions, and then the resulting ordinary and partial differential equations were solved simultaneously. The mathematical model used for the upstream, downstream and horizontal flow sections is the axial dispersion model and for the pump, liquid- gas separator and static mixer sections is the model of a series stirred tank. The mass transfer between the phases and the reaction kinetics is considered. Model sensitivity analysis was also performed for gas and liquid velocity variations as well as $CH_4$ to total inlet air ratio and the optimum operating conditions were evaluated. For the initial conditions assumed, the substrates in the gas and liquid phases and the cells have uniform dispersions through all sections from the outset, and also oxygen and methane in the liquid and gas phases have reached equilibrium state. The initial concentration of the cell is 1 g/L. Excessive oxygen in the environment causes a side reaction and has an inhibitory effect on cell growth. The effect of increasing and decreasing the inlet oxygen component on the rate of cell growth can be observed by changing the ratio of inlet air to inlet methane. As the volume of air increases from 0.5 to 0.6, the cell growth increases, further increase in air volume results in decreased cell growth due to oxygen inhibition or methane restriction. The best condition for the produc-tion of biomass by the microorganisms in the given operating conditions are gas flow rate of 1.5 L/min, a liquid flow rate of 15 L/min and the ratio of 0.6 for methane to total inlet gas Rahnama, et al. (2012); Ghoddosi, et al. (2019).

### 3.3. Mesh- independent results

In order to investigate the independence of the results of the simulation with respect to mesh, meshing with different numbers must be performed. By decreasing the mesh size to the point that the difference between the obtained results are less than 0.5%, the solution is independent of the computational mesh. The number of elements was consider-

ed as 6770 to 810707. In table 2, the biomass concentration for the number of elements is examined. Considering the aver-age biomass concentration obtained in the bioreactor, the concentration of this component was slightly changed and the error percentage was less than 0.5%. So, the responses obtained are independent of the mesh and the number of 261128 elements is considered as the number of appropriate elements.

**Table 2.** The results with respect to the number of elements.

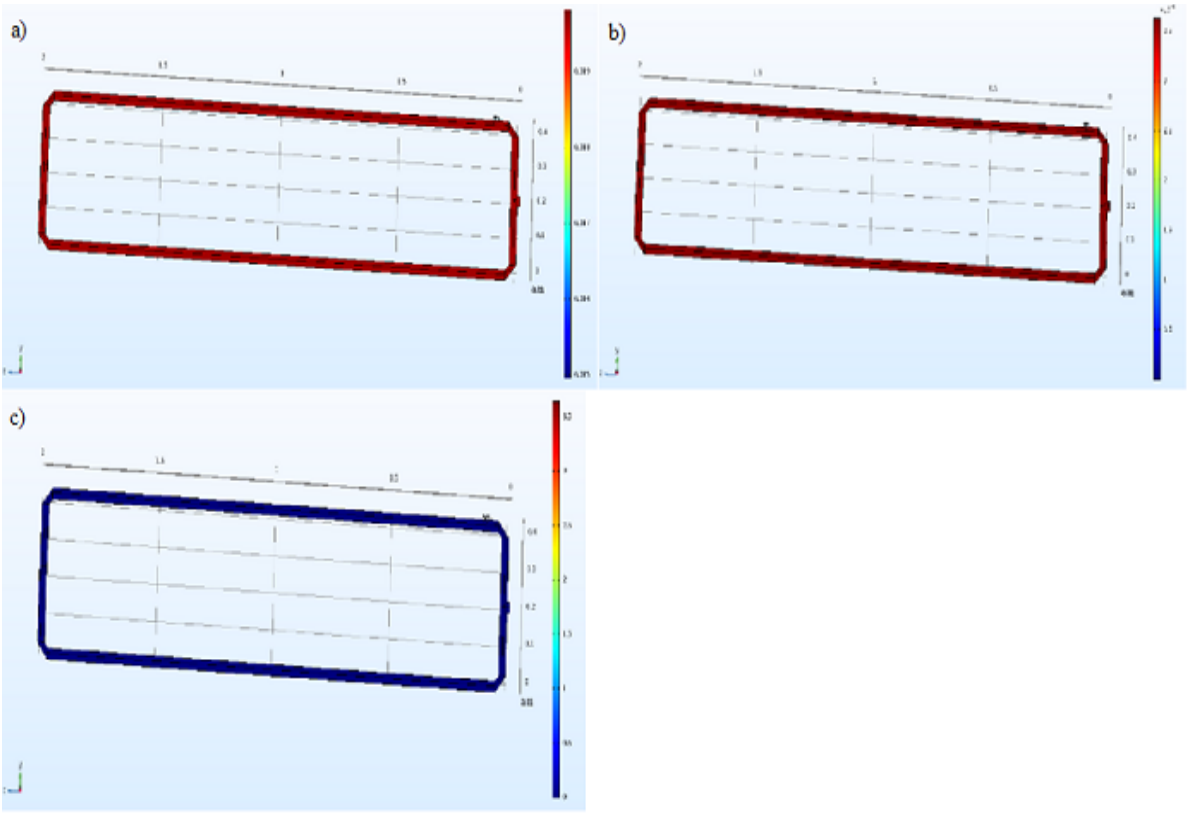
| Element's number | 6770 | 62811 | 261128 | 810707 |
|---|---|---|---|---|
| Biomass concentration ($mol/m^2$) | 0.052147 | 0.064958 | 0.065955 | 0.065956 |

### 3.4. Investigation of methane and air concentration contours

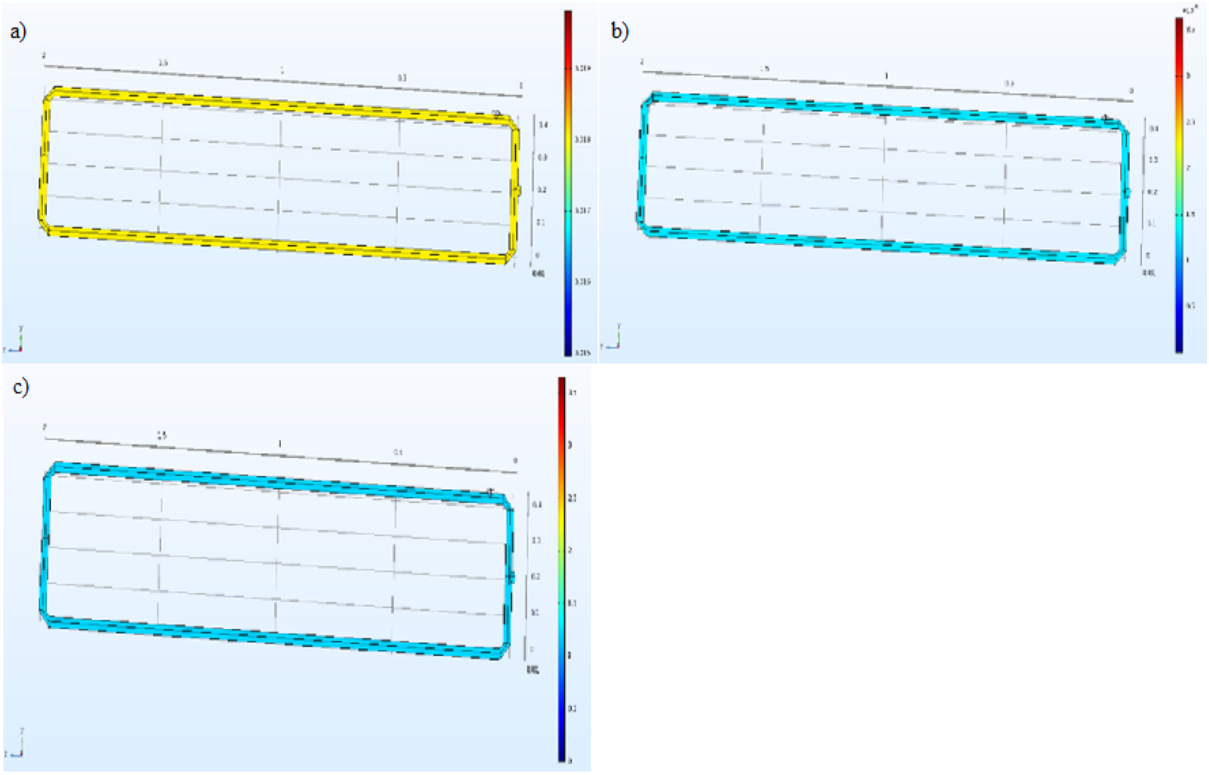
*3.4.1. Methane and air concentrations in the ratio of 50% methane and 50% air*

Figure 5 (I) (a-c) shows the contours of methane, air and biomass concentration in g/L, in the ratio of 50% methane and 50% air at 100 hours, respectively. In figure 5 (II) (a-c) the contours of $CH_4$, air and biomass concentration in g/L, in the ratio of 50% $CH_4$ and 50% air at 200 h are shown respectively. Also figure 5 (III) (a-c) shows the contours of methane, air and biomass concentration in g/L, in the ratio of 50% methane and 50% air at 250 hours, respectively.

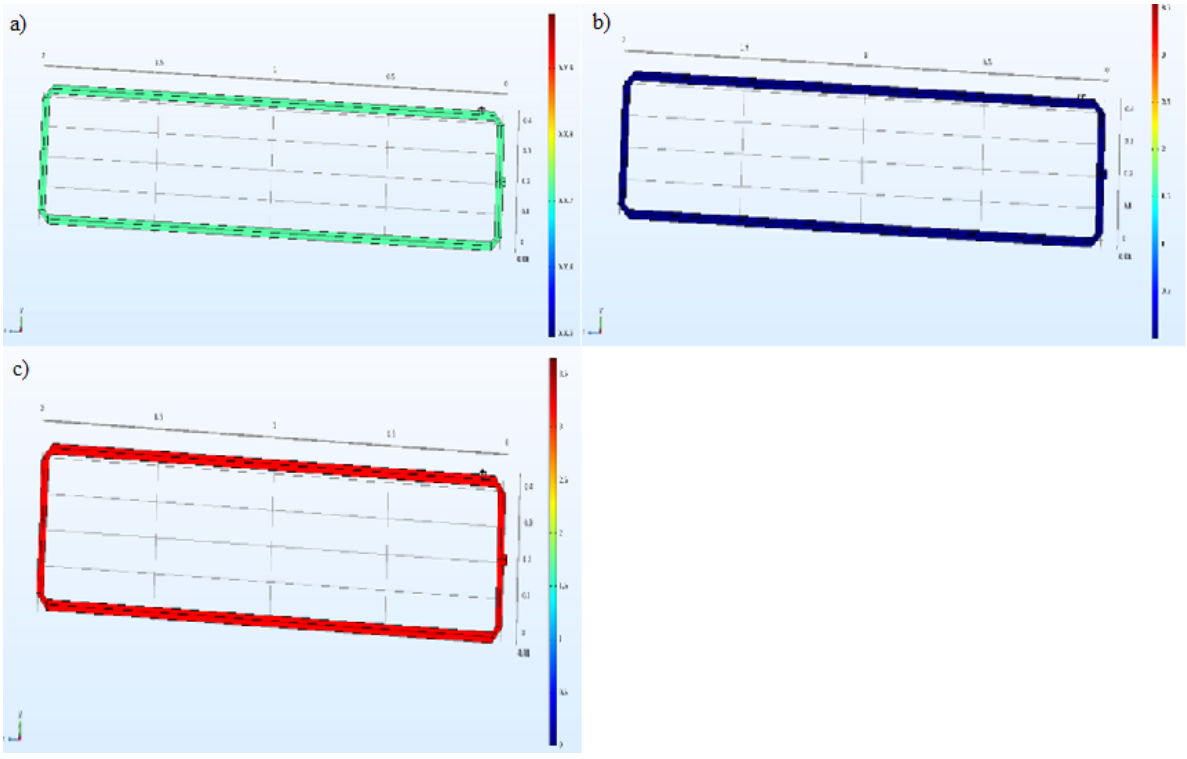
5 (I)



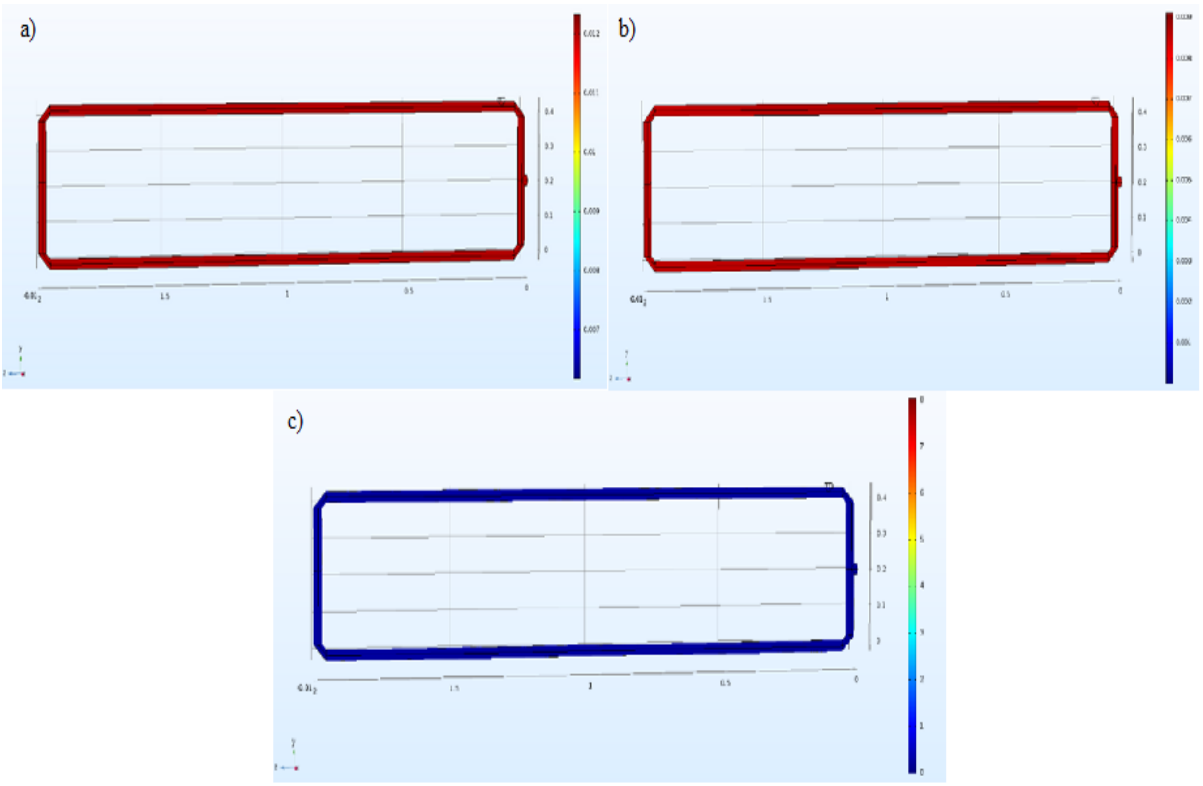

5 (II)



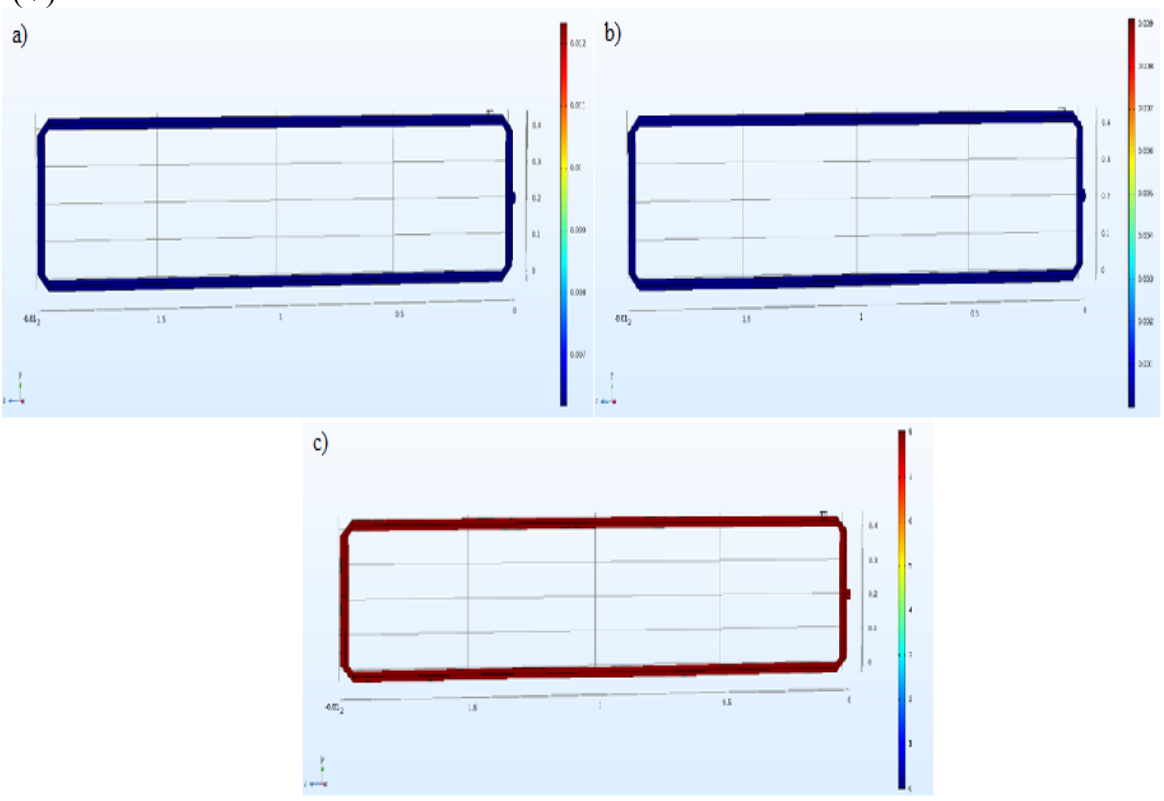

5 (III)



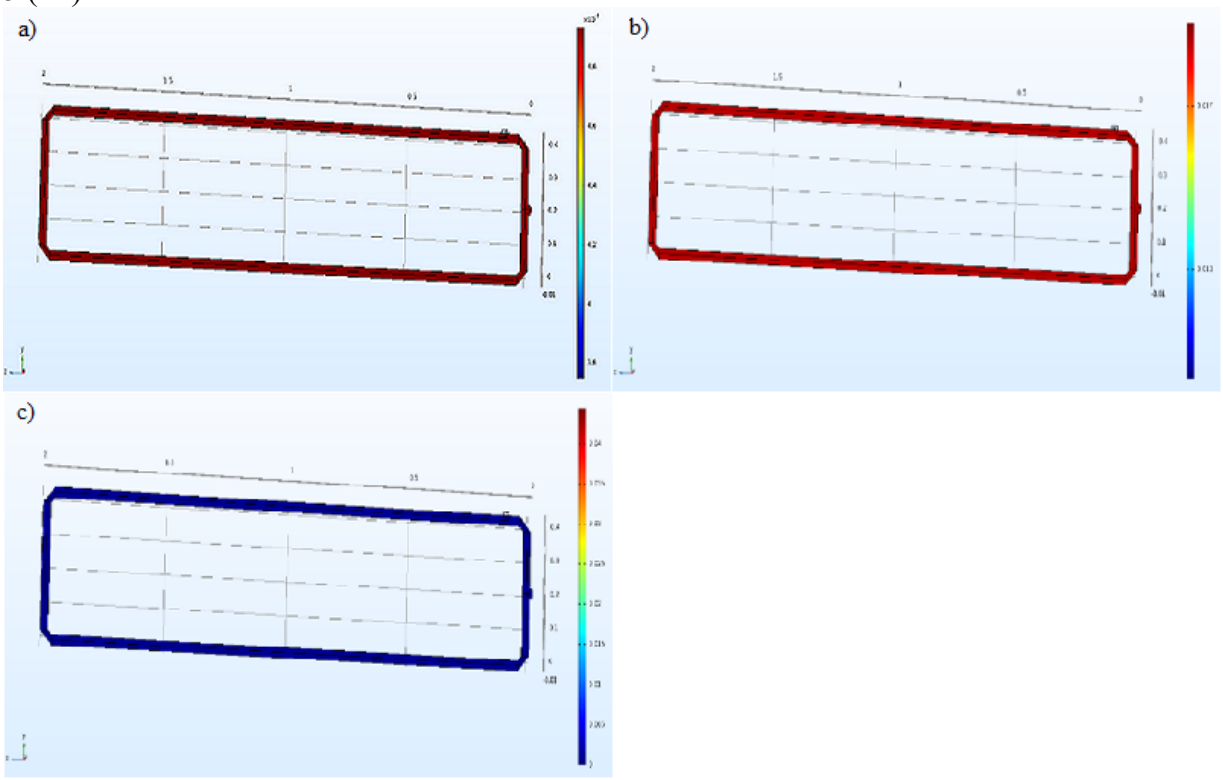
5 (IV)




5 (V)



5 (VI)




5 (VII)

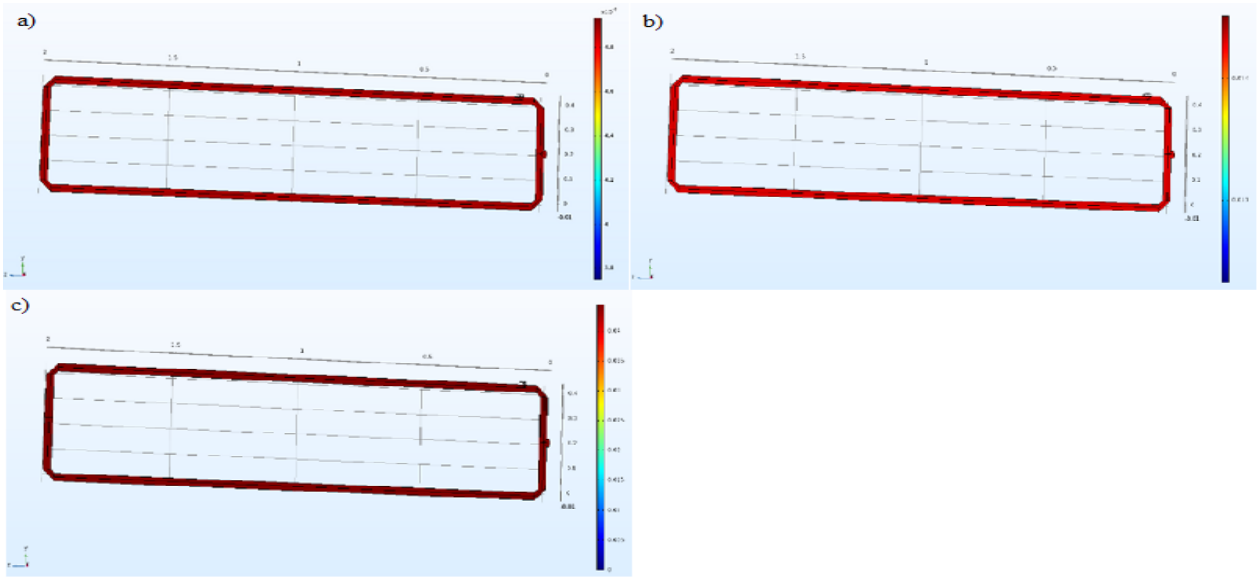



**Figure 5.** The concentration contours (g/L) in 50% methane and 50% air at 100 (I), 200 (II), 250 (III) hours for methane (a), air (b) and biomass (c). The concentration contours (g/L) in 80% methane and 20% air at 250 (IV), 400 (V) hours for methane (a), air (b) and biomass (c). The concentration contours (g/L) in 20% methane and 80% air at 250 (VI), 400 (VII) hours for methane (a), air (b) and biomass (c).

**Table 3.** Average change in component's concentration over time at different air and methane. ratios (g/L)

| CH4 / air | t = 100 hr. | t = 200 hr. | t = 250 hr. | t = 400 hr. |
|---|---|---|---|---|
| 0.8 / 0.2 | CH4 = 0.0123(g/l)<br>O2 = 0.009(g/l)<br>Biomass = 8.66e-4(g/l) | CH4 = 0.0122(g/l)<br>O2 = 0.00899(g/l)<br>Biomass = 0.039(g/l) | CH4 = 0.0108(g/l)<br>O2 = 0.0067(g/l)<br>Biomass =1.41(g/l) | CH4 = 0.0062(g/l)<br>O2 = 4.98e-6(g/l)<br>Biomass =2.51(g/l) |
| 0.5 / 0.5 | CH4 = 0.01964 (g/l)<br>O2 = 0.00355 (g/l)<br>Biomass = 0.00632 (g/l) | CH4 = 0.0181(g/l)<br>O2 = 0.00115(g/l)<br>Biomass =1.28(g/l) | CH4 = 0.017267(g/l)<br>O2 = 7.765e-7(g/l)<br>Biomass =3.243(g/l) | CH4 = 0.01724 (g/l)<br>O2 = 3.72e-7(g/l)<br>Biomass =8.245(g/l) |
| 0.2 / 0.8 | CH4 = 0.00495(g/l)<br>O2 = 0.01445(g/l)<br>Biomass =7e-5(g/l) | CH4 = 0.00494(g/l)<br>O2 = 0.01442(g/l)<br>Biomass =3.82e-4(g/l) | CH4 = 0.00493(g/l)<br>O2 = 0.01441(g/l)<br>Biomass =0.0021(g/l) | CH4 = 0.0049(g/l)<br>O2 = 0.01440(g/l)<br>Biomass =0.044 (g/l) |

*3.4.2. Methane and air concentration in 80% methane and 20% air ratio*

In figure 5 (IV) (a-c) the contours of methane, air and biomass concentration (g/L) in the ratio of methane/air 80/20 at 250 and 400 (V) h are shown respectively.

*3.4.3 Methane and air concentration in 20% methane and 80% air*

In figure 5 (VI) (a-c) the contours of methane, air and biomass concentration in g/L, in the ratio of 20% methane and 80% air at 250 hours are shown respectively. Figure 5 (VII) (a-c) shows the contours of methane, air and biomass concentration in g/L, in the ratio of 20% methane and 80% air at 400 hours, respectively.

In 2010, Yazdian et al. designed a horizontal loop bioreactor for biomass production using CFD software. In this study, parameters affecting bacterial growth rate such as inlet air velocity, inlet fluid velocity, bubble diameter and viscosity were investigated. The contours of methane and air concentration changes as well as biomass concentration change were studied at 0.5, 1, 1.5 and 4 s. In optimum conditions, the highest growth rate for the bacteria was obtained with 50% methane to 50% air ratio. It was also observed that in the horizontal sections of the bioreactor, the rate of biomass production was much higher than that in the vertical sections Yazdian, et al. (2010).

Finally, table 3 presents the variation in the concentration of methane and air components along with the biomass concentration in the liquid phase in g/L at different times in different ratios of methane and air. The biomass concentration increased over time in each ratio of methane to air, but at the same time, the air and methane concentrations showed a decreasing trend in the liquid phase. In addition, as the concentration of inlet air increases, the biomass production rate decreases.

## 4. Conclusions

Despite wide application in different fields, the high cost of PHB production has made it economic unprofitable comparing to synthetic polymers. Separation cost, carbon source used, fermentation process and carbon substrate efficiency are some of the factors affecting PHB production cost. Production of Polyhydroxy Butyrate is usually increased under nutrient deficiency conditions. Therefore, after reaching a high cell density, it's production should be increased by eliminating or reducing a nutrient such as nitrogen in the bacterium. In this study, a vertical loop bioreactor was simulated by COMSOL software to produce biomass from methanotroph methanocystis hirsutas bacterium purchased from Microbial Bank of Iran Scientific and Industrial Research Organization using natural gas. The Mund's kinetic model is considered for the kinetics of cell mass growth. The simulated environment is a laboratory-scale 3-L bioreactor. Reactor simulation was performed in 3D by determining the reactor dimensions, along with flow parameters, growth

kinetics constants and mass transfer coefficient in the liquid phase. The amount of PHB produced from methane (municipal gas) by the bacterium Methylocystis hirsute was 3 g/L using a loop air system at optimum conditions for 250 hours of fermentation. By comparing these results with other studies of PHB production from methane, this bacterial species introduced as a novel and efficient type. According to the relationship of growth rate for the biomass, the process of changing the concentration of the components is as follows,

The upward trend of increasing methane and air concentrations in the liquid phase due to their transfer from the gas phase to the liquid-gas interface. Constant concentration of these components due to equilibrium concentration with liquid phase. Decreasing trend for both components and increasing biomass concentration due to biomass growth response. The highest biomass growth was observed for the ratio of 50% methane and 50% air, which was 3 g/L at 250 h with 0.5% error reported in the thesis.

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# COMPARATIVE EFFECT OF DIFFERENT NANOPARTICLES ON STRUCTURAL, THERMAL AND BARRIER PROPERTIES OF POLY(ETHYLENE TEREPHTHALATE) IN FOOD PACKAGING SECTOR

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**ABSTRACT**

In the present study, the effect of nanoclay and nanomica on the structural, thermal and barrier properties of poly (ethylene terephthalate) was investigated. The morphology of the papered nanocomposites (Clay and Mica) was illustrated by X-Ray Diffraction (XRD), Transmission electron microscopy (TEM) and Atomic force microscopy (AFM). According to Dynamic Mechanical Thermal Analyzer (DMTA) results, the samples' tanδ values ranged from 0.4 to 0.6. The results of Differential scanning calorimetry (DSC) revealed that the incorporation of nanoparticles increased both the crystallization temperature (Tc) and the degree of crystallization (Xc). Then, the higher aspect ratio of nanomica compared to nanoclay led to higher levels of Xc. A significant water vapor permeability decrease (maximum reduction at 1% loading level of nanomica) of nanocomposites was attributed to an increase in the tortuosity of water vapor molecules path diffusing into the nanocomposites. PET/mica nanocomposites presented larger tortuosity factors compared to PET/clay. As a result, improved barrier properties of nanocomposites were obtained in the case of food packaging.

## 1.Introduction

Over past decades, polymers have been augmented with micro-fillers to obtain higher stiffness and strength, improve fire resistance, or cost reduction. Though, the incorporation of these particles may have some detrimental effects, for instance, opacity and brittleness. Nanoparticles have been used to overcome the restrictions of micro-scale fillers (Müller et al., 2017). In addition, very low loading levels of nanoparticles (<10 wt.%) in comparison to micro-fillers (usually 25-40 wt.%) are required to achieve improvements in nanocomposites (Chouit et al., 2014; Nilagiri Balasubramanian & Ramesh, 2018; Sravanthi, Mahesh, & Rao, 2021).

Size, shape, structure, specific surface area, concentration and adequate dispersion of nanoparticles can affect the ultimate properties of the nanocomposite (Coetzee, Venkataraman, Militky, & Petru, 2020; Ferdous, Sarker, & Adnan, 2013). There can be three categories of nanoparticles in terms of size and shape to polymer reinforcement. The first nanoparticles possess one dimension in the nanometer scale (≤ 100 nm), such as layered nanographite and platelet-like shaped montmorillonite (nanoclays); the second with two dimensions in

the nano-scale, elongated structures, for instance, carbon nanotubes or fibers; and the third has all three dimensions in the nano-scale such as $SiO_2$, spherical silica and titanium oxide (Winkler, Notter, Meyer, & Naegeli, 2018).

Several research studies have investigated different effects of nanoparticles on the properties of PET films. The results showed that the incorporation of nanoparticles could increase the crystallization rate of nanocomposites due to the heterogeneous nucleation role of nanoplatelets (Madakbaş, Türk, Şen, & Kahraman, 2017; Papageorgiou et al., 2014). Some research studies have also discussed the effect of nanoparticles on the crystallization and viscoelastic behavior of PET (Ghanbari, Heuzey, Carreau, & Ton-That, 2013; X. Zhang, Zhao, Mohamed, Kuo, & Xin, 2020). On thermal properties of PET nanocomposites, it has been found that the glass transition point of PET nanocomposite could increase (Wang, Wei-huWang, Zhang, Xu, & Li, 2016) or decrease (Lima, Costa, Sousa, Arruda, & Almeida, 2021) or without change (Farhoodi et al., 2012) in comparison with pure polymer.

Nano reinforcements are nano-scale particles that are dispersed into a specific polymer matrix through processing. One of the most important properties is the ratio of the largest to the smallest dimension of nanofiller, known as the aspect ratio (Sahani & Sharma, 2020). High aspect ratios of nanoparticles lead to increased surface areas with improved, reinforcing properties (Dasgupta & Ranjan, 2018; Sahani & Sharma, 2020). In the case of food packaging applications, the aspect ratio has considerable attention in improving packages' boundary properties. Higher aspect ratios make better boundary properties against gasses (oxygen, water vapor, ethylene, etc.). Consequently, because of the sensitivity of some food (for example, oils, dairy products, fruit and vegetables, etc.) to gases and water, nano-packaging can play a critical role in inhibiting food quality degradation, development of rancid off-flavors, changes in color, shelf life reduction and impair of nutritional quality (Boskovic, Glisic, Djordjevic, & Baltic, 2019; Pillai & Ray, 2015; C. Zhang et al., 2018).

The properties of the bulk polymer will be significantly affected by changes in the size and number of nanoparticles per unit volume (Luo, Wu, & Zhi, 2016). The relation between aspect ratio, volume percent and barrier property of nanocomposites is shown in equation (1) (Choudalakis & Gotsis, 2009):

$$\frac{P}{P'} = \frac{1}{1 + \frac{1}{2\phi}.\left(\frac{L}{W}\right)} \quad (1)$$

where $P$ is the permeability of nanocomposite, $P'$ is the permeability of neat polymer, φ is volume percent, and $L/W$ is the aspect ratio of nanocomposites.

Tortuosity factor ($\tau$) is determined as the distance travelled by a permeant molecule ($d'$) to the thickness of the specimen ($d$) (see equation 2).

$$\tau = \frac{d'}{d} = 1 + \frac{1}{2\phi}.\left(\frac{L}{W}\right) \quad (2)$$

Therefore, the larger aspect ratio of nanoparticles leads to an increase in the content of d′ and, ultimately, enhancement in the polymer's tortuosity factor and barrier properties.

This study aimed to evaluate how nanoparticles with different aspect ratios, including nanoclay and nanomica, at different concentrations (0, 1, 3 and 5% wt.) can affect the structure, thermomechanical and barrier properties of PET nanocomposites.

## 2. Materials and methods

### 2.1. Materials

Neat polyethylene terephthalate (PET) (blow molding grade) with an intrinsic viscosity of 0.82 dL/g was purchased from Tondgooyan Petrochemical Company (Iran). Somasif MAE and Closite 20A nanoparticles were provided by CBC Company (Japan) and Southern Clay Co. (USA), respectively. The characteristics of selected nanoparticles (as recorded by the company) have been described in Table 1.

| Nanoparticle | Commercial name/type | Shape | Code [a] | Density | Organic modifier |
|---|---|---|---|---|---|
| **Nano clay** | Closite20A | platelet-like | NC | 1.77 g/ml | 2M2HT [b] |
| **Nano mica** | Somasif MAE | platelet-like | NM | 1.98 g/ml | MT2EtOH [c] |

[a] NC= Nanoclays (PET1%NC, PET3%NC and PET5%NC), NM= Nano Mica (PET1%NM, PET3%NM and PET5%NM)
[b] 2M2HT: dimethyl, dehydrogenated tallow, quaternary ammonium
[c] methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium
(Tallow: ~65% C18; ~30% C16; ~5% C14)

PET was reinforced with 1D platelet-like shape of organo-modified layered silicates (Closite20A) (platelet size approx. 1 x 200 nm) and 1D platelet-like shape of organo-modified layered silicates (Somasif MAE) (platelet size approx. 1 x 200 nm).

## 2.2. Methods

### *2.2.1. PET nanocomposites preparation*

PET granules and nanoparticles powders were dried overnight at 140°C. The mixing process was developed using a Lab mixer (HAAKE Rheomix 600®) at 60 rpm. Further, PET nanocomposites incorporated with 1, 3 and 5 wt. % of both mica and clay nanoparticles were prepared using melt blending in a lab-scale counter-rotating twin-screw extruder (Collin ESC-T10 model). The extruder contained 5 heater zones fixed at 250, 270, 275, 270, 265, and a die zone set at 265°C (with the screw speed of 90 rpm). Before extrusion, nanocomposite components were dried in an oven at 170°C for 5 h. The prepared profiles were water cooled and after that, milled in conventional milling equipment. Pure PET specimen (coded as PET0) was fabricated by the same process (Farhoodi et al., 2012).

### *2.2.2. DSC analysis*

The thermal properties of PET samples were measured by differential scanning calorimeter 200 F3 Maia® (NETZSCH, Germany) equipment. Nanocomposite specimens' melting behavior has been investigated using heating and cooling tests in the range of 25-270°C at a heating rate of 10°C/min. The first heating run was utilized to delete the thermal history, and all reported data on the melting properties of the prepared specimens were obtained from the second heating curve. The degree of crystallinity (Xc) of prepared PET samples were determined by the equation (3):

$$X_c = \left(\frac{\Delta H_m}{\Delta H_{m0}}\right) \times 100 \quad (3)$$

where $\Delta H_m$ is the melting enthalpy of the samples, and $\Delta H_{m0}$ is the melting enthalpy of 100% crystalline PET ($\Delta H_{m0}$=140 J/g) (Lima et al., 2021).

### *2.2.3. Transmittance*

The transparency of films was measured using a lux meter (Testo 540 pocket-sized lux meter, UK). To do this, the device bubble was placed under an optical source with minimum volatility, and the lux was noted. Then, in stable condition, the specimen was placed on the bubble, and the lux was received. From the two numbers obtained, the transparency of the samples was reported as percentages.

### *2.2.4. Dynamic mechanical analysis (DMA)*

A Polymer Laboratories DMTA (Polymer Laboratories, Loughborough, UK) was employed for DMTA experiments. A frequency of 1 Hz and a temperature range of 25 to 270°C was used with single cantilever bending of samples for these experiments (around 30×10×2 $mm^3$ in size).

### *2.2.5. X-ray diffraction (XRD)*

An X-ray diffractometer (Siemens D5000-Germany) with Cu Kα radiation in the wavelength of 1.5409°A (operational tube voltage and current 30 kV and 30 mA, respectively) was used to record the XRD patterns at room temperature. The samples were scanned in 2θ scan mode by increasing the

temperature from 2 to 80 with a step size of 0.04°C.

In Equation 4, the relation between the angular and layer spacing values according to the Bragg's law is shown.

$$\lambda = 2d \sin\theta \quad (4)$$

where d is spacing between diffraction lattice planes and θ is the measured diffraction angle. The Scherrer equation is widely used to calculate full-width data at half maximum (FWHM) intensity, expected for that average crystallite size:

$$L = \frac{K.\lambda}{\beta_m.\cos\theta} \quad (5)$$

where $\lambda$ is the X-ray wavelength, $\beta_m$ is the line width of the 'pure' diffraction profile resulting from small crystallite size, θ is the diffraction angle, and K is a constant almost equal unity and depended on crystallite shape.

### *2.2.6. Transmission Electron Microscopy (TEM)*

Ultra-thin sections of the nanocomposites at a thickness of 70-100 nm were prepared with a microtome (Leica Ultra-cut UCT, Vienna, Austria) using a diamond knife. The morphology of PET/clay and PET/mica nanocomposites were revealed by transmission electron microscopy (TEM, Philips-EM208S electron).

### *2.2.7. Atomic Force Microscopy (AFM)*

A DualScope scanning probe-optical microscope (DME, Denmark) equipped with a DS 95-50-E scanner and an AC probe was used to prepare tapping-mode AFM micrographs. The tapping-mode AFM has been recently developed by changing the cantilever probe's phase angle to create a second image, referred to as the phase-contrast image. During tapping-mode, the phase lag between the cantilever drive frequency and its response can often create significantly more contrast than the topographic image and is sensitive to material surface characteristics, for instance, viscoelasticity, stiffness, and chemical composition. In general, variations in phase angle through scanning are dependent on energy dissipation during tip-sample interaction and can be a result of variations in topography, tip-sample molecular interactions and deformation at the tip-sample contact (Jiang et al., 2003). In this study, nanoparticles dispersion has been found by the phase images of PET nanocomposites. Measurements were performed in the air at room temperatures. Height and phase images were recorded at the same time.

### *2.2.8. Water Vapor Permeability Test*

The values of water vapor transmission were determined using anhydrous calcium chloride as a desiccant (dish method) according to the ASTM-E 96. Circular trials with a thickness of 0.16±0.01 mm and an area of 28.7 $cm^2$ were prepared by compression molding then dried at 120°C for 24 h. The water vapor permeability (WVP) was calculated at 30°C and 75% relative humidity (RH). The permeability of water vapor P [g m/ Pa $m^2$ h] is measured from Eq. (6):

$$P = \frac{\frac{m}{t.A}}{\frac{P_s(RH_1 - RH_2)}{100}}.L \quad (6)$$

where *m* is the weight gain of the polymer film, *t* is the time for gaining weight, *A* is the test area, *L* is the thickness of the film, *Ps* is the saturation vapor pressure at the test temperature, $RH_1$ is the relative humidity in the test chamber, $RH_2$ is the relative humidity at the dry side of the film, which was considered to be zero. Highly dispersed nanoparticles have the ability to increase the tortuosity of the system significantly. According to the Nielsen model, the ratio of permeability of pure polymer to nanocomposite polymer has been defined as the tortuosity factor (Tan & Thomas, 2017).

## 2.3. Statistical analysis

Data analysis was carried out using SPSS statistical software version 23 (SPSS Inc., Chicago IL). To recognize any significant difference between samples, analysis of variance (two-way ANOVA) followed by Bonferroni test was employed. The level of

significance was set at $p<0.05$. All tests were run in triplicate.

## 3. Results and discussion

### 3.1. Transparency

Regarding the effect of nanoparticles' presence in the polymer structure, maintaining the transparency of the prepared nanocomposites is one of the parameters that should be considered during production. According to the observation of prepared films and the results from lux meter (Table 2), the highest transparency was observed in neat PET samples (89.5 ± 2.24%), while the 5% nanomica samples had the minimum values (61.6 ± 1.95%). The transparency of samples was significantly affected by the type and percentage of nanoparticles ($p<0.05$). Significant differences were also observed between all nanoparticle levels. The incorporation of nanoclay (NC) in comparison with nanomica (NM) made a minor change in the transparency of the nanocomposites ($p< 0.05$).

**Table 2.** The effect of nanoparticles on the transparency of the films.

| Sample | Transparency (%) | SD |
|---|---|---|
| **PET0** | 89.50[a] | 2.24 |
| **PET1%NC** | 81.02[b] | 1.62 |
| **PET3%NC** | 75.00[c] | 2.25 |
| **PET5%NC** | 70.32[d] | 2.25 |
| **PET1%NM** | 72.02[e] | 1.44 |
| **PET3%NM** | 67.91[f] | 2.04 |
| **PET5%NM** | 61.06[g] | 1.95 |

Appropriate dispersion of clay and mica nanoparticles in 1 wt.% and thoroughly exfoliated structure (according to the result of XRD and TEM) at low loading levels of nanoparticles caused more transparent films. By increasing the amount of nanoparticles in the polymer matrix, the exfoliation morphology is reduced, and the probability of increasing the sizes of the nanoparticles and flocculated structure increased. Consequently, darkening of PET/mica and PET/clay nanocomposite films was observed at the levels of 3 and 5%. Lin et al. (2020) indicated that most nanoparticles have dimensions less than the visible light wavelength, especially clay platelets with exfoliated nanostructures. So, no significant changes in the transparency of the prepared films are expected (Lin, Bilotti, Bastiaansen, & Peijs, 2020). Nevertheless, a significant decrease in the films' transmittance will be observed when the nanoparticles are not entirely dispersed in the polymer matrix. Other possible reasons are due to the nature of the nanoparticles and the probable crystallization of the prepared nanocomposites (Dadashi, Mousavi, Emam-Djomeh, & Oromiehie, 2014). Considering that the films used in this study entered the cold water immediately after leaving the thermal press, there was no crystallisation chance. Therefore, changing the degree of crystallinity could not be an effective factor in this study's transparency.

### 3.2. Investigation of thermal properties by DSC

Measurements of glass transition temperature (Tg), crystallization temperature (Tc), enthalpy of crystallization (ΔHC), melting temperature (Tm), enthalpy of melting (ΔHm), degree of crystallinity (Xc) and their data analysis are

summarized in Table 3. As seen in Table 3, the reduction in Tg for nanocomposites in comparison to pristine PET is not significant ($p > 0.05$). Unchanged Tg of the nanocomposites could be due to weak cross-linking reaction among PET chains and nanofillers' surface. In the case of PET 5% nanomica, there was almost 3°C decrease in Tg, which could be a result of polymer chains destruction at high shears during the extrusion process in the presence of hard nanomica particles. In several research studies, a decrease in Tg of PET nanocomposites was detected in the presence of MMT (A Greco, Corcione, Strafella, & Maffezzoli, 2010; Antonio Greco, Gennaro, & Rizzo, 2012; Lima et al., 2021). Soon et al. (2009) also reported that molecular weight reduction of polymer after extrusion caused the reduction in Tg; nevertheless, the decline is not very significant (K. H. Soon et al., 2009). In this study, incorporation of mica and clay nanofillers in PET structure increased both the crystallization temperature (Tc) and the degree of crystallization (Xc). As observed in Table 3, the nanocomposites containing 1, 3 and 5% nanoparticles have higher crystallization temperatures than neat PET, and the peak width ($\Delta Tc$) for all of them is narrower than neat PET ($p < 0.05$). The narrowest crystallization peak was detected in 3% nanomica, 13.1°C narrower than the $\Delta TC$ of PET0 sample. DSC curves of the cooling and heating process of neat PET, PET/clay and PET/mica nanocomposites are shown in Fig. 1. As identified from the cooling curves (a, c), all nanocomposites' crystallization temperatures are higher than pristine PET. The crystallization peak width reduction can verify the increase of the overall crystallization rate of the produced nanocomposites. Nanocomposites, with 1, 3 and 5% loading of C20A and MAE, showed higher Xc than the PET0 ($p < 0.05$) and the highest Xc was observed at 1 wt.% loading of mica nanoparticles (Table 3). Incorporation of 1% w/w nanoclay and nanomica caused an 11.94% and 16.63% increase in the degree of crystallinity, respectively.

**Table 3.** The characteristic values of DSC analysis of samples.

| Sample | $T_g$ (°C) | $T_m$ (°C) | $\Delta T_m$ (°C) | $\Delta H_m$ (J/g) | $X_C$ (%) | $T_c$ (°C) | $\Delta T_c$ (°C) | $\Delta H_C$ (J/g) |
|---|---|---|---|---|---|---|---|---|
| **PET0** | $80.5^{a} \pm 0.05$ | $249.2^{a} \pm 0.1$ | $20.1^{a} \pm 0.2$ | $35.48^{a} \pm 0.31$ | $33.48^{a} \pm 0.2$ | $187.5^{a} \pm 0.7$ | $24.3^{a} \pm 0.14$ | $37.73^{a} \pm 0.3$ |
| **PET1%NC** | $80.87^{a} \pm 0.09$ | $249.8^{a} \pm 0.08$ | $20.8^{a} \pm 0.15$ | $48.14^{b} \pm 0.13$ | $45.42^{b} \pm 0.18$ | $190.2^{b} \pm 0.28$ | $20.0^{b} \pm 0.56$ | $47.92^{b} \pm 0.09$ |
| **PET3%NC** | $79.27^{a} \pm 0.35$ | $250.3^{ab} \pm 0.12$ | $19.3^{b} \pm 0.24$ | $42.15^{c} \pm 0.2$ | $39.77^{c} \pm 0.09$ | $192.1^{c} \pm 0.14$ | $17.6^{c} \pm 0.08$ | $41.70^{c} \pm 0.1$ |
| **PET5%NC** | $79.25^{a} \pm 0.27$ | $251.0^{b} \pm 0.2$ | $19.3^{b} \pm 0.12$ | $40.32^{d} \pm .15$ | $38.05^{d} \pm 0.1$ | $192.3^{c} \pm 0.42$ | $17.3^{c} \pm 0.07$ | $40.35^{d} \pm 0.05$ |
| **PET1%NM** | $80.08^{a} \pm 0.21$ | $245.07^{c} \pm 0.2$ | $23.49^{c} \pm 0.3$ | $53.10^{e} \pm 0.42$ | $50.11^{e} \pm 0.21$ | $204.5^{d} \pm 0.01$ | $12.54^{d} \pm 0.07$ | $46.29^{e} \pm 0.12$ |
| **PET3%NM** | $79.64^{a} \pm 0.3$ | $249.48^{d} \pm 0.35$ | $23.12^{c} \pm 0.39$ | $44.61^{f} \pm 0.09$ | $42.10^{f} \pm 0.3$ | $207.60^{e} \pm 0.4$ | $11.20^{f} \pm 0.04$ | $43.34^{f} \pm 0.23$ |
| **PET5%NM** | $77.53^{a} \pm 0.22$ | $252.60^{e} \pm 0.14$ | $25.08^{d} \pm 0.12$ | $44.93^{f} \pm 0.11$ | $42.40^{f} \pm 0.15$ | $209.13^{f} \pm 0.6$ | $12.25^{d} \pm 0.07$ | $44.84^{g} \pm 0.08$ |

$^{a-f}$ significantly different ($p < o.o5$).

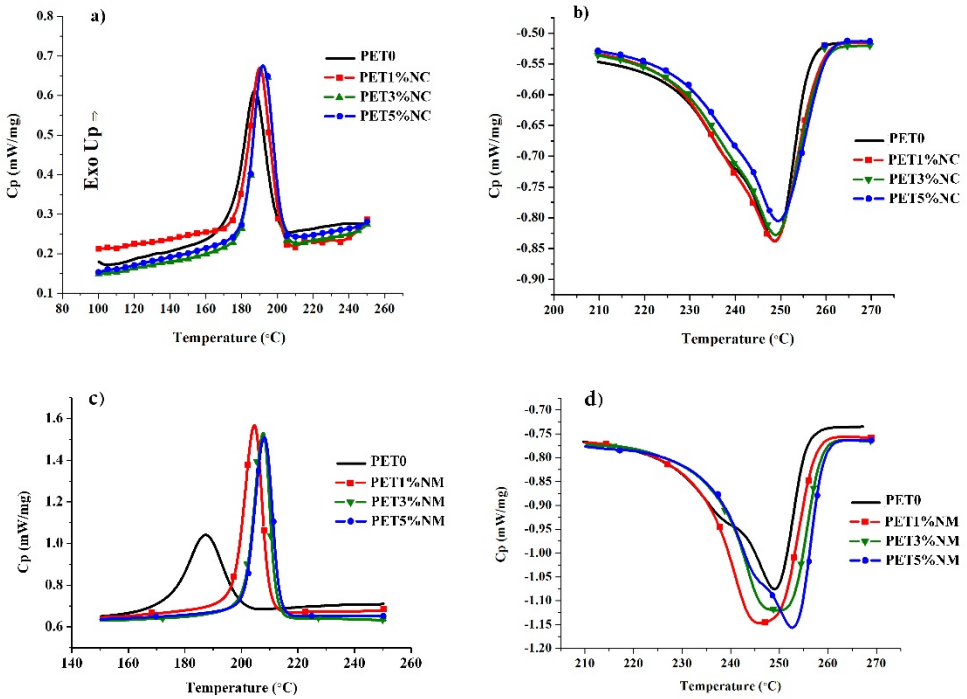


**Figure 1.** DSC curves of neat PET, PET/clay and PET/mica nanocomposites (a, c) the cooling process, (b, d) the heating process

However, when the content of C20A and MAE reached 3 and 5%, Xc was reduced. The existence of impenetrable crystals on the amorphous polymer region can hinder the diffusion of gases like water vapor through the polymer matrix. The crystallization behavior of nanocomposites is typically related to nanofillers loading levels and their dispersion in the polymer matrix and the relative dominating status of two effects of nanoparticles (nucleation effect and growth restriction effect). Crystallinity degree measurements indicated that the nucleation effect becomes stronger at low nanoparticle contents (1 wt.% C20A and MAE). So, the Xc of these specimens are the highest levels compared to other samples. Whereas, by increasing the nanoparticles loading, the situation could be changed in which the restriction of macromolecules motions effect dominates. In other words, at high loading levels of nanoparticles, the mobility of polymer chain segments is significantly restricted because of the physical barrier properties of nanofillers against the motion of polymer chains (Wan, Chen, Chua, & Lu, 2004). Thus, the crystallization process of nanocomposites can initiate earlier or at higher temperatures compared to pristine PET. Accordingly, the nucleation rate and, after that, the overall crystallization kinetics promote. The results revealed that synthetic mica on crystallization is more sensible than nanoclays. The higher aspect ratio of nanomica compared to nanoclay could lead to higher levels of Xc. Lima et al. (2021) reported that by incorporation of 2.5% and 7.5% closite 20A into PET, higher crystallization temperature in comparison to the neat polymer was obtained (Lima et al., 2021). Another study also revealed an increase in $T_c$ by incorporation of 1, 2 and 3% nanoclay into the PET matrix (Guan et al., 2008). Fig. 1 shows a slight increase in the melting points of clay nanocomposites compared to the neat PET. The melting peaks in PET/mica nanocomposites are broader ($p< 0.05$), especially for PET 5% nanomica ($\Delta Tm$ =25.08˚C in comparison to 20.1˚C for PET 0). The melting point and $\Delta Tm$ of clay nanocomposites have been not changed

significantly in comparison with neat PET. This indicates that the crystal forms and the crystal structure of PET have not been changed by incorporation of nanoclay to pet; that is, the melting temperature of PET is constant.

This result is a consequence of solid nanoparticles' presence leading to broader crystallite size distribution and imperfections in the crystallite growth process. Therefore, different sizes of the crystallites with different thermal stabilities formed in nanocomposites can widen the melting peaks.

### 3.3. Dynamic mechanical analysis

Fig. 2 (a, c) display the storage modulus (E') and (b, d) tanδ of PET/clay and PET/mica nanocomposites against temperature, respectively. Tanδ indicates the relative significance of both viscous and elastic behaviors of materials, whereby tanδ<1 exhibits stronger elastic behavior and materials may behave like solids. While, tanδ>1 exhibits stronger viscous behavior and materials' behavior is like liquids more (Bertolo, Martins, Horn, Brenelli, & Plepis, 2020).

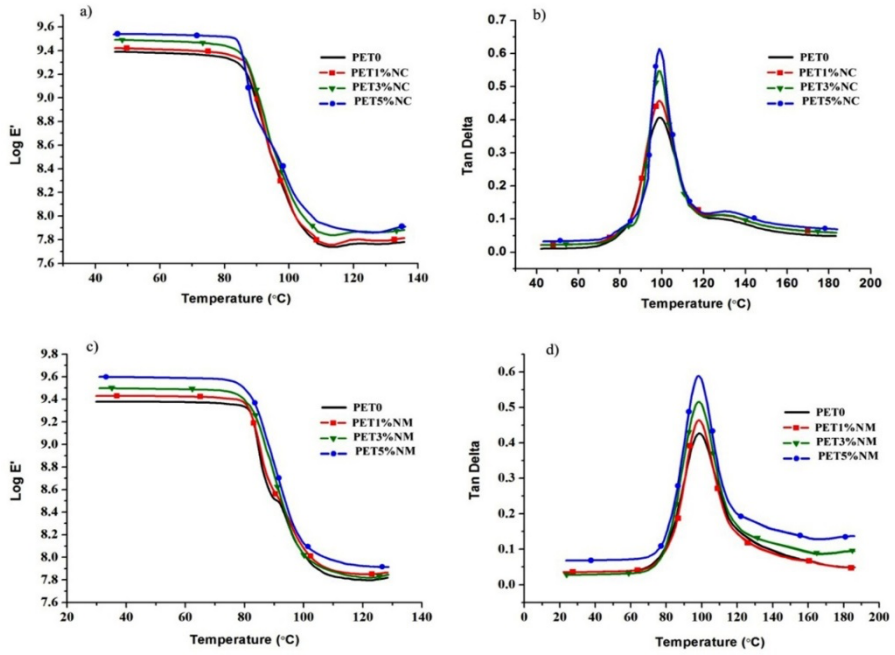


**Figure 2(a, c) Storage modulus (E') and (b, d) tanδ of neat PET and its nanocomposites**

As can be seen in Table 4, tanδ values of the obtained samples are in the range of 0.4 - 0.6, indicating that neat PET sample and PET nanocomposites specimens behave like elastic solids more than viscous liquids. The position of tanδ peak does not alter significantly for PET/clay and PET/mica nanocomposites compared to PET0 specimens (indicating insignificant changes in $T_g$ values). According to the data analysis, the difference between neat PET and 5% nanocomposites was significant ($p< 0.05$), but the type of nanoparticles made no significant difference between tanδ values. By increasing the nanoparticles loading, the tanδ peak magnitudes enhance. It can verify that the pure PET has lower damping capability in comparison with the prepared nanocomposites, and this reduction is more significant for nanocomposite comprising 5 wt.% nanoparticles.

As found, the mean storage modulus varied between 2.15 to 3.90 GPa at 40°C and 0.06 to 0.083 GPa at 140°C depending on the loading of nanofillers from 0 to 5 wt.%. Analysis of our finding showed a significant difference in storage modulus between all treatments at 40°C ($p< 0.05$), but no difference was observed at 140°C ($p> 0.05$). This increasing trend is more sensible in PET/mica.

**Table 4.** E' at 40°C and 140°C and Tanδ peak values for the neat PET and PET/clay and PET/mica nanocomposites.

| Sample | E' at 40 °C (GPa) | E' at 140 °C (GPa) | temp of tanδ at peak (°C) | Tan δ peak value |
|---|---|---|---|---|
| **PET0** | $2.15^{a} \pm 0.2$ | $0.060^{a} \pm 0.01$ | $98.76^{a} \pm 0.1$ | $0.406^{a} \pm 0.13$ |
| **PET1%NC** | $2.63^{b} \pm 0.1$ | $0.061^{a} \pm 0.02$ | $98.50^{a} \pm 0.15$ | $0.457^{a} \pm 0.1$ |
| **PET3%NC** | $3.09^{c} \pm 0.11$ | $0.073^{a} \pm 0.01$ | $98.57^{a} \pm 0.09$ | $0.547^{ab} \pm 0.07$ |
| **PET5%NC** | $3.38^{d} \pm 0.3$ | $0.073^{a} \pm 0.025$ | $98.59^{a} \pm 0.14$ | $0.614^{b} \pm 0.06$ |
| **PET1%NM** | $2.63^{e} \pm 0.12$ | $0.07^{a} \pm 0.01$ | $98.36^{a} \pm 0.1$ | $0.464^{a} \pm 0.1$ |
| **PET3%NM** | $3.14^{f} \pm 0.2$ | $0.066^{a} \pm 0.01$ | $98.79^{a} \pm 0.08$ | $0.515^{ab} \pm 0.12$ |
| **PET5%NM** | $3.90^{g} \pm 0.15$ | $0.083^{a} \pm 0.03$ | $97.80^{a} \pm 0.1$ | $0.588^{b} \pm 0.07$ |
| $^{a-f}$ significantly different (p< o.o5). | | | | |

Soon et al. (2009) reported that the modulus, particularly above the glass transition, was enhanced by the incorporation of 1, 2 and 5% synthetic mica (Somasif MTE and Somasif MAE) to PET matrices (K. H. Soon et al., 2009). The formation of a network structure among polymer chains and nanoparticles leads to increased storage modulus beyond the Tg point, which enhances the rigidity of the nanocomposites. Other research studies have also reported this fashion (Francis, Joy, Aparna, & Vijayan, 2014; Majdzadeh-Ardakani, Zekriardehani, Coleman, & Jabarin, 2017).

### 3.4. X-Ray Diffraction (XRD)

To reveal information about Intercalation/exfoliation of PET/clay and PET/mica Wide-angle XRD (WAXD) measurements on neat PET, pure nanofillers and nanocomposites were carried out. Table 5 summarizes the parameters of XRD curves. The d-spacing values of the PET/mica, PET/clay and nanocomposite sheets were calculated by the Bragg equation. The performance of filling is obviously dependent on the larger interlayer distance of nanoparticles in PET nanocomposites. The polymer chains need to be inserted within the interlayer of the nanoparticle structure to formation of intercalated or exfoliated structures in which an efficient interaction among nanoparticles and polymer matrix is necessary.

**Table 5.** Values of 2theta and d-spacing for PET nanocomposites.

| | 2θ | d-spacing (nm) |
|---|---|---|
| **Nanoclay Powder** | 5.712 | 1.53 |
| **PET1%NC** | 2.58 | 3.50 |
| **PET3%NC** | 3.13 | 2.85 |
| | 5.875 | 1.51 |
| **PET5%NC** | 3.038 | 2.96 |
| | 5.639 | 1.56 |
| **Nanomica Powder** | 4.82 | 1.83 |
| **PET1%NM** | 5.84 | 1.51 |
| **PET3%NM** | 2.68 | 3.29 |
| | 5.91 | 1.49 |
| **PET5%NM** | 2.65 | 3.33 |
| | 5.81 | 1.52 |

Fig. 3 (a, b) demonstrates the WAXD profiles for the PET/clay and PET/mica nanocomposites, respectively. A mixed morphology of intercalated/exfoliated structure has been formed in all nanocomposites. For nanoclay powder, a peak emerged at about 2θ= 5.712 (d = 1.53 nm), confirming the basal interlayer spacing of the clay. The maximum decrease in the 2theta value obtained in the systems incorporated with 1% of nano-clays, which corresponds with semi-exfoliation (exfoliation of a certain number of tactoids). A shift to lower angles of the characteristic diffraction peak in 3 and 5 wt.% PET/clay suggests an increase in interlayer spacing of the clay, which is referred to as intercalation. At 1 wt.% Closite, the organoclay layers have been randomly dispersed in the polymer (for instance, exfoliated). While increasing the concentration of Closite to 3 and 5% suggests intercalated to exfoliated structure in which polymer molecules have entered the distance between organoclay layers.

As seen in Fig. 3b, at PET 1% nanomica, there seems to be a peak at 2θ< 2, which has not been revealed in the WAXD analysis. The presence of this peak can be a result of more opening of mica layers at low loading levels. The presence of the peaks at about 2θ=2.68 (d=3.29 nm) for PET 3% nanomica and 2θ=2.65 (d=3.33 nm) for PET 5% nanomica can indicate the opening of the mica layers by polymer chains and formation of intercalation structure. Nevertheless, WAXD should not be used as a stand-alone procedure for the characterization of polymer nanocomposites. In some cases (for instance, large amounts of clay, random orientation of the layers, inhomogeneous distribution of surfactant in the interlayer space or a distribution of interlayer distance) it may lead to a misleading interpretation of the nanostructure. For these reasons, it is recommended that the results of this test be compared with direct observations of the layers by Transmission Electron Microscopy (TEM) or Atomic Force Microscopy (AFM) (K. H. Soon et al., 2009).

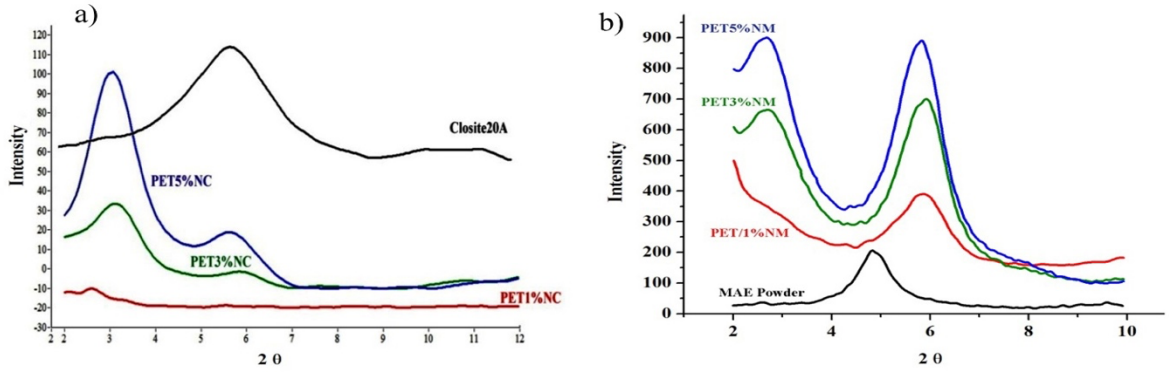


**Figure 3.** WAXD profiles for Closite20A powder and PET/clay (a) and MAE powder and PET/mica nanocomposites (b)

### 3.5. Transmission Electron Microscopy (TEM)

The TEM micrographs confirm the results of WAXD analysis. TEM micrographs of nanocomposites containing 1 and 3 wt.% nanoclay and nanomica are shown in Fig. 4. The individual delaminated silicate layers especially nanoclay marked in a circle in Fig. 4b. In Fig. 4 (c, d), micrographs of PET/mica nanocomposites are shown. As can be seen, when the content of nanomica is 1%, the mica layers are completely dispersed in the polymer matrix, and the exfoliated structure is obtained, whereas 3% nanomica gives

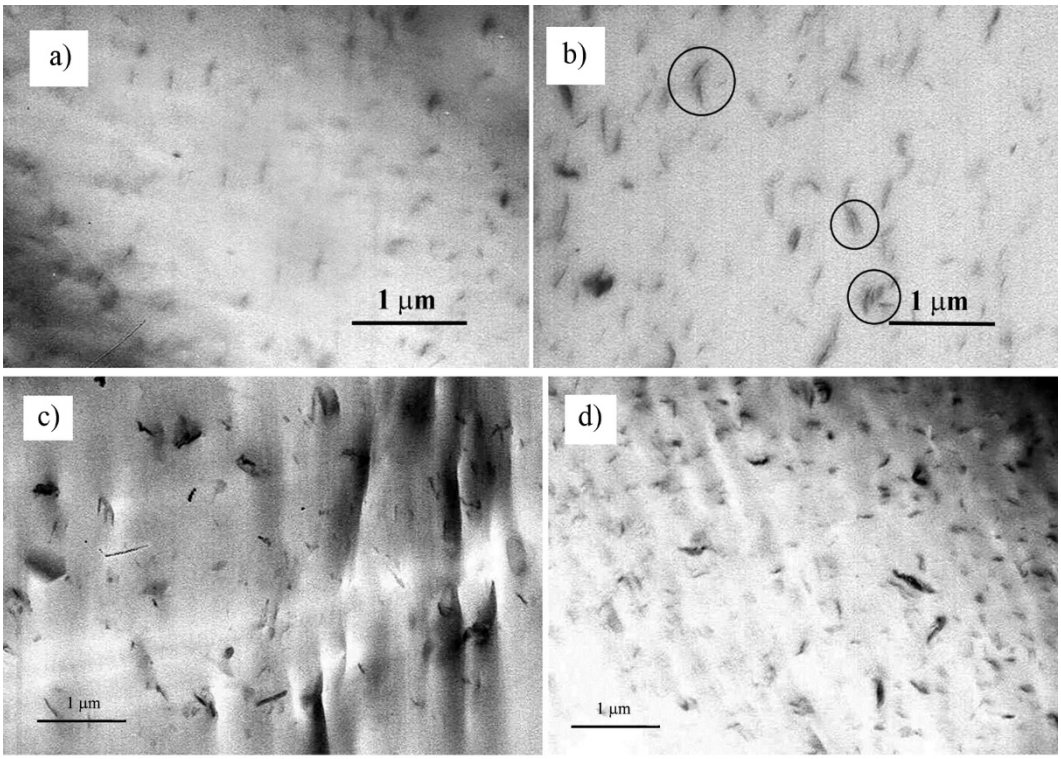


**Figure 4.** TEM micrographs 1 (a) and 3% (b) nanoclay and 1 (c) and 3% (d) nanomica

detected at 1%wt. Closite revealed a more exfoliated structure. A partially exfoliated/intercalated structure with dispersed tactoids for 3% Closite was observed. Intercalated/exfoliated structures of 3% intercalated/exfoliated morphology. The results of the dispersion of nanoparticles in our micrographs are in accordance with previous investigations (K. Soon et al., 2012; K. H. Soon et al., 2009).

### 3.6. Atomic Force Microscopy (AFM)

The height and phase images of PET nanocomposites are illustrated in Fig. 5 and 6. As shown, phase In Fig. 6, micrographs of 1% PET/mica nanocomposites are shown. As can be seen, when the content of nanomica is 1% (Fig. 6a), the mica layers are completely dispersed in the polymer matrix, and the exfoliated structure is obtained, whereas 3% nanomica gives intercalated/exfoliated morphology (Fig. 6b). The results of the dispersion of nanoparticles in our micrographs are in accordance with previous investigations (Bizarria et al., 2007; K. H. Soon et al., 2009).

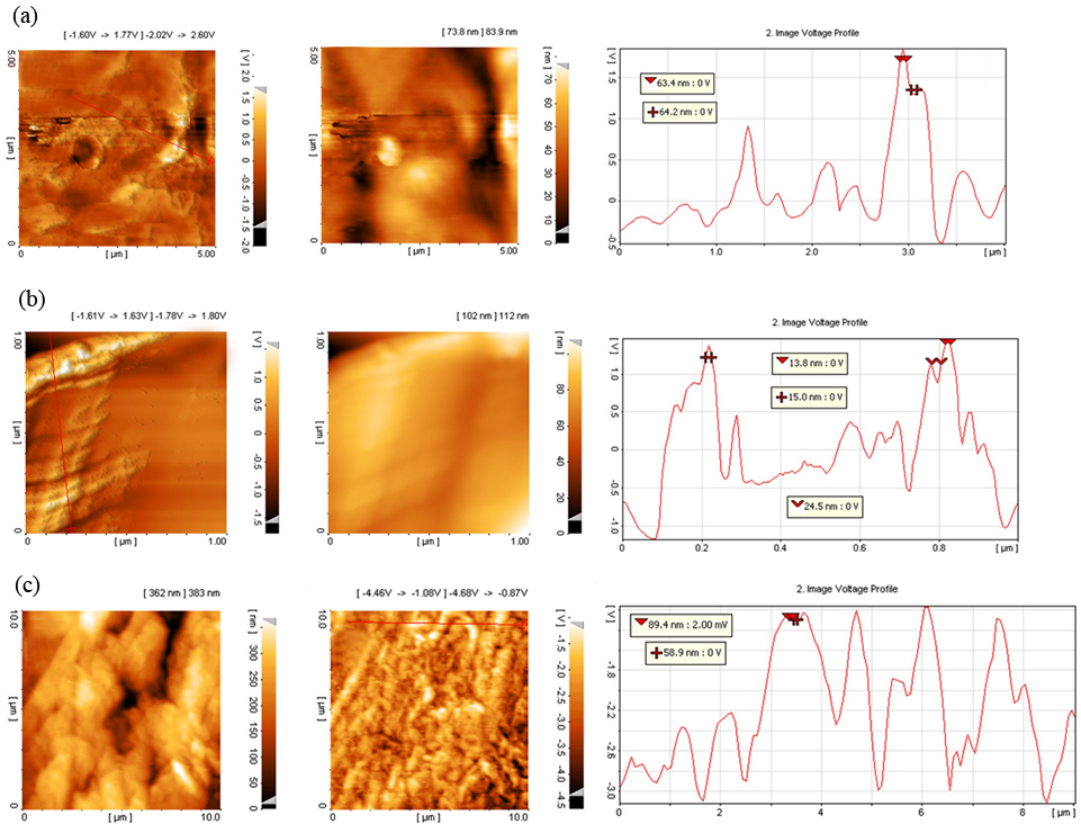


**Figure 5.** The height and phase images of PET 1% wt. (a), 3% wt. (b) and 5% wt. (c) nanoclay

### 3.7. Water Vapor Permeability (WVP)

Since water is one of the most critical factors in food spoilage reactions, water vapor transmission rate (WVTR) and water vapor permeability (WVP) are the key properties in polymeric food packaging. WVTR and WVP of nanocomposite films are influenced by various factors, including hydrophilicity and hydrophobicity of the components, nanocomposites preparation method, type and distribution of additives, tortuosity and ultimately order in the polymer structure (Sarfraz, Gulin-Sarfraz, Nilsen-Nygaard, & Pettersen, 2021). The distance travelled by a permeant molecule in the polymer matrix is known as the tortuosity factor. The decrease in WVP of nanocomposites is attributed to an increase in the tortuosity of water vapor molecules path diffusing into the nanocomposites (Farhoodi, Mohammadifar, Mousavi, Sotudeh-Gharebagh, & Emam-Djomeh, 2017; Saxena et al., 2020). The increased tortuosity of water vapor molecules leads to improved barrier properties of nanocomposites.

The results of measuring the permeability of pure PET films and their nanocomposites are shown in Fig. 7a. As can be seen, each of the nanoparticles has different behaviors. The presence of nanoparticles led to a significant permeability decrease of polymer matrix with a maximum reduction of 64% in 1% PET/mica nanocomposite compared to neat PET. As presented in Fig. 7b, PET/mica nanocomposites have larger tortuosity factors than PET/clay. According to data analysis, there was a significant difference between type and the percentage of nanoparticles on WVP and

tortuosity factor of PET/clay and PET/mica nanocomposites ($p<0.05$). As a result, nanomica had a more significant effect on reducing permeability and increasing tortuosity factor than nanoclay. As previously mentioned, significant differences were observed at all levels, especially at 1% wt nanoparticles.

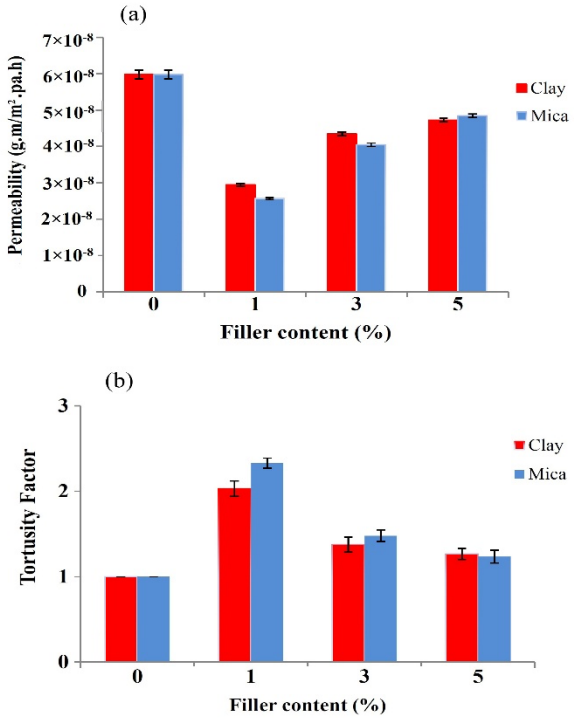


**Figure 7.** Permeability and tortuosity factor of neat PET films and their nanocomposites

In the case of food packaging, the incorporation of high aspect ratio nanoparticles could increase the tortuosity factor. This factor is a function of the aspect ratio of nanoparticles and the nanofiller volume fraction in the composite. Platelet-like nanoparticles can particularly affect barrier properties of a polymer. Their performance is improved when their degree of exfoliation and their aspect ratio increased (Cerisuelo, Gavara, & Hernández-Muñoz, 2015; Yeh et al., 2020).

As previously mentioned, and was observed in our obtained results of XRD, TEM, and AFM, low loading levels of nanoparticles (1% wt.) have been randomly dispersed in the polymer matrix. Therefore, a typical exfoliated structure was formed, and the maximum WVP properties happened. While at higher loading levels of nanoparticles, the ratio of intercalated to exfoliated structure increased. At high loading levels, the probability of increasing the nanoparticles sizes and flocculated structure increased. In similar research studies, the WVP was significantly reduced for PET nanocomposites; moreover, exfoliated structure improved polymer properties compared to intercalation morphology (Antonio Greco, Esposito Corcione, & Maffezzoli, 2010).

High aspect ratios of nanoparticles could provide large surface areas with better reinforcing effects. A higher aspect ratio of nanoparticles enhances the tortuosity factor. Therefore, due to the more significant aspect ratio of nanomica compared to nanoclay, the

tortuosity factor improved more, particularly at 1% nanoparticles loading levels. Besides, diffusion of moisture is prevented by impermeable crystals, especially at 1 wt% contributed to higher Xc.

## 4. Conclusions

In this study, the effect of incorporating different levels of nanoclay and nanomica with varying ratios of aspect into the PET matrix on the characteristics of prepared nanocomposites and their influence on barrier properties was investigated. PET/clay and PET/mica nanocomposites were prepared via melt blending. For all the prepared nanocomposites, transparency decreased significantly with increasing nanoparticle content. In addition, nanomica compared to nanoclay made more significant changes in the polymer matrix's transparency. The observations of XRD, TEM, and AFM showed that the exfoliation morphology is reduced by increasing the amount of nanoparticles in the polymer matrix. The probability of increasing the nanoparticles sizes and flocculated structure increased. Consequently, darkening of PET/mica and PET/clay nanocomposite films was observed at the levels of 3 and 5%. According to DSC, the addition of mica and clay nanoparticles in PET structure increased both the crystallization temperature (Tc) and the degree of crystallization (Xc). As a result, the stiffer structure with reduced amorphous regions compared with pure PET was obtained, which increased the tortuosity factor that led to the improvement of barrier properties. Furthermore, the results revealed that the higher aspect ratio of nanomica compared to nanoclay led to higher Xc levels. DMTA results showed an insignificant reduction in Tg for nanocomposites in comparison to pure PET. The magnitudes of tanδ peak in prepared nanocomposites enhanced, which can verify that the pure PET has lower damping capability than nanocomposites. This reduction is more significant for nanocomposite containing 5 wt.% nanoparticles. Concerning barrier properties in food packaging, it was observed that the presence of high aspect ratio nanoparticles in the PET matrix decreased WVP and increased the tortuosity factor. In other words, the increased tortuosity of water vapor molecules led to improved barrier properties of nanocomposites. PET/mica nanocomposites have larger tortuosity factors than PET/clay due to the higher aspect ratio of mica nanoparticles. Taken all the above data together, incorporation of higher aspect ratio nanoparticles, particularly at low loading levels, can significantly reinforce the thermomechanical and barrier properties of the PET matrix.

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# PHYSIOCHEMICAL AND SENSORIAL ATTRIBUTES OF APRICOT FORTIFIED WHEAT BISCUITS

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**ABSTRACT**

This study envisages the application of apricot kerned, a by-product of apricot fruits, in partial substitution of wheat flour (WF) by apricot kernel flour (AKF) in the proportion of 5, 10 and 15 % in making biscuit with three formulations F1, F2, F3, respectively and the fourth one was a control biscuit without AKF. Physicochemical and functional properties of WF, AKF and their blends were studied. The effect of AKF addition on the physical and sensory properties of composite biscuits was also investigated. Obtained results showed that the incorporation of AKF affect significantly the physicochemical characteristics and the functional properties of the flours comparing to the WF as control. A significant difference ($p < 0.05$) in physical characteristic between biscuits fortified with AKF and control was showed except for spread factor and percentage of spread factor. Furthermore, sensory evaluation revealed that the cookies containing AKF were acceptable by the panellists at all concentrations ($p < 0.05$) and based on the sensory evaluation, biscuits supplemented with 5 % of AKF got the highest scores compared to other samples. Thus, AKF can be incorporated at a rate of 5 % to prepare acceptable quality biscuits.

## 1.Introduction

Feeding differs from person to another depending on the religions and eating habits of each person, but all human beings eat to protect their health with the nutrients they find in food and especially in fruits and vegetables. Among the most famous fruits, the apricot, which is one of the most important fruits because of its constitution which gives it a considerable place in the human diet and its use in other non-food products. Scientific investigations state that apricot is a fruit of high nutritional density in terms of sugars (more than 60%), proteins (8%), crude fibers (11.50%), crude fat (2%), total minerals (4%), vitamins (vitamin A, C, K and B) and reasonable amounts of organic acids (malic and citric acids) (Tabasum *et al.*, 2018). Like the fruit, apricot kernel has remarkable nutritional value. In fact, apricot kernels, especially rich in lipid, protein and total dietary fiber, are potentially valuable in human nutrition. The

chemical and nutritional properties of apricot kernels were widely studied by many research groups (Aziz *et al.*, 2020). Various pharmacological effects of apricot kernel have been reported, including antibacterial, antifungal, anti-tumor, anti-coagulant, anti-inflammatory, antidiabetic, anticancer, antiparasitic, antiaging, anti-atherosclerotic, anti-anginal, hepatoprotective, renoprotective, antioxidant (Gupta *et al.*, 2018; Ramadan *et al.*, 2020). Furthermore, apricot kernels can contribute considerably in the prevention and treatment of chronic health disorders (Chen *et al.*, 2020). Those benefit health effects are due to the presence of bioactive components including polyphenol, fatty acid, sterol derivatives, carotenoids, cynogenic glycosides and volatile component (Tabasum *et al.*, 2018). With a production of 209 204 tons in 2019, Algeria is considered a major apricot producer country (FAO, 2019) and the major apricot productions in Algeria is mainly consumed as fresh or dried fruit, or transformed into jam or juice. In all of these uses, apricot kernels have always been considered waste. Recently, more attention has focused on the use of agricultural wastes due to their availability, biodegradability and above all, their lower cost (Melini *et al.*, 2020). This is the reason why, man invested in the valorization of apricot fruits through the recovery and use of their seeds (Dilucia *et al.*, 2020). However, the use of apricot kernels in the food industry is very rare; they are used as a flavoring in food gums. The main use of apricot kernels lies in the extraction of its oil which is already commonly used in cosmetics (soaps, ointments, creams, shampoo) and in medicine for its beneficial effects on health proven in several scientific studies due, mainly to its antioxidant properties (Chen *et al.*, 2020). Nevertheless, the use of apricot kernel in human food is limited due to the presence of amygdalin (cyanogenic glycoside also known as laetrile or vitamin B17) considered a toxic compound when consumed in amounts exceeding the prescribed doses (Jaswal *et al.*, 2018). Despite this, several studies have raised the possibility of adding apricot kernel flour to certain food preparations, given their beneficial effects on health (Dhen *et al.*, 2018; Aziz *et al.*, 2020). Thus, the main objective of the current study is the contribution to the valorization of apricot kernel from the Algerian production as flour in the manufacture of a biscuit. To achieve this, flour blends were made by partially substituting wheat flour by AKF at substitution percentages of (5, 10 and 15) % which were analyzed for their physicochemical and functional properties. The prepared biscuits were characterized in terms of physical characteristics and sensory features.

## 2. Materials and methods

### 2.1. Preparation of apricot kernel flour (AKF)

The pits were recovered from the apricots (*Prunus armeniaca*) in the region of Oum Tboul (Algerian-Tunisian border) during the month of August 2020. The fruits were collected in a meticulous manner, by taking from all sides of the orchards (left, right, up, down, face exposed to the sun and not exposed to the sun). The recovered pits were washed with tap water before being dried in the sun for 12 hours. At the end of drying, the pits were shelled and the kernels were thus collected and crushed to obtain the apricot kernel flour (AKF) with 500 µm particle size. The resulting AKF was stored in plastic bags in a freezer to prevent oxidation of the product until the time of analysis.

### 2.2. Flour blends preparation

The AKF was mixed with wheat flour in different proportions (AKF-Wheat flour) of 00: 100, 05-95, 10-90, 15-85, respectively. The resulting flours, besides WF and AKF, were analysed for their physicochemical and functional properties.

### *2.2.1. Physicochemical characteristics of wheat flour, AKF and their blends*

Physicochemical analyses were carried out on WF, AKF and their blends. Flours were analysed for their:

- Moisture content: was assessed by heating the samples at 105 ± 2 °C until constant weight;
- pH: measured with pH meter (*Crison*);
- Acidity: the principle of the measurement is based on the titration of the alcoholic extract of the flour, using an alkaline solution titrated in the presence of phenolphthalein. Results were expressed in grams of sulfuric acid ($H_2SO_4$) per 100 grams of dry matter;
- Conductivity: measured with conductivity meter;
- Total ash: was determined by calcinations at 550 °C and finally;
- Total sugar: was determined by extraction with an alcoholic solvent (ethanol) followed by measurement of the absorbance at 485 nm.

Analysis was performed in the quality laboratory and conformity, Annaba (Algeria) in four replications.

### *2.2.2. Functional properties of flour blends*

Some functional properties of flours were determined in order to know their behaviour once incorporated in food matrices (biscuit in the current study). The most important of them are water absorption capacity (WAC) and oil absorption capacity (OAC) which were evaluated according to the method described previously by Diomande et al. (2017). The hydrophilic-lipophilic ratio (RHL) as defined by Marie et al. (2015) was calculated by making the ratio of the WAC to the OAC. It is a report which allow to evaluate the comparative affinity of flours for water and for oil.

## 2.3. Preparation of biscuits

The biscuits were prepared according to the recipe given by Sheikh et al. (2019). Briefly, for 100 g of flour, 30 g of fine sugar, 20 mL of milk, one egg, 25 g of margarine, 1 g of sodium bicarbonate, 1 g of vanilla and 1 g of salt have been added. The ingredients were mixed as with any cake. The dough was rolled out into a thin sheet of uniform thickness and was cut using a cookie cutter to obtain discs 30 mm in diameter and 5 mm in thickness. The pieces have been placed on a baking tray and are baked at 180 °C for 10 to 15 minutes in a baking oven. The well-baked cookies were removed from the oven, allowed to cool at room temperature for 30 minutes, wrapped and stored at room temperature in polypropylene bags for further studies. Four biscuits formulations were prepared in accordance with Table 1. Control biscuits were formulated without the addition of AKF (B100).

**Table 1.** Experimental design of biscuit formulations

| Formulation | B100 | B95 | B90 | B85 |
|---|---|---|---|---|
| **Wheat flour (g)** | 100 | 95 | 90 | 85 |
| **AKF (g)** | - | 5 | 10 | 15 |
| **Egg** | 1 egg | 1 egg | 1 egg | 1 egg |
| **Fat (margarine) (g)** | 25 | 25 | 25 | 25 |
| **sodium bicarbonate (g)** | 1 | 1 | 1 | 1 |
| **Vanilla (g)** | 1 | 1 | 1 | 1 |
| **Salt (g)** | 1 | 1 | 1 | 1 |

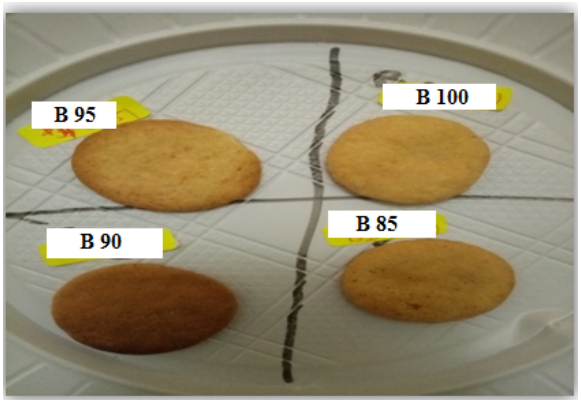


**Figure 1.** Produced Biscuit

### 2.4. Characterization of AKF fortified biscuits

#### *2.4.1. Physical properties*

The diameter (D) and the thickness (T) of biscuits were measured, using a caliper, on five pieces according to the protocol described by Sheikh et al. (2019), results were expressed in cm. The ration of the diameter to the thickness represents the spreading factor (SF). The percentage of the SF (%SF) was represented as the ration of the SF of the biscuits prepared with flour blends to the SF of the Control biscuits. As for the volume, this was determined according to the following equation: $V\ (cm^3) = (D^2\pi T)/4$, while the density was expressed by the ration between the weight and the volume of biscuit.

#### *2.4.2. Sensory evaluation*

The samples of biscuits were organoleptically scored by semi-trained subjects. For this purpose, judges were selected from the faculty staff (21 panellists, between teachers and students comprising male and female) of the department of agronomic sciences, faculty of natural and life sciences (University Chadli Bendjedid, El-Tarf, Algeria) were invited, according to their motivation and interest, to taste the control and fortified biscuits and to attribute a point based on a five-point hedonic scale (1: Don't like at all; 2: Don't like very much; 3: Indifferent; 4: Like a little and 5: Like a lot). The samples of cookies with different compositions were presented in plates divided into 4 parts, in each of them, a biscuit of each formulation is placed. The panelists were asked to evaluate the sensory attributes (General aspect, color, flavor, aroma, friability and overall acceptability).

### 2.5. Statistical analysis

The results are presented as a mean ± standard deviations calculated with Microsoft Office Excel 2007. Results obtained from different experiments were subjected to the analysis of variance (ANOVA). The significant difference between the means was tested against the critical difference at 5 % significance level ($\alpha = 0.05$). The statistical analysis was performed using Minitab version 17 Software (Minitab Inc., State College, PA, United States). The means were compared and the differences were revealed using LSD test (Fisher's test).

## 3. Results and discussions

### 3.1. Effect of AKF addition on the physicochemical characteristics of flours

The physiochemical characteristics of the wheat and apricot kernel flours and their blends are summarized in Table 2. At 5% significance level, significant differences were recorded between all flour samples for all studied parameters. In fact, the AKF had the highest pH (6.38), acidity (0.035 g/L), dry matter (94.270 %), ash (3.50 %), conductivity (795.50 µS/cm) and total sugar (2.49 g/100g). Whereas, the same sample had shown a lower water content comparing to the WF (control) and flour blends (WF-AKF).

### *3.1.1. pH*

The pH is a parameter which determines the aptitude of foods to be preserved. The addition of AKF had affected significantly ($p < 0.05$) the pH of wheat and composite flours. WF had the lowest pH (6.33) while AKF had the highest (6.38), wheat flours with the addition of 5, 10 and 15% AKF had intermediate pH of 6.35, 6.36 and 6.36, respectively. Indeed, the results revealed that pH of composite flours increased with decrease in proportions of WF from 100 % to 85 %. Our results were in accordance with those of Nabil et al. (2020) who indicated a decrease of pH in flour supplemented with cladode flour at different proportions 0, 25, 50, 75, and 10 0%. The same authors reported a pH of whole wheat flour of 6.05 which was slightly lower than that observed in the current study. Chandra et al. (2015a) have found that the pH of composite flours increased with increase in the level of composition of rice flour, green gram flour and potato flour and that the lowest pH value of 5.516 was noted, as in this study, in wheat flour.

### *3.1.2. Fat acidity*

The result showed that mixing AKF with WF raised significantly ($p < 0.05$) the samples acidity with a percentage of 34.29, 34.29 and 31.43 % for samples C95, C90 and C85, respectively. AKF was clearly stood out by a higher acidity of 0.035 g/L compared to the other samples. The fat acidity developed in AKF was approximately 1.6 fold higher than that in WF, this makes AKF more sensitive to the oxidation phenomenon thus reducing its shelf life. Increasing the fatty acidity of composite flours is undesirable, since they will be more sensitive to oxidation, which will have repercussions on the shelf life just like AKF. The fat acidity of millet, wheat and corn flours, expressed as mg potassium hydroxide in 100 g of the product, was 47.5, 12 and 15 mg KOH/100 g dry matter, respectively **(**Goyal *et al.*, 2017). Goyal et al. (2017) observed an increase in fat acidity of these flours during storage and explained this by action of lipases, which causes bitterness and can make meal unacceptable. Authors also stated that the milling of grains leads to tissue damage, thus contact between enzymes and the fat substrate increases. Information on the fatty acidity of AKF is not available in the literature.

### *3.1.3. Moisture content*

Before preparation of biscuits, moisture content of wheat, apricot kernel flour and their blends at different levels were analyzed for their moisture content (Table 2). The moisture of AKF was significantly ($p < 0.05$) different from the wheat and blend flours. The AKF with 5.74 % moisture content makes it more stable and prevent the microorganism's proliferation. Depending upon the blending ratio, the water content ranged from 11.66 % to 12.16 % (dry basis). The respective moisture of composite flours was 12.16 %, 11.94 %, 11.78 % and 11.66 % for C100, C95, C90 and C80. Blending of AKF up to 15 % was found to decrease significantly ($p < 0.05$) the water content. Similar trends were reported previously by Chandra et al. (2015b). They used the blends of rice, green gram and potato flour which resulted in diminution of water content of composite flours. The water content of AKF in the current study was higher than the 2.18 – 2.47 % of moisture reported by Gezeret et al. (2011) in apricot kernels from five varieties collected from Malatya location of Turkey, whereas, that of wheat flour was close to the 13.01 % recorded by Legesse and Emire (2012).

### *3.1.4. Ash*

The ash content of the AKF (3.5 %) differed significantly ($p < 0.05$) from that of WF and blend flours. However, the ash rate of WF and blends revealed non-significant differences ($p > 0.05$). From the results of table 2, it can be seen that incorporation of AKF at different levels in wheat flour significantly increased ($p < 0.05$) the ash content from 0.66 % in wheat flour to 0.70 % in C85 sample (wheat flour with 15% of AKF). The observed increase in ash contents of flour enriched with AKF can be explained by the intake of ash AKF which was very rich in minerals in comparison to wheat flour. Similarly, Sheikh et al. (2019) observed an increase in ash content of the AKF fortified

biscuits. The authors explained this situation by the relatively higher ash content of AKF than wheat flour. This could justify the results obtained from the flour samples substituted with different rates of AKF. The same trend was found by Legesse and Emire (2012) who noted that blending of the mango kernel flour with wheat flour up to 30 % raised significantly the ash content from 0.85 for wheat flour to 1.13 g/100g for flour added with 30 % of mango kernel flour. It was established that in the mineral fraction, Ca, K, Mg, Na and P are as major minerals in apricot kernels (Gezer et al., 2011). Özcan et al. (2010) have stated the same situation.

**Table 2.** Proximate composition and functional properties of wheat flour composite flours (WF – AKF)

| Combination | $C_{100}$ | $C_{95}$ | $C_{90}$ | $C_{85}$ | $C_0$ |
|---|---|---|---|---|---|
| **pH** | 6.33±0.00$^{d}$ | 6.35±0.01$^{c}$ | 6.36±0.01$^{b}$ | 6.36±0.00$^{b}$ | 6.38±0.00$^{a}$ |
| **Fat acidity g/L of $H_2SO_4$** | 0.022±0.00$^{c}$ | 0.023±0.00$^{b,c}$ | 0.023±0.00$^{b,c}$ | 0.024±0.00$^{b}$ | 0.035±0.00$^{a}$ |
| **Moisture content (%)** | 12.16±0.01$^{a}$ | 11.94±0.03$^{b}$ | 11.78±0.02$^{c}$ | 11.66±0.01$^{d}$ | 5.74±0.03$^{e}$ |
| **Dry matter (%)** | 87.84±0.01$^{e}$ | 88.06±0.03$^{d}$ | 88.22±0.02$^{c}$ | 88.34±0.01$^{b}$ | 94.270.03$^{a}$ |
| **Ash (%)** | 0.66±0.01$^{b}$ | 0.68±0.01$^{b}$ | 0.69±0.005$^{b}$ | 0.70±0.01$^{b}$ | 3.50±0.01$^{a}$ |
| **Conductivity (µS / cm)** | 730.25±0.96$^{d}$ | 730.50±0.58$^{d}$ | 737.00±0.82$^{c}$ | 755.25±0.96$^{b}$ | 795.50±1.29$^{a}$ |
| **Total sugar (g / 100g)** | 1.45±0.01$^{e}$ | 1.48±0.01$^{d}$ | 1.52±0.01$^{c}$ | 1.58±0.01$^{b}$ | 2.49±0.02$^{a}$ |

*Each value in the table is the mean ± standard deviation (n = 4); Different letters in the same line indicate a significant difference ($p < 0.05$); Results are ranked in descending order: a>b>c>d;*
***$C_{100}$**: Wheat Flour **(100%)** or control biscuit .*
***$C_{95}$**: Wheat Flour **(95%)** + AKF **(5%)** .*
***$C_{90}$**: Wheat Flour **(90%)** + AKF **(10%)**.*
***$C_{85}$** : Wheat Flour **(85%)** + AKF **(15%)**.*
***$C_0$**: Wheat Flour **(0%)** + AKF **(100%)**.*

### *3.1.5. Electrical Conductivity*

From the Table 2, it appears that the AKF is the most mineralized (795.50 µS/cm) and its incorporation in WF had significantly ($p < 0.05$) increased the conductivity of the composite's flours with a percentage of 8.17, 7.35 and 5.06 % for wheat flour incorporated with 5, 10 and 15% of AKF, respectively. The conductivity of AKF which is reported for the first time in this research is much higher than the 2.86 µS/cm reported by Benmeziane-Derradji et al. (2020) in the lentils flour. The conductivity of the flour is proportional to the ash content recorded with correlation coefficient $R^2$ of 0.77.

### *3.1.6. Total sugar*

The addition of AKF impacted significantly ($p < 0.05$) the total sugar of composite flours. In fact, the rate of total sugar increased as the substitution percentage from 5 to 15 % increased. The AKF had the highest rate of 2.49 %, which explain the elevation of the total sugar content of flour blends as the WF had the lowest content of 1.45 %. Alpaslan and Hayta (2006) have reported a mean total sugar content of 2.86 % in kernels from different cultivars in Turkey which is close to what was recorded in the current study. The same authors have partitioned the average sugar content into 0.77, 0.81 and 1.28 % for glucose, fructose and sucrose,

respectively. Yarilgaç et al. (2008) have identified the sugar in some Turkish and Foreign Apricot (*Prunus armeniaca* L.) varieties. They found that the main sugar was sucrose in the majority of apricot varieties. Kernels of Turkish varieties contained 2.20-5.30 g/100 g sucrose, 0.40-3.40 g/100 g maltose, 0.90-3.64 g/100g glucose and 0.57-5.58 g/100 g fructose. Sugar contents of seeds belonging to foreign varieties were 3.30-4.67 g/100 g sucrose, 1.50-2.52 g/100 g maltose, 3.38-3.72 g/100 g glucose and 1.86-2.93 g/100 g fructose. The authors concluded that sugar contents significantly, differed by varieties. Differences in proximate composition with other results might be due to differences in varieties, soils, climatic environment, and physical conditions as stated by Nabil et al. (2020).

### 3.2. Effect of AKF on the functional properties of flours

Food proteins are the main constituent responsible for the functional properties of foods. Protein has both hydrophilic and hydrophobic properties therefore, can interact with water and oil in foods. Functional properties are of primary importance in food processing, including solubility, water and oil absorption capacities and many others. These properties are involved in food texture and organoleptic characteristics and are essential in the manufacture of products such as dairy and meat products, cookies, confectionery, drinks and salad dressings. These tests are carried out with the aim of simulating a possible incorporation into a food matrix.

**Table 3.** Functional properties of different flours

| Combination | $C_{100}$ | $C_{95}$ | $C_{90}$ | $C_{85}$ | $C_0$ |
|---|---|---|---|---|---|
| **WAC (%)** | 107.92±0.33 $^{e}$ | 109.18±0.06 $^{d}$ | 110.26±0.10 $^{c}$ | 111.76±0.47 $^{b}$ | 147.66±0.27 $^{a}$ |
| **OAC (%)** | 86.94±0.18 $^{e}$ | 88.30±0.17 $^{c}$ | 87.56±0.16 $^{d}$ | 89.91±0.16 $^{b}$ | 101.60±0.13 $^{a}$ |
| **HLR** | 1.24±0.01 $^{b,c}$ | 1.24±0.00 $^{b,c}$ | 1.26±0.00 $^{b}$ | 1.24±0.00 $^{c}$ | 1.45±0.00 $^{a}$ |

*Each value in the table is the mean ± standard deviation (n = 4)*; *Different letters in the same line indicate a significant difference (p < 0.05); Results are ranked in descending order: a>b; WAC: Water Absorption Capacity; OAC: Oil Absorption Capacity; HLR: Hydrophilic-Lipophilic Ratio*
***$C_{100}$**: Wheat Flour **(100%)** or control biscuit.*
***$C_{95}$**: Wheat Flour **(95%)** + AKF **(5%)**.*
***$C_{90}$**: Wheat Flour **(90%)** + AKF **(10%)**.*
***$C_{85}$**: Wheat Flour **(85%)** + AKF **(15%)**.*
***$C_0$**: Wheat Flour **(0%)** + AKF **(100%)**.*

From Table 3, it can be seen that AKF is distinguished by significantly ($p < 0.05$) higher WAC and OAC of 147.66 % and 101.60 %, respectively compared to those of WF or composite flours. The high OAC which supposes the lipophilic nature of the constituents of the AKF is very useful in flours as fats act as trap for flavors and different smells. Indeed, Seena and Sridhar (2005) indicated that high OAC, as that recorded for the AKF in the current study, is desired in ground meat formulations, flavor retention, amelioration of palatability, extension of shelf life of bakery or meat products, meat replaces and extenders, doughnuts, pancake, baked goods and soups. Whereas, high WAC is desirable feature in food such as sausages, custards and dough because these are assumed to absorb water without dissolution of proteins thereby attaining body thickening and viscosity, according to the same authors. In addition, Okpala et al. (2013) indicated that flours with high WAC, as recorded in this study, would be useful in bakery products, as this could prevent staling by reducing moisture loss. The WF had respective lowest values of 107.92 and 86.94 % for WAC and OAC. The addition of AKF had a positive significant ($p < 0.05$) effect on the WAC of composite flours, which was increased by 1.15,

2.12 and 3.44 % for the replacement percentage of 5, 10 and 15 %, respectively, along with a positive significant ($p < 0.05$) impact on the OAC which increase by 1.50, 0.74 and 3.30 % for the composite flours at replacement level of 5, 10 and 15 %, respectively. The increase in WAC may be due to different types of hydrophilic carbohydrates and a varied protein structure provided by AKF, and that in OAC may be due to the presence of non-polar side chains provided by AKF which link the hydrocarbon side chain of the oil (Seena and Sridhar, 2005). Our observations were lower than the earlier findings of Thakur et al. (2019) in wild apricot protein isolate which had higher water absorption and oil absorption capacities (2.45 mL/g and 2.52 mL/g, respectively) and those of Benmeziane-Derradji et al. (2020) in raw lentil flour (136 % for WAC and 130.67 % for OAC). The HLR varied significantly ($p < 0.05$) between flours; However, all flours recorded ratios greater than 1 which assumes that all flours have much more affinities towards water than oil, the AKF had the highest ratio of 1.45. Benmeziane-Derradji et al. (2020) obtained the same results on raw and roasted lentil flours.

### 3.3. Physical characteristics of biscuits

Biscuits prepared with varying levels of replacement of WF by AKF were assessed for diameter, thickness, spread ratio, percentage of spread ration, volume and density (Table 4). In the statistical processing of the results with the Fisher's LSD test, significant differences ($p < 0.05$) in the diameter, thickness, volume, density and weight were recorded between fortified biscuits and the control. Indeed, the diameter and the density of the biscuits increased as the inclusion of AKF increased in the biscuit formulation. This increase may be due to the presence of AKF by providing fibers which led to the leavening of the dough during baking. Our results don't agree with those of Ashraf et al. (2018) who reported a decrease in the diameters of nut crackers when foam mat dried apricot powder was substituted in wheat flour; and those of Chen et al (2020), who stated when the effects of spray-dried apple fiber was compared to wheat and oat brans in cookies, that as the concentration of apple fiber increased, the diameter of cookies decreased, and their thickness increased, oppositely, the diameters of oat bran supplemented cookies did not vary significantly as the addition percentage increased. As for the weight, there was no significant differences ($p > 0.05$) in all samples except the biscuit B85 which presented the highest weight (5 g). The same sample had the highest volume and the density, this can be explained by the increased protein network due to the presence of 15% AKF as stated by Sheikh et al. (2019). Our results were in agreement with Awad and skokry (2018) who pointed out that the increasing levels of pumpkin powder in cake manufacture (0 - 15 %) significantly increased the weight of biscuits samples and they attributed this increment in weight to the pumpkin fiber content which increased the water absorption capacity. However, the addition of the AKF didn't affect ($p > 0.05$) the spread factor and the percentage of the spread factor of enriched biscuits comparing to the control. According to Masmoudi et al. (2021) and Adeola and Ohizua (2018), the spread ratio is considered as one of the most important parameters in determining the quality of cookies, also, the higher the spread ratio of biscuit the more desirable it is. Hence, based on spread ratio, biscuit prepared from the flour blend containing 5 % of AKF may be the most preferred. This assertion is confirmed in Table 4 where there were no significant ($p > 0.05$) differences in the sensory attributes of this biscuit sample (B95) and the one adjudged to be the most acceptable. The same sample had the highest overall acceptability. Our results corroborate with those of Adeola and Ohizua (2018) on the biscuit prepared from blending 45 % cooking banana, 10 % pigeon pea and 45 % sweet potato, which was the most preferred based on spread ratio and the sensory evaluation. The current findings for diameter are coherent with results of Aziz et al (2020) who reported that the diameter of the cookies increases in supplemented cookies with apricot powder (10

%) comparing to the control. Masmoudi et al. (2021) have noted similar results with an insignificant decrease of the spread factor of biscuits enriched with jujube (*Zizyphus lotus* L.) flour and fiber concentrate compared to the control, while Jothi et al (2014) have indicated a significant ($p < 0.05$) decrease in spread factor with the addition of various amounts of composite flour (Gluten-free wheat flour, rice flour, Bengal gram flour, potato flour and Italian millet flour). Authors explained this decrease by the fact that composite flours apparently formed aggregates with increase numbers of hydrophilic sites available that contributed for the limited free water in biscuit dough. Furthermore, Özboy-Özbaş et al. (2010) indicated that spread ratio values of the resistant starch supplemented cookies decreased significantly ($p < 0.01$) as the percentage of resistant starch increased, however, a slight increase in the spread ratios of resistant starch/apricot kernel flour supplemented cookies were observed up to 20 % level. The spread factor is correlates with texture, grain finesse, bite and overall mouth feel of the biscuits (Jothi et al. 2014).

**Table 4.** Influence of AKF on the physical characteristics of biscuits

| Formulation | B100 | B95 | B90 | B85 |
|---|---|---|---|---|
| **Diameter(cm)** | $3.200\pm0.274^{b}$ | $3.300\pm0.274^{a,b}$ | $3.300\pm0.274^{a,b}$ | $3.700\pm0.447^{a}$ |
| **Thickness (cm)** | $0.800\pm0.274^{a,b}$ | $0.600\pm0.274^{b}$ | $0.900\pm0.224^{a,b}$ | $1.000\pm0.354^{a}$ |
| **Spread Factor** | $4.400\pm1.475^{a}$ | $5.43\pm2.44^{a}$ | $3.900\pm1.194^{a}$ | $4.132\pm1.711^{a}$ |
| **Percentage of Spread Factor** | $100\pm0.00^{a}$ | $130.96\pm75.58^{a}$ | $99.30\pm49.02^{a}$ | $94.4\pm21.01^{a}$ |
| **Volume ($cm^3$)** | $6.67\pm3.05^{a,b}$ | $5.25\pm2.52^{b}$ | $7.88\pm2.67^{a,b}$ | $11.16\pm5.48^{a}$ |
| **Density** | $8.081\pm1.396^{b}$ | $8.586\pm1.393^{a,b}$ | $8.591\pm1.395^{a,b}$ | $10.87\pm2.48^{a}$ |
| **Weight (g)** | $3.60\pm0.55^{b}$ | $3.60\pm0.55^{b}$ | $3.60\pm0.55^{b}$ | $5.00\pm1.00^{a}$ |

*Each value in the table is the mean ± standard deviation (n = 5); Different letters in the same line indicate a significant difference ($p < 0.05$); Results are ranked in descending order: a>b;*
***B100:*** *Control biscuit (without AKF)*
***B 95:*** *5% AKF fortified biscuit*
***B90:*** *10% AKF fortified biscuit*
***B85:*** *15% AKF fortified biscuit*

### 3.4. Sensory assessment

For each new food product developed, a sensory evaluation is necessary to determine its acceptability by consumers for whom the product is intended. The effect of AKF incorporation on the organoleptic characteristics of biscuits is presented in Table 5 and figure 2 (spider plot according to the sensory hedonic test). As it can be seen, the AKF addition had no statistically significant ($p > 0.05$) effect on the color and smell of biscuits, whereas a significant impact ($p < 0.05$) on the general aspect, flavor, friability and overall acceptability of the biscuits was noted. The current outcomes were contradictory to those of Ashraf et al. (2019) who recorded a significant difference between all sensory attributes (color, texture, taste and overall acceptability) of nut crackers fortified with apricot flour. The authors noted that the overall organoleptic assessment showed that up to 18 % of apricot powder did not influence the consumer acceptance and that this treatment scored more in terms of color, texture, taste and overall acceptability when compared with control. Overall, the study found that scores for the different sensory attributes were elevated with increasing incorporation of AKF. These results were in line with what was found by Chandra et al. (2015b) and Sheikh et al. (2019) where the authors reported that the scores attributed to the biscuits made from composite flour were higher compared to the scores of the

control biscuit (made from wheat flour only). Our results showed that after control biscuit, which got a minimum score for all studied attributes and samples B90 and B85, which got an intermediate scores for all sensory attributes, the biscuit B95 got a maximum score for all the assessed sensory parameters. The overall acceptability on a five-point hedonic scale was 4.048 for biscuits at 5 % addition level which differ significantly ($p < 0.05$) from the control rated as poor (2.857) and B90 and B85 with intermediate overall acceptability of 3.190 and 3.333, respectively. Based on this and on the other attributes scores, the samples can be classified in the following decreasing order: B95 > B90 - B85 > B100.

**Table 5.** Effect of Apricot Kernel Flour (AKF) Addition on Sensory Properties of biscuits

| Biscuit | General aspect | Color | Flavor | Aroma | Friability | Overall acceptability |
|---|---|---|---|---|---|---|
| $B_{100}$ | 3.048±1.071$^{b}$ | 3.333±1.238$^{a}$ | 2.714±1.384$^{b}$ | 3.143±1.153$^{a}$ | 2.810±1.250$^{b}$ | 2.857±1.315$^{b}$ |
| $B_{95}$ | 4.048±1.024$^{a}$ | 3.810±1.209$^{a}$ | 3.952±0.669$^{a}$ | 3.905±0.768$^{a}$ | 4.143±0.793$^{a}$ | 4.048±0.805$^{a}$ |
| $B_{90}$ | 3.048±1.203$^{b}$ | 3.095±1.375$^{a}$ | 3.476±1.167$^{a,b}$ | 3.667±0.856$^{a}$ | 3.667±1.065$^{a,b}$ | 3.190±1.209$^{a,b}$ |
| $B_{85}$ | 3.190±1.250$^{a,b}$ | 3.3667±1.317$^{a}$ | 2.905±1.221$^{b}$ | 3.381±1.117$^{a}$ | 3.524±1.123$^{a,b}$ | 3.333±0.913$^{a,b}$ |

*Different letters in the same column indicate a significant differences ($p < 0.05$); Results are ranked in descending order : a>b*
***B100:*** *Control biscuit (without AKF)*
***B 95:*** *5% AKF fortified biscuit*
***B90:*** *10% AKF fortified biscuit*
***B85:*** *15% AKF fortified biscuit*

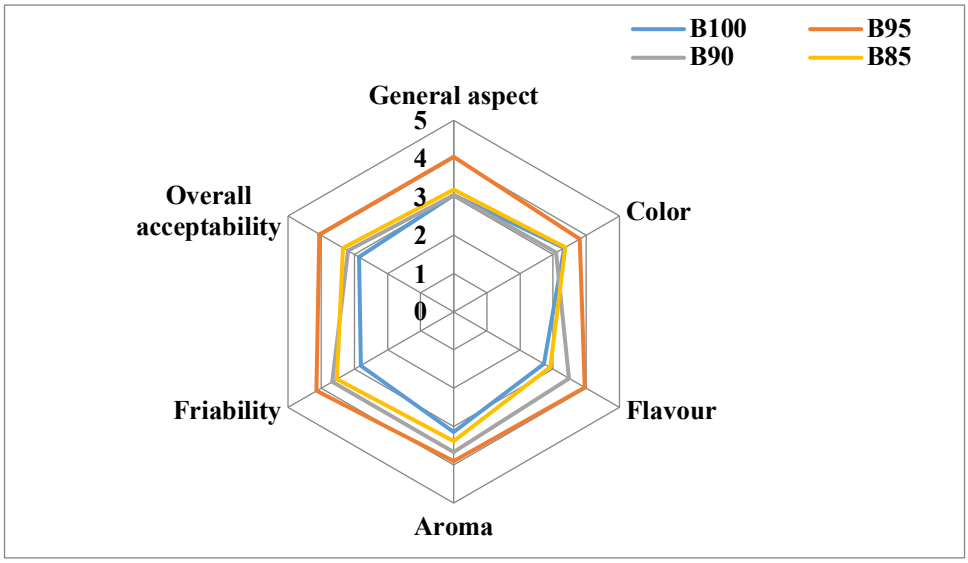


**Figure 2.** Sensory evaluation of apricot supplemented cookies.
***B100:*** *Control biscuit (without AKF)*
***B 95:*** *5% AKF fortified biscuit*
***B90:*** *10% AKF fortified biscuit*
***B85:*** *15% AKF fortified biscuit*

As it can be seen in figure 2, the tasters liked more the biscuit B95 for all studied sensory attributes (general aspect, color, flavor, aroma, friability, overall acceptability). The samples B90 and B85 had the same or very close appreciation for their sensory attributes with a low appreciation for the general appearance of the B90 compared to B85. The flavor of the control and the enriched biscuit at 15 % was the

least appreciated than the fortified biscuit at 5 % and 10 %. Color is a major sensory attribute which impacts consumer preference. Although, there was a significant difference ($p < 0.05$) between samples in term of color, the enriched biscuit at 5 % was the most preferred. This had a slight but a significant and positive influence on biscuit acceptability. Finally, the control (B100) had the lowest appreciation degree, so the least accepted by tasters, comparing to the fortified biscuits. In addition, the figure showed that there was no significant differences ($p > 0.05$) in terms of aroma and color of biscuits, but the enriched biscuits present distinctly different general aspect, flavor, friability and overall acceptability. Based on the results of the sensory analysis, fortification of biscuits with apricot flour clearly increased their level of appreciation by consumers, which encourages the making of biscuit from wheat flour enriched with AKF. Dhen et al. (2018) have found that a partial substitution of wheat flour in bread up to 12 % by AKF, yields in satisfactory overall consumer acceptability. However, bread containing 24 % of AKF was noted comparatively lower, which might be due to excessive amounts of AKF which negatively affected the aroma, taste, and texture of bread.

## 4. Conclusions

According to the results obtained from this study, it can be concluded that the AKF has been used advantageously in the preparation of biscuits, which constitutes a way of upgrading apricot kernels, considered until now as waste. The addition of apricot kernel flour into wheat flour up to 15 % (w/w) enabled acceptable biscuit in terms of physical and sensory properties. Referring to the sensory evaluation, the control biscuit had the lowest score while, biscuit with substitution rate at 5 % of AKF had the highest score when compared with other treatments. Therefore, AKF might be accepted when used up to 5 % to obtain an acceptable biscuit in view of sensory quality as well as to improve the nutritive value. Thus, give to producers an alternative way to diversify the production and healthy choice option to the consumers.

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# INFLUENCE OF METEOROLOGICAL CONDITIONS ON THE QUALITY OF GRAPES AND AROMA-RELEASING ENZYME ADDITION ON THE CHEMICAL COMPOSITION, AROMATIC COMPLEX AND ORGANOLEPTIC PROFILE OF RED WINES

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*Organoleptic characteristics.*

**ABSTRACT**

In the period 2013 – 2015 the influence of the meteorological conditions of the year on the harvest quality and aroma-releasing enzyme addition before the alcoholic fermentation on the chemical composition, aromatic profile and organoleptic features of red wines were investigated. The objects of the study were the clone Pamid 5/76, candidate–clones Gamza 52-9-4 and Gamza 52-9-5 and the varieties Kaylashki Rubin and Trapezitsa (obtained by interspecific hybridization). The sugar accumulation dynamics in the grapes was monitored during the ripening phase, in order to determine the time of technological maturity. The latest ripening variety was Kaylashki Rubin, and it demonstrated the most gradual sugar accumulation and acidity reduction. The experimental wines had different composition and organoleptic characteristics, depending on the potential and specifics of the variety and the harvest. Kaylashki Rubin wines had the highest alcohol content and sugar-free extract and the lowest - Pamid 5/76. The amount of total phenolic compounds and anthocyanins in wines was increasing in the order Pamid 5/76 < Trapezitsa < Gamza 52-9-4 < Gamza 52-9-5 < Kaylashki Rubin. The positive effect of the added aroma-releasing enzyme on the content of esters in wines and their aromatic characteristics was confirmed. No effect of the enzyme addition on the amount of total aldehydes and higher alcohols was observed. No correlation was found between the studied components of the aromatic composition of the wines and their tasting evaluation.

## 1. Introduction

Wine is a beverage containing a large number of organic compounds in different quantitative ratios. It consists of substances coming from grapes as they are contained in it; coming from grapes but undergoing a change in the course of the alcoholic fermentation; formed during alcoholic fermentation; formed during the wine aging (Chobanova, 2012).

The content of the various components is a function of many factors – variety, soil and weather conditions, region and method of cultivation, conditions of vinification, processing and storage of wine.

The substances contained in wine, depending on their organoleptic influence and technological significance, could be divided into volatile and non-volatile (extractive) ones. The group of volatile compounds includes ethyl alcohol, volatile acids, esters, aldehydes, higher alcohols, terpenes, etc., which form the wine aroma (bouquet). Their amount in wine is about 10 times higher than in grapes because they are formed mainly during the alcoholic

fermentation. The extractive ones are non-volatile components of organic and inorganic origin, including carbohydrates, organic acids, phenolic compounds, nitrogen and mineral substances, etc. (Chobanova, 2012).

The wine titratable acidity is an indicator varying within wide ranges for the individual varieties. From the organic acids, the tartaric and malic acids are prevailing, which in a certain ratio with the other components form the taste. After the malolactic fermentation, the malic acid decreases merely to traces and wine acquires a mild and pleasant taste. Small amounts of citric, succinic, glycolic, oxalic and other acids are also found (Dimov and Getov, 2003; Kučerová and Široký, 2011; Wilkes, 2016).

Phenolic compounds have a direct or indirect effect on wine quality, especially red wines. Its main characteristics (colour, taste, clarity, stability) are largely related to the presence of phenolic substances and their transformations. The phenolic compounds content in wine depends mainly on the phenolic reserve of the variety, the method of cultivation and the conditions in the growing area, the applied technology of winemaking, ect. (Getov, 2002; Stoyanov *et al.*, 2004; Bai *et al.*, 2013; Kekelidze *et al.*, 2014; Gardin and Altindisli, 2015). Grapes are rich in phenols, which are localized in solids (skins, seeds, rachis) and are a source of flavonoid phenolic substances (including anthocyanins), while the fleshy part is rich in non-flavonoid compounds. Few substances of the phenolic complex appear during the alcoholic fermentation, as well as from the transformation of other products, resulting from the evolution of the natural phenols of the grapes (Chobanova, 2012; Bai *et al.*, 2013).

The aroma is one of the important factors determining wine nature and quality. The wine contains more than 800 volatile components, but only a few dozen of them have an effect on the aroma. The varietal aroma of wines is determined by the content of monoterpenes, norisoprenoids, methoxypyrazines and thiols. However, the main aroma-determining compounds are derived from yeast metabolism. About 90% of the free volatile compounds are esters and higher alcohols, which are formed during the alcoholic fermentation and depend on the conditions under which it occurs – temperature, aeration, yeast strain (Selli *et al.*, 2004; Antalick *et al.*, 2015; Mina and Tsaltas, 2017; Manolache *et al.*, 2018). Ethyl esters are among the key components responsible for the fruity aroma of wines, having a positive effect on their quality. In red wines, after the malolactic fermentation and as a result of the metabolism of the lactic acid bacteria, diethyl succinate is in the highest rates and is determinant of aroma formation (Gil *et al.*, 2006; Manolache *et al.*, 2018).

The climate changes and weather conditions of the year also affect the chemical composition of wines, their aromatic complex, organoleptic profile and aging potential. At higher temperatures during the grape ripening period, methoxypyrazine levels decrease that leads to fading of the herbaceous and herbal nuances of the wine. Hints of unripe green peppers and peas appear. The content of norisoprenoids and monoterpenes that cause the fruity and floral nuances in the aroma is higher when the grapes are exposed to more light and ripen at higher temperatures (Pons *et al.*, 2017). Severe water deficiency during maturation decreases the levels of the volatile thiol precursors (Peyrot des Gachons *et al.*, 2005). Under the conditions of water stress, that delay the grapes ripening, the levels of methoxypyrazines reach rates that adversely affect the wine aroma. The combined effect of the temperature and limited water status causes changes in the vine metabolism that sometimes delay the ripening process (Pons *et al.*, 2017).

The objective of the study was to investigate the influence of the meteorological conditions of the year on the grapes composition and the addition of aroma-releasing enzyme before the alcoholic fermentation on the chemical composition, aromatic profile and organoleptic features of red wines from clones and varieties, cultivated

under the soil and climate conditions of the region of Pleven, Central Northern Bulgaria.

## 2. Material and methods

### 2.1. Materials

#### *2.1.1. Plant material*

The study was carried out at the Institute of Viticulture and Enology (IVE) - Pleven, during the period 2013 – 2015. The objects of the study were the clone Pamid 5/76, candidate–clones Gamza 52-9-4 and Gamza 52-9-5 and the varieties Kaylashki Rubin and Trapezitsa that were selected at the IVE by means of intraspecific hybridization and were characterized by higher practical resistance to diseases and low winter temperatures (Nakov *et al.*, 2011; Ivanov *et al.*, 2011; Ivanov *et al.*, 2012).

The town of Pleven is located in the Northern wine-growing region (the Danube Plain, Central Northern Bulgaria) and is characterized by a typical continental climate, and the soils include all types of black soils.

The vineyards were fruit-bearing, grown at the Experimental Base of the Institute. The experimental vine plots covered areas of 0.2 ha of each studied clone and variety. Pamid clone and Gamza candidate-clones were cultivated, at the ground, on improved Guyot training and planting distance 2.2 m between the rows and 1.2 m in the row. Kaylashki Rubin and Trapezitsa varieties were cultivated on stem Moser training, with stem height of 1.2 m. at planting distance 3.00 m/1.20 m. All varieties were grafted to Berlandieri x Riparia SO4 rootstock.

The sugar accumulation dynamics in the grapes of the selected clones and varieties was monitored during the ripening phase, in order to determine the time of technological maturity and grapes harvest. The change in the sugar concentration was determined refractometrically (under field conditions) and by hydrometer of Dujardin (under laboratory conditions). The titratable acids content was determined by titration with NaOH.

In 2013, Trapezitsa grapes were not harvested because of the poor sanitary status and not enough yield, as a result of a hail storm in the spring that affected the trial plantation.

#### *2.1.2. Grapes processing and vinification*

Every year the grapes from the studied clones and varieties were processed in the Experimental Winery of IVE – Pleven. The classical technology for production of dry red wines was applied under the conditions of micro-vinification (Yankov, 1992) – removing the berries, crushing, sulfating (50 mg/kg $SO_2$), adding pure culture dry wine yeast *Saccharomyces cerevisiae Vitilevure CSM* in the amount of 20 g/hl, fermentation temperature 28°C, separation of solid particles, further sulfating, storage. During the study period, when the must from the samples was insufficient in sugar content, to produce wines with the optimum alcohol concentration, a proportional adjustment was made for sugars with pure sucrose.

The grapes of the studied clones and varieties was divided in equal quantities into two technological variants, 30 kg each: V1 – control; V2 - with the addition of aroma-releasing enzyme *Zymovarietal Aroma G* in the amount of 3 g/100 kg before the alcoholic fermentation.

After the completion of the process, determined by chemical analysis of sugars, the young wines were decanted and further sulfated to 30 mg/l free $SO_2$.

### 2.2. Methods

#### *2.2.1. Grapes chemical composition*

The chemical composition of grape juice was determined according to the following methods (Ivanov *et al.*, 1979): sugars, g/l - hydrometer of Dujardin; glucose and fructose, g/l – iodometric method; titratable acids (TA), g/l – titration with NaOH; pH - pH-meter; glucoacidimetric index (GAI) – calculation method as the ratio of sugars (%) and TA (g/l).

#### *2.2.2. Wine chemical composition*

The main indicators of wines chemical composition were analyzed by conventional methods used in the winemaking practice (Ivanov et al., 1979; Chobanova, 2007): sugars,

g/l – Schoorl's method; alcohol, vol. % - distillation method, Gibertini apparatus with densitometry of the distillate density; total extract (TE), g/l - Gibertini apparatus with densitometry, density of alcohol-free sample; sugar-free extract (SFE), g/l - calculation method (the difference between TE and sugars); titratable acids (TA), g/l - titration with NaOH; pH – pH-meter; total phenolic compounds (TPC), g/l – method of Singleton et Rossi; monomeric anthocyanins (mg/l) – method of Singleton et Rossi by pH changing. The colour spectral characteristics were also determined: intensity I [abs. units] – method of Somers; tint T [abs. units] – method of Glories.

The aromatic profile of wines included the following indicators and methods of analysis (Ivanov *et al.* 1979): total aldehydes (mg/l) - bisulphite method; total esters (mg/l) - a method of saponification with NaOH; total higher alcohols (mg/l) - modified method of Komarovsky - Felenber.

The annual trial results presented were the arithmetic mean of two simultaneous samples. In cases where a significant difference in the amount of the analyzed indicator was found, a third sample was prepared and the two closest rates were taken into account.

***2.2.3. Wine organoleptic analysis***

The organoleptic characteristics of the experimental wines were determined by a 9-member tasting committee using a 100-score scale (Tsvetanov, 2001), by the indicators: colour (clarity, hue, intensity); aroma (purity, intensity, finesse, harmony); taste (purity, intensity, flesh, harmony, durability, aftertaste); general impressions. The tasting results represented an average of the committee members' scores, as the highest and lowest were discarded.

***2.2.4. Statistical analysis***

Statistical processing of the results of the analyzes was performed including mean and standard deviation (±SD) using the Excel 2007 program from the Microsoft Office package.

The weather characteristics of the years from the study period (Table 1) were determined by the methods of mathematical statistics (Sirakov, 1981).

**Table 1.** Precipitation (P%) and average daily air temperature for the experimental years.

| Year | | 2013 | 2014 | 2015 |
|---|---|---|---|---|
| N | P% | 43 (moderate wet) | 14 (very wet) | 17 (very wet) |
| T° | | 94 (very cool) | 97 (very cool) | 29 (moderately hot) |

P – probability; N – precipitations; T°- average air temperature

## 3. Results and discussion

### 3.1. Sugar accumulation dynamics in the grapes

The sugar accumulation dynamics in the grapes of the studied red clones and varieties, during the ripening phase, for the study period, is shown in Figures 1, 2, 3. The influence of the meteorological conditions of the year on the process, composition and quality of the grapes has been defined.

The data on the changes in the sugar and acid content of grapes, 2013 harvest are presented in Figure 1 and show normal course of ripening. That was due to the favorable weather conditions during this period (July-August-September) – hot summer, without precipitation. The high temperatures in August had determined the good sugar accumulation and the rapid acidity reduction. In 2014, all varieties showed a delay in ripening due to the adverse climatic factors during the period – cool and rainy summer (Figure 2). The preconditions for the deterioration of the sanitary condition of the grapes created by the meteorological conditions necessitated an earlier grape harvest. The sugar accumulation dynamics in 2015 is presented in Figure 3. The graphic data revealed normal ripening of all varieties. The change in the weather conditions in September, manifested by the lower temperatures, rainfall and hail, created

prerequisites for the deterioration of the sanitary condition of the grapes.

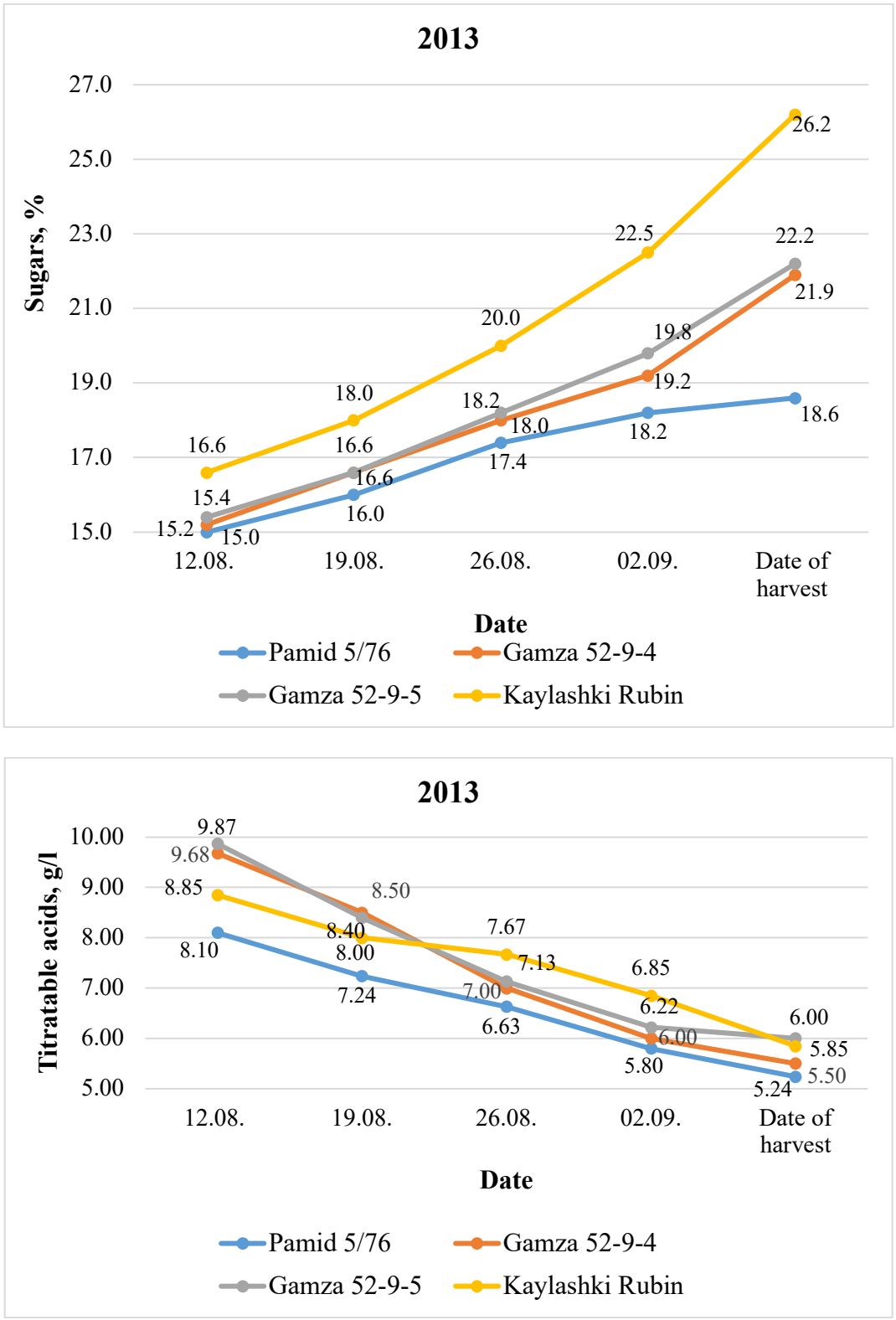
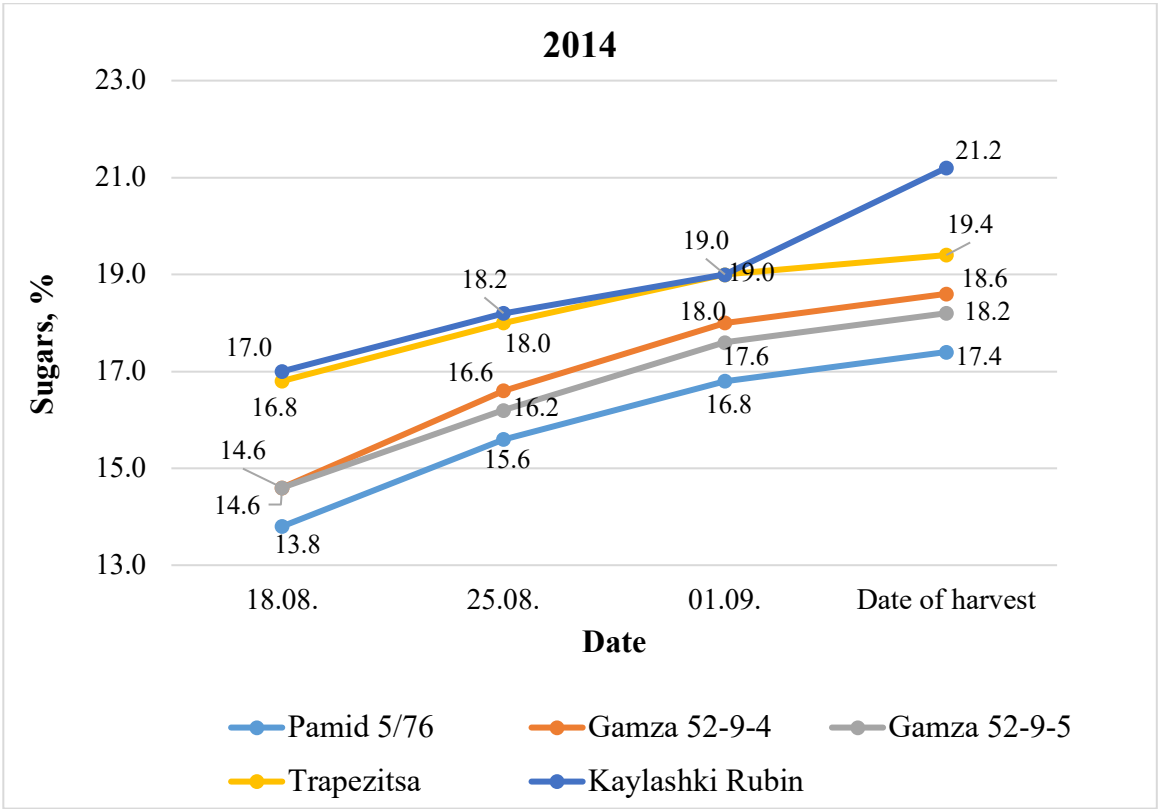



**Figure 1.** Changes in sugars and titratable acids during the grape ripening period of the studied red clones and varieties, 2013.



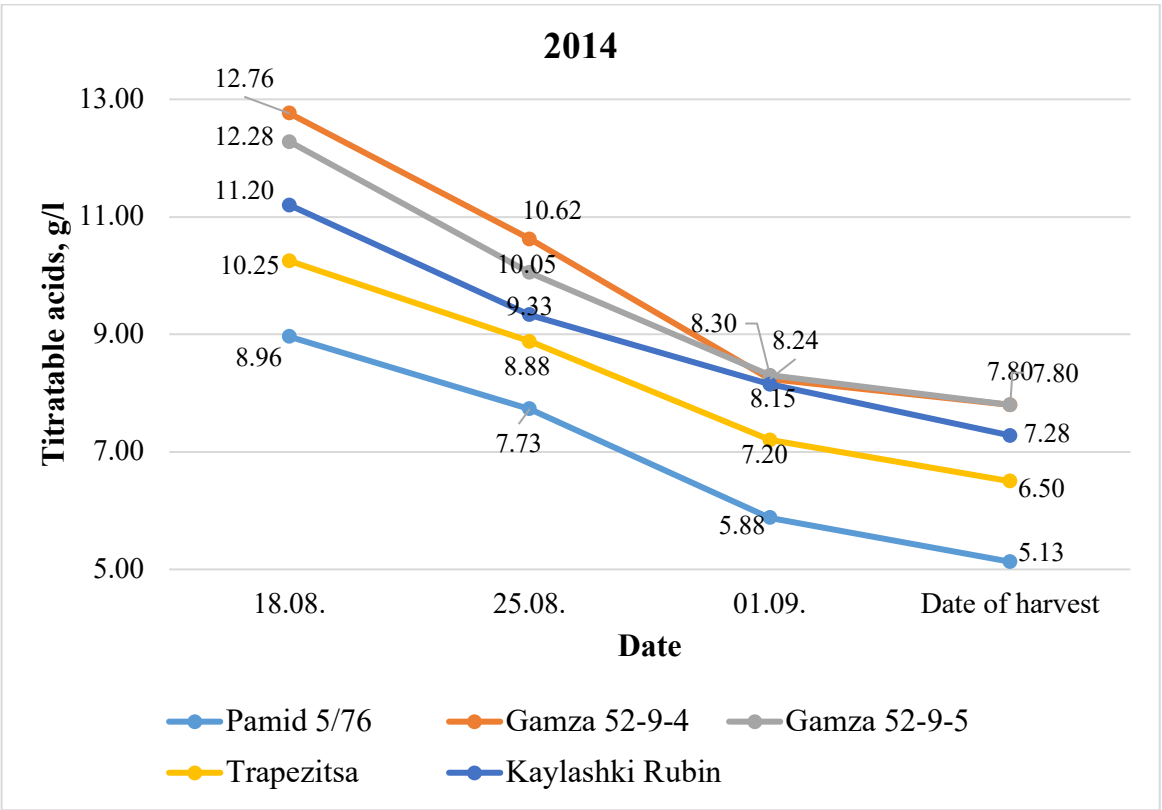
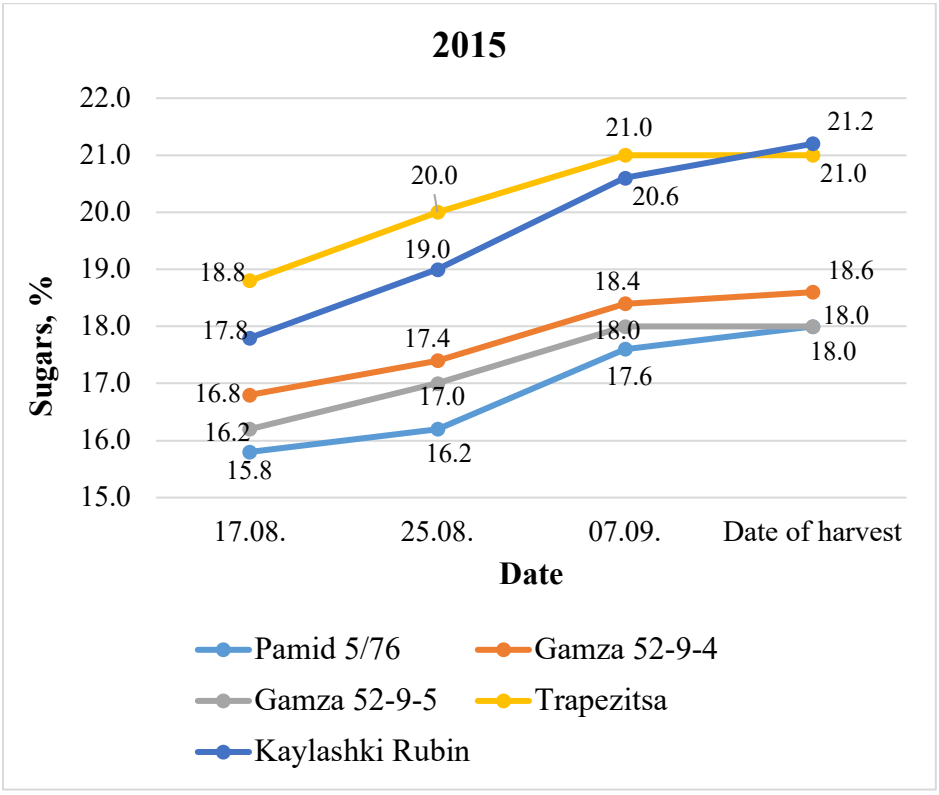




**Figure 2.** Changes in sugars and titratable acids during the grape ripening period of the studied red clones and varieties, 2014.



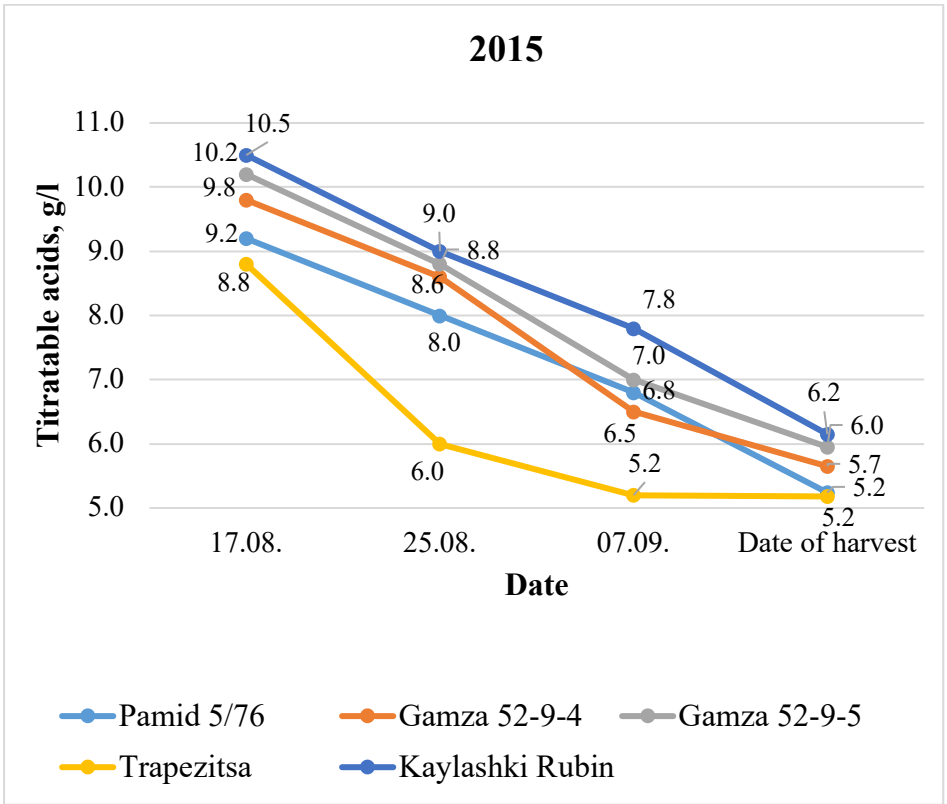




**Figure 3.** Changes in sugars and titratable acids during the grape ripening period of the studied red clones and varieties, 2015.

The studied red clones and varieties had different ripening times, therefore their harvest took place at different intervals. Usually, all of them reached their technological maturity in the second half of September. The grapes of Pamid 5/76 was characterized by the lowest sugar content and acidity, which was considered a varietal feature. Kaylashki Rubin was the latest ripening, as it had the most gradual sugar accumulation and titratable acids reduction. Both Gamza candidate-clones were with similar rates in terms of the studied parameters. In 2015, Trapezitsa variety was distinguished as the one with the highest rates of sugar accumulation and simultaneous decrease of titratable acids.

### 3.2. Grapes chemical composition

The data on the grapes composition are presented in Table 2. The general trend for the period was that the lowest sugar accumulation was reported for Pamid 5/76 (mean 180.00±6.00 g/l), the highest for Kaylashki Rubin (mean 228.67±23.69 g/l). Grapes from 2013 harvest, had high sugar content, ranging from 219.00 to 262.00 g/l, except Pamid 5/76 (186.00 g/l). The highest sugar rates were reported for Kaylashki Rubin. In 2014, the sugar concentrations in grapes varied from 174.00 (Pamid 5/76) to 212.00 g/l (Kaylashki Rubin). No significant difference was observed in the sugar amounts in both Gamza candidate-clones. In grapes from 2015 harvest, the sugar concentration ranged from 180.00 (Pamid 5/76, Gamza 52-9-5) to 212.00 g/l (Kaylashki Rubin). The content of monosaccharides glucose and fructose was also determined in the grape juice of all varieties. Their ratio was less than 1, with quantitative predominance of fructose.

The data revealed lower titratable acids in grapes of Pamid 5/76 (mean 5.22±0.08 g/l) and Trapezitsa (mean 5.84±0.93 g/l), which was their varietal feature (Table 2). Higher acidity was reported in Kaylashki Rubin variety (mean 6.43±0.75 g/l) and both Gamza candidate-clones – Gamza 52-9-4 (mean 6.32±1.29 g/l), Gamza 52-9-5 (mean 6.58±1.05 g/l). Characteristic of 2013 harvest was that, despite the higher sugar content of Gamza 52-9-5 grapes and Kaylashki Rubin, higher acids, respectively, 6.00 g/l and 5.85 g/l, were also analyzed. In 2014, the rates ranged from 5.13 (Pamid 5/76) to 7.80 g/l (Gamza 52-9-4 and 52-9-5), and in 2015 from 5.18 (Trapezitsa) to 6.15 g/l (Kaylashki Rubin).

On the basis of the found sugars and titratable acids content in the grapes, the glucoacidimetric index (GAI) was determined for each of the clones and varieties studied. Its values were indicative of the grapes quality and its purpose in wine production. With the highest value of the indicator is Kaylashki Rubin (mean 3.61±0.80). The calculated values for 2013 harvest were higher than 3, with Kaylashki Rubin reaching 4.48. It was a proof that the grapes were suitable for the production of high quality wines in terms of chemical composition and tasting characteristics. In 2014 harvest, GAI ranged from 2.33 (Gamza 52-9-5) to 3.39 (Pamid 5/76), i.e. the grapes not from all varieties had good enough indicators for the production of quality wines. The GAI for 2015 harvest varied from 3.02 (Gamza 52-9-5) to 4.05 (Trapezitsa), indicating the good characteristics of grapes for producing wines with optimal composition and organoleptic profile (Table 2, Table 3).

The data from the chemical composition of the grapes during the study period show that for Pamid 5/76, Gamza 52-9-5 and Kaylashki Rubin, the conditions in 2013 were the most favorable for obtaining the harvest with the highest sugar accumulation, optimal acid content and highest GAI values. These indicators were a prerequisite for the production of wines with normal chemical composition and characteristics (Table 3). For Gamza 52-9-4 and Trapezitsa the most favorable in terms of composition and quality of grapes was 2015. Poor weather conditions during ripening in 2014, were the reason for the deteriorating chemical parameters of the grapes from the studied clones and varieties - insufficient sugar accumulation and reported lower GAI values (Table 2).

**Table 2.** Chemical composition of grapes from the studied clones and varieties

| Variety, clone | Vintage | Date of harvest | Sugars, g/l | Glucose , g/l | Fructose, g/l | Titratable acids, g/l | GAI | pH |
|---|---|---|---|---|---|---|---|---|
| Pamid 5/76 | 2013 | 13/09/ | 186.00 | 75.00 | 111.30 | 5.24 | 3.55 | 3.19 |
| | 2014 | 12/09/ | 174.00 | 77.00 | 87.00 | 5.13 | 3.39 | 3.35 |
| | 2015 | 10/09/ | 180.00 | 80.35 | 99.65 | 5.30 | 3.40 | 3.31 |
| | *mean± SD* | | *180.00 ±6.00* | *77.45 ± 2.70* | *99.32 ±12.15* | *5.22 ±0.08* | *3.45 ±0.09* | *3.28 ±0.08* |
| Gamza 52-9-4 | 2013 | 19/09/ | 219.00 | 92.10 | 126.90 | 5.50 | 3.98 | 3.24 |
| | 2014 | 12/09/ | 186.00 | 81.00 | 105.00 | 7.80 | 2.38 | 3.30 |
| | 2015 | 10/09/ | 186.00 | 87.08 | 98.92 | 5.65 | 3.29 | 3.22 |
| | *mean± SD* | | *197.00 ±19.05* | *86.73 ±5.56* | *110.27 ±14.72* | *6.32 ±1.29* | *3.22 ±0.80* | *3.25 ±0.04* |
| Gamza 52-9-5 | 2013 | 19/09/ | 222.00 | 82.54 | 139.46 | 6.00 | 3.70 | 3.21 |
| | 2014 | 12/09/ | 182.00 | 77.40 | 104.60 | 7.80 | 2.33 | 3.28 |
| | 2015 | 10/09/ | 180.00 | 81.68 | 98.32 | 5.95 | 3.02 | 3.08 |
| | *mean± SD* | | *194.67 ±23.69* | *80.54 ±2.75* | *114.13 ±22.16* | *6.58 ±1.05* | *3.02 ±0.68* | *3.19 ±0.10* |
| Kaylashki Rubin | 2013 | 25/09/ | 262.00 | 121.35 | 140.65 | 5.85 | 4.48 | 3.27 |
| | 2014 | 19/09/ | 212.00 | 94.00 | 118,00 | 7.28 | 2.91 | 3.10 |
| | 2015 | 16/09/ | 212.00 | 80.00 | 132.00 | 6.15 | 3.45 | 3.23 |
| | *mean ±SD* | | *228.67 ±23.69* | *98.45 ±21.03* | *130.22 ±11.43* | *6.43 ±0.75* | *3.61 ±0.80* | *3.20 ±0.09* |
| Trapezitsa | 2013 | - | - | - | - | - | - | - |
| | 2014 | 12/09/ | 194.00 | 81.00 | 113,00 | 6.50 | 2.98 | 3.26 |
| | 2015 | 02/09/ | 210.00 | 89.10 | 120.90 | 5.18 | 4.05 | 3.29 |
| | *mean± SD* | | *201.67 ±28.87* | *85.05 ±5.73* | *116.95 ±5.58* | *5.84 ±0.93* | *3.51 ±0.75* | *3.27 ±0.02* |

### 3.3. Wine chemical composition

After the completion of the alcoholic fermentation, the wines obtained from the experimental variants were subjected to chemical and organoleptic analysis. Data on their composition and tasting scores are presented in Table 3 a, b.

The alcohol content of the samples varied over a wide range. During the study period, it was observed the trend for the highest ratio in Kaylashki Rubin wines - from 12.66 to 14.24 vol.% (mean 13.19±0.91 vol.%, V1 and mean 13.15±0.73 vol.%, V2). It was followed by the wines of both Gamza candidate-clones. With the lowest alcohol were the Pamid 5/76 samples - from 12.12 to 12.47 vol.% (mean 12.22±0.15 vol.%, V1 and mean 12.29±0.15 vol.%, V2). The differences in the alcohol ratios were not significant between the different variants of the studied clones and varieties. All experimental wines showed a gradual increase of the rate in V2, except Gamza 52-9-5 (2014, 2015) and Kaylashki Rubin (2013).

The complete alcoholic fermentation was confirmed by the residual sugars rates in wines, that for 2013 vintage was within the range from 1.98 (Gamza 52-9-4, V1) to 3.54 g/l (Kaylashki Rubin, V1), for 2014 vintage - from 0.97 (Pamid 5/76, V1) to 2.05 g/l (Kaylashki Rubin,

V2) while for 2015 vintage – from 1.10 (Pamid 5/76, V1, V2) to 2.05 g/l (Gamza 52-9-4, V2).

The sugar-free extract (SFE) had been an important indicator of the wine composition, which was also relevant to their tasting qualities. Its rates were within the typical range for red wines of the respective varieties. The differences found in the SFE content of the samples were due to the varietal specifics and potential. That explained the lower rates for Pamid 5/76 (mean 20.14±0.51 g/l, V1 and mean 20.49±0.44 g/l, V2) and the higher ones for Kaylashki Rubin (mean 23.97±0.11 g/l, V1 and mean 23.97±0.73 g/l, V2). The differences between the variants of one variety were minimal, however, in all experimental wines V2 was characterized by a higher extract. That was also reflected in the tasting score from the organoleptic analysis of the wines. These variants, with the exception of Pamid 5/76 (2013, 2015), Gamza 52-9-4 (2014), Gamza 52-9-5 (2015) and Trapezitsa (2015) received more scores because of their better density and harmony in the taste (Table 3 a, b). The data showed that the samples from 2015 had the highest SFE rate. An exception was observed for Pamid 5/76 and Kaylashki Rubin, where higher rates were reported respectively in the variants from 2014 and 2013 vintage. During the study period it was found a relatively constant trend in the content of the indicator in the experimental wines. From the inter-varietal variants (2013, 2014), the highest SFE rates had Kaylashki Rubin samples, followed by both Gamza candidate-clones. From 2015 the highest SFE rates were found for Kaylashki Rubin and Trapezitsa (Table 3 a, b).

The titratable acidity of the experimental samples also varied within the typical range for red wines and corresponded to the specifics of the variety. Usually lower rates were observed in wines from Pamid 5/76 (mean 4.44±0.45 g/l, V1 and mean 4.28±0.20 g/l, V2) and Trapezitsa (mean 4.31±0.26 g/l, V1 and mean 4.76±0.47 g/l, V2). The titratable acids of the samples, 2013 harvest, were from 4.00 to 5.63 g/l, for 2014 - from 4.13 to 6.95 g/l, for 2015 - from 4.43 to 6.08 g/l (Table 3 a, b). The results did not show significant differences between the variants of the clones and varieties. In 2013, except Gamza 52-9-5, the samples with higher acidity were assessed with fewer points during the tasting. In 2014 the variants of Pamid 5/76, Gamza 52-9-5 and Kaylashki Rubin with lower acid content were evaluated with higher scores during the organoleptic analysis. That trend was also observed in all samples in 2015 (Table 3 a, b).

All wines had normal volatile acidity ranging from 0.42 to 0.66 g/l (2013), from 0.62 to 0.66 g/l (2014) and from 0.60 to 0.70 g/l (2015) that did not deteriorate their organoleptic properties (Table 3 a, b).

The content of TPC and anthocyanins were important indicators of the red wine composition influencing their organoleptic characteristics (Chobanova, 2012). Their rates were directly related and determined by the potential and specifics of the variety, thus they increased in the order Pamid 5/76 < Trapezitsa < Gamza 52-9-4 < Gamza 52-9-5 < Kaylashki Rubin (Table 3 a, b). The total phenolic compounds in the experimental samples ranged from 1.00 to 2.73 g/l (2013), from 0.98 to 2.33 g/l (2014) and from 0.94 to 2.28 g/l (2015). The amount of anthocyanins in the variants varied from 134.78 to 466.50 mg/l (2013), from 122.00 to 279.36 mg/l (2014) and from 117.43 to 288.25 mg/l (2015). The samples from 2013 vintage had the highest content of the analyzed indicators. No significant differences were found in their quantity between the variants of each variety. V2 wines contained more TPC and anthocyanins, but they did not always have better tasting qualities.

**Table 3a.** Chemical composition of the experimental wines from the studied clones.

| Indicators / Wines | Variant | Vintage | Alcohol, vol. % | Total extract, g/l | Sugar, g/l | SFE, g/l | Titratable acids g/l | Volatile acids, g/l | pH | TPC, g/l | Anthocyanins, mg/l | Colour intensity, I [abs. units] | Colour tint, T [abs. units] | Tasting score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pamid 5/76 | 1 | 2013 | 12.12 | 22.00 | 2.12 | 19.88 | 4.00 | 0.66 | 3.17 | 1.00 | 134.78 | 7.20 | 0.60 | 79.11 |
| | | 2014 | 12.14 | 21.70 | 0.97 | 20.73 | 4.90 | 0.62 | 3.31 | 0.98 | 122.00 | 7.64 | 0.61 | 76.43 |
| | | 2015 | 12.39 | 21.10 | 1.10 | 19.80 | 4.43 | 0.66 | 3.30 | 0.94 | 117.43 | 7.76 | 0.66 | 79.86 |
| | | *mean ±SD* | *12.22 ±0.15* | *21.60 ±0.46* | *1.40 ±0.63* | *20.14 ±0.51* | *4.44 ±0.45* | *0.65 ±0.02* | *3.26 ±0.08* | *0.97 ±0.03* | *124.74 ±8.99* | *7.53 ±0.29* | *0.62 ±0.03* | *78.47 ±1.80* |
| | 2 | 2013 | 12.21 | 22.80 | 2.15 | 20.65 | 4.13 | 0.54 | 3.12 | 1.22 | 142.77 | 7.54 | 0.58 | 77.78 |
| | | 2014 | 12.20 | 22.00 | 1.17 | 20.83 | 4.20 | 0.66 | 3.32 | 1.05 | 130.35 | 7.88 | 0.66 | 79.57 |
| | | 2015 | 12.47 | 20.90 | 1.10 | 20.00 | 4.50 | 0.62 | 3.32 | 0.98 | 124.48 | 7.81 | 0.61 | 79.00 |
| | | *mean ±SD* | *12.29 ±0.15* | *21.90 ±0.95* | *1.47 ±0.59* | *20.49 ±0.44* | *4.28 ±0.20* | *0.61 ±0.06* | *3.25 ±0.11* | *1.08 ±0.12* | *132.53 ±9.34* | *7.74 ±0.18* | *0.62 ±0.04* | *78.78 ±0.91* |
| Gamza 52-9-4 | 1 | 2013 | 12.84 | 23.40 | 1.98 | 21.42 | 5.32 | 0.42 | 3.36 | 2.19 | 339.93 | 9.63 | 0.54 | 80.67 |
| | | 2014 | 12.28 | 23.60 | 1.45 | 22.15 | 5.63 | 0.62 | 3.42 | 1.61 | 218.10 | 9.56 | 0.68 | 75.00 |
| | | 2015 | 12.51 | 24.50 | 1.74 | 22.76 | 5.55 | 0.60 | 3.32 | 1.55 | 226.89 | 9.37 | 0.60 | 79.14 |
| | | *mean ±SD* | *12.54 ±0.28* | *23.83 ±0.58* | *1.72 ±0.26* | *22.11 ±0.67* | *5.50 ±0.16* | *0.55 ±0.11* | *3.37 ±0.05* | *1.78 ±0.35* | *261.64 ±67.94* | *9.52 ±0.13* | *0.61 ±0.07* | *78.27 ±2.93* |
| | 2 | 2013 | 12.92 | 24.40 | 2.42 | 21.98 | 5.13 | 0.54 | 3.34 | 2.32 | 352.05 | 9.88 | 0.56 | 81.78 |
| | | 2014 | 12.34 | 23.80 | 1.45 | 22.35 | 5.40 | 0.66 | 3.37 | 1.72 | 226.30 | 9.75 | 0.69 | 74.57 |
| | | 2015 | 12.56 | 24.90 | 2.05 | 22.85 | 5.40 | 0.60 | 3.34 | 1.69 | 237.50 | 9.42 | 0.58 | 79.86 |
| | | *mean ±SD* | *12.60± 0.29* | *24.37 ±0.55* | *1.97 ±0.49* | *22.39 ±0.44* | *5.31 ±0.15* | *0.60 ±0.06* | *3.35 ±0.02* | *1.91 ±0.35* | *271.95 ±69.59* | *9.68 ±0.24* | *0.61 ±0.07* | *78.74 ±3.73* |
| Gamza 52-9-5 | 1 | 2013 | 13.08 | 23.20 | 2.25 | 20.95 | 5.10 | 0.54 | 3.33 | 2.33 | 358.18 | 10.05 | 0.52 | 76.44 |
| | | 2014 | 12.32 | 23.20 | 1.00 | 22.20 | 5.55 | 0.64 | 3.39 | 1.70 | 224.00 | 9.71 | 0.69 | 76.14 |
| | | 2015 | 12.55 | 24.64 | 1.27 | 23.37 | 5.18 | 0.62 | 3.28 | 1.68 | 234.47 | 9.45 | 0.60 | 77.57 |
| | | *mean ±SD* | *12.65 ±0.39* | *23.68 ±0.81* | *1.51 ±0.66* | *22.17 ±1.21* | *5.28 ±0.24* | *0.60 ±0.05* | *3.33 ±0.05* | *1.90 ±0.37* | *272.22 ±74.63* | *9.74 ±0.30* | *0.60 ±0.08* | *76.72 ±0.75* |
| | 2 | 2013 | 13.15 | 24.10 | 2.15 | 21.95 | 5.63 | 0.42 | 3.36 | 2.30 | 345.17 | 9.74 | 0.56 | 83.00 |
| | | 2014 | 12.27 | 23.40 | 1.14 | 22.26 | 5.33 | 0.62 | 3.39 | 1.76 | 228.20 | 9.73 | 0.68 | 78.29 |
| | | 2015 | 12.48 | 25.21 | 1.71 | 23.50 | 5.33 | 0.60 | 3.33 | 1.71 | 248.32 | 9.72 | 0.61 | 77.29 |
| | | *mean ±SD* | *12.63 ±0.46* | *24.23 ±0.91* | *1.67 ±0.51* | *22.57 ±0.82* | *5.43 ±0.17* | *0.51 ±0.10* | *3.36 ±0.03* | *1.92 ±0.33* | *273.90 ±62.54* | *9.73 ±0.01* | *0.62 ±0.06* | *79.53 ±3.05* |

**Table 3b.** Chemical composition of the experimental wines from the studied varieties.

| Indicators / Wines | Variant | Vintage | Alcohol, vol. % | Total extract, g/l | Sugar, g/l | SFE, g/l | Titratable acids g/l | Volatile acids, g/l | pH | TPC, g/l | Anthocyanins, mg/l | Colour intensity, I [abs. units] | Colour tint, T [abs. units] | Tasting score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kaylashki Rubin | 1 | 2013 | 14.24 | 27.60 | 3.54 | 24.06 | 5.54 | 0.66 | 3.36 | 2.72 | 456.52 | 11.16 | 0.62 | 85.00 |
| | | 2014 | 12.66 | 25.80 | 1.95 | 23.85 | 6.95 | 0.64 | 3.14 | 2.28 | 273.22 | 10.12 | 0.60 | 75.43 |
| | | 2015 | 12.68 | 25.56 | 1.55 | 24.01 | 6.08 | 0.66 | 3.11 | 2.26 | 288.25 | 10.15 | 0.64 | 82.00 |
| | | *mean ±SD* | *13.19 ±0.91* | *26.32 ±1.11* | *2.35 ±1.05* | *23.97 ±0.11* | *6.19 ±0.71* | *0.65 ±0.01* | *3.20 ±0.14* | *2.42 ±0.26* | *339.33 ±101.77* | *10.48 ±0.59* | *0.62 ±0.02* | *80.81 ±4.89* |
| | 2 | 2013 | 14.00 | 27.60 | 3.00 | 24.60 | 5.00 | 0.66 | 3.35 | 2.73 | 466.50 | 11.37 | 0.60 | 85.89 |
| | | 2014 | 12.72 | 26.20 | 2.05 | 24.15 | 6.80 | 0.66 | 3.15 | 2.33 | 279.36 | 10.17 | 0.67 | 76.71 |
| | | 2015 | 12.74 | 25.80 | 1.63 | 24.17 | 6.00 | 0.68 | 3.15 | 2.28 | 285.93 | 10.10 | 0.63 | 83.14 |
| | | *mean ±SD* | *13.15 ±0.73* | *26.53 ±0.95* | *2.23 ±0.70* | *23.97 ±0.73* | *5.93 ±0.90* | *0.67 ±0.01* | *3.22 ±0.11* | *2.45 ±0.25* | *343.93 ±106.20* | *10.55 ±0.71* | *0.63 ±0.03* | *81.91 ±4.71* |
| Trapezitsa | 1 | 2013 | - | - | - | - | - | - | - | - | - | - | - | - |
| | | 2014 | 12.48 | 22.40 | 1.10 | 21.30 | 4.13 | 0.66 | 3.27 | 1.68 | 220.14 | 9.66 | 0.65 | 75.43 |
| | | 2015 | 12.60 | 24.90 | 1.27 | 23.63 | 4.50 | 0.66 | 3.23 | 1.77 | 250.10 | 9.88 | 0.62 | 80.14 |
| | | *mean ±SD* | *12.54 ±0.08* | *23.65 ±1.77* | *1.18 ±0.12* | *22.46 ±1.65* | *4.31 ±0.26* | *0.65 ±0* | *3.25 ±0.03* | *1.72 ±0.06* | *235.12 ±21.18* | *9.77 ±0.15* | *0.63 ±0.02* | *77.78 ±3.33* |
| | 2 | 2013 | - | - | - | - | - | - | - | - | - | - | - | - |
| | | 2014 | 12.52 | 23.40 | 1.61 | 21.79 | 4.43 | 0.62 | 3.31 | 1.65 | 222.10 | 9.70 | 0.67 | 76.71 |
| | | 2015 | 12.64 | 25.40 | 1.64 | 23.76 | 5.10 | 0.70 | 3.24 | 1.85 | 256.74 | 9.93 | 0.60 | 79.43 |
| | | *mean ±SD* | *12.58 ±0.08* | *25.40 ±1.41* | *1.62 ±0.02* | *22.77 ±1.39* | *4.76 ±0.47* | *0.66 ±0.06* | *3.27 ±0.05* | *1.75 ±0.14* | *239.42 ±24.49* | *9.81 ±0.16* | *0.63 ±0.05* | *78.07 ±1.92* |

The TPC content in wine influenced its taste indicators, and the amount of anthocyanins determined the colour characteristics (Chobanova, 2012; Kekelidze *et al.*, 2014). However, the experimental data obtained did not show correlation with the scores from the organoleptic analysis (Table 3 a, b).

The colour properties of red wines were greatly determined by the concentration of TPC and anthocyanins (Chobanova, 2012; Bai *et al.*, 2013). The wines from the variants containing more anthocyanins had higher rates of intensity and accordingly the colour indices were rated higher when tasting. In accordance with the varietal specifics, the variants of Pamid 5/76 had the lowest colour intensity (mean 7.53±0.29 [abs. units], V1 and mean 7.74±0.18 [abs. units], V2) and the highest – those of Kaylashki Rubin (mean 10.48±0.59 [abs. units], V1 and mean 10.55±0.71 [abs. units], V2). The colour tint indicated the level of evolution of the red colour and its values in the experimental samples ranged from 0.52 to 0.62 [abs. units] (2013), from 0.61 to 0.69 [abs. units] (2014) and from 0.58 to 0.66 [abs. units] (2015), which is within the normal range for young red wines (Table 3 a, b).

### 3.4. Wine aromatic composition

Data on the aromatic composition of the experimental red wines during the study period are presented in Table 4. The content of total esters, total aldehydes and total higher alcohols was analyzed in all samples.

**Table 4.** Aromatic composition of the experimental wines from the studied clones and varieties.

| Indicators / Wines | Variant | Vintage | Total esters, mg/l | Total aldehydes, mg/l | Total higher alcohols, mg/l |
|---|---|---|---|---|---|
| Pamid 5/76 | 1 | 2013 | 176.00 | 93.00 | 419.50 |
| | | 2014 | 140.80 | 17.60 | 378.00 |
| | | 2015 | 246.40 | 33.00 | 499.00 |
| | | *mean±SD* | *187.73 ±53.77* | *47.87 ±39.84* | *432.17 ±61.48* |
| | 2 | 2013 | 193.60 | 114.00 | 382.50 |
| | | 2014 | 158.40 | 11.00 | 342.00 |
| | | 2015 | 264.00 | 22.00 | 520.00 |
| | | *mean±SD* | *205.33 ±53.77* | *49.00 ±56.56* | *414.83 ±93.30* |
| Gamza 52-9-4 | 1 | 2013 | 88.00 | 90.82 | 455.00 |
| | | 2014 | 105.60 | 35.20 | 329.00 |
| | | 2015 | 264.00 | 26.40 | 526.00 |
| | | *mean±SD* | *152.53 ±96.93* | *50.81 ±34.93* | *436.67 ±99.77* |
| | 2 | 2013 | 105.60 | 74.00 | 436.00 |
| | | 2014 | 123.20 | 35.20 | 353.00 |
| | | 2015 | 316.80 | 35.20 | 558.00 |
| | | *mean±SD* | *181.86 ±117.18* | *48.13 ±22.40* | *449.00 ±103.12* |
| Gamza 52-9-5 | 1 | 2013 | 70.40 | 73.80 | 508.50 |
| | | 2014 | 105.60 | 33.00 | 326.00 |
| | | 2015 | 264.00 | 52.80 | 550.00 |
| | | *mean±SD* | *146.67 ±103.13* | *53.20 ±20.40* | *461.50 ±119.17* |
| | 2 | 2013 | 88.00 | 101.40 | 448.50 |
| | | 2014 | 140.60 | 74.80 | 328.00 |
| | | 2015 | 299.20 | 35.20 | 470.00 |
| | | *mean±SD* | *175.93 ±109.94* | *70.47 ±33.31* | *415.50 ±76.53* |

| | | | | | |
|---|---|---|---|---|---|
| Kaylashki Rubin | 1 | 2013 | 140.80 | 81.10 | 466.50 |
| | | 2014 | 123.20 | 48.40 | 374.00 |
| | | 2015 | 228.80 | 46.20 | 494.00 |
| | | *mean±SD* | *164.27 ±56.57* | *58.57 ±19.55* | *444.83 ±62.86* |
| | 2 | 2013 | 176.00 | 80.00 | 531.00 |
| | | 2014 | 158.40 | 39.60 | 432.00 |
| | | 2015 | 228.80 | 37.40 | 556.00 |
| | | *mean±SD* | *187.73 ±36.64* | *52.33 ±23.98* | *506.33 ±65.58* |
| Trapezitsa | 1 | 2013 | - | - | - |
| | | 2014 | 158.00 | 22.00 | 345.00 |
| | | 2015 | 176.00 | 26.40 | 500.00 |
| | | *mean±SD* | *167.00 ±12.73* | *24.20 ±3.11* | *422.50 ±109.60* |
| | 2 | 2013 | - | - | - |
| | | 2014 | 176.00 | 26.40 | 376.00 |
| | | 2015 | 193.60 | 17.60 | 474.00 |
| | | *mean±SD* | *184.80 ±12.45* | *22.00 ±6.22* | *425.00 ±69.30* |

The esters in wine were formed by chemical reaction as a result of esterification processes occurring during the grapes ripening and wine aging or biologically from the yeast during the alcoholic fermentation (Chobanova, 2012; Antalick et al., 2015). During the study period, no strict correlation was found in the amount of total esters in the experimental samples per varieties and vintages. In 2013, the ester content varied in the range from 70.40 to 193.60 mg/l, as it was the lowest in Gamza 52-9-5 (V1) and the highest in Pamid 5/76 (V2). In 2014, their rates ranged from 105.60 (Gamza 52-9-4, V1 and Gamza 52-9-5, V1) to 176.00 mg/l (Trapezitsa, V2). The 2015 samples were distinguished for the highest ratio of esters ranging from 176.00 (Trapezitsa, V1) to 316.80 mg/l (Gamza 52-9-4, V2). An increase in their amount in V2 compared to the control (V1<V2) was also observed due to the application of the aroma-releasing enzyme. Of all V2 with the highest ester content were Pamid 5/76 (mean 205.33±53.77 mg/l) and Kaylashki Rubin (mean 187.73±36.64 mg/l). These results had confirmed the positive effect of the enzyme on the ester content of the wines and their aromatic characteristics.

Aldehydes were found in very small quantities in grapes. In wine they appeared (mainly acetaldehyde) along several major pathways – an intermediate reaction and normal product of the alcoholic fermentation, enzymatic oxidation of alcohols by yeast, non-enzymatic oxidation of alcohols by the action of the oxygen from the air, oxidative deamidation of amino acids, and decarboxylation of keto acids (Chobanova, 2012).

The total aldehydes content in the experimental wines was below 100 mg/l and it did not adversely affect their organoleptic characteristics. The concentration ranged from 73.80 to 114.00 mg/l (2013), from 11.00 to 74.80 mg/l (2014) and from 17.60 to 52.80 mg/l (2015). The samples of Trapezitsa variety had the lowest concentrations during the study period (mean 24.20±3.11 mg/l, V1 and mean 20.00±6.22 mg/l, V2). Differences were observed within the variants of the varieties, but from different vintages. The 2013 samples contained the most total aldehydes. In 2013, Pamid 5/76, V2 and Gamza 52-9-5, V2 contained the most aldehydes. In 2014, V1 of Pamid 5/76 and Kaylashki Rubin had higher aldehyde content, while in Gamza 52-9-5 and Trapezitsa it was V2. In Gamza 52-9-4, V1 and V2 had similar rates. In 2015, a greater amount

of aldehydes was analyzed in V1 of Pamid 5/76, Gamza 52-9-5, Kaylashki Rubin and Trapezitsa. The data obtained and their analysis did not reveal a correlation between the influence of the applied enzyme on the aldehydes ratio.

The major part of higher alcohols was formed during the alcoholic fermentation by the degradation of sugars or amino acids, and a small part of them during the aging of wines (Chobanova, 2012; Mina and Tsaltas, 2017; Manolache *et al.*, 2018). The samples from the studied harvests were characterized by high content of higher alcohols, ranging from 382.50 to 531.00 mg/l (2013), from 326.00 to 432.00 mg/l (2014) and from 470.00 to 558.00 mg/l (2015). It was the lowest in wines from 2014 vintage and the highest in the wines from 2015. In 2013, the smallest amount was recorded in the samples of Pamid 5/76 variety, and the highest in the samples from Kaylashki Rubin. Their ratios in the wines from both Gamza candidate-clones were close. In the studied red wines, with the exception of Kaylashki Rubin, the content of total higher alcohols was higher in the control. In 2014, the amount of higher alcohols in the samples from both Gamza candidate-clones was similar and the lowest, and the highest was in the variants of Kaylashki Rubin. With the exception of Pamid 5/76, their ratio was lower in the control. The wines of 2015 vintage had similar higher alcohol content, as with Pamid 5/76, Gamza 52-9-4 and Kaylashki Rubin the control V1 had a lower rate.

The results from the analysis of the wine aromatic composition did not show a strict correlation between the studied components and the organoleptic profile, respectively the tasting evaluation (Table 3 a, b, Table 4). With Pamid 5/76, V1 (2015) was the best evaluated. It had high rates of esters and higher alcohols and low of aldehydes. From the wines of both Gamza candidate-clones 52-9-4 and 52-9-5 with the best organoleptic characteristics were determined the samples from V2 (2013), despite the low concentration of esters and the high rates of higher alcohols. From Kaylashki Rubin variants, V2 (2013), which had high rates of esters, aldehydes and higher alcohols, received the most points (85.89 points). From Trapezitsa, V1 (2015), having higher rates of esters and higher alcohols, was rated the highest (80.14 points).

## 4. Conclusions

On the basis of the obtained results it could be summarized:

- Under the growing conditions in the region of Pleven, the studied red clones and varieties reached their technological maturity in the middle and second half of September. The latest ripening variety was Kaylashki Rubin that had the most gradual sugar accumulation and reduction of acidity.
- The lowest sugar accumulation and titratable acids were reported for Pamid 5/76 and the highest for Kaylashki Rubin. Grapes from 2013 vintage had the highest sugar content due to favorable weather conditions during the ripening phase, and GAI rates, which showed that it was suitable for the production of wines of optimum quality in terms of chemical composition and tasting features.
- The experimental wines had different composition and organoleptic characteristics depending on the potential and the specifics of the variety and the vintage.
- The highest alcohol content had Kaylashki Rubin wines, followed by the wines of both candidate-clones of Gamza, and the lowest – Pamid 5/76 samples.
- Pamid 5/76 samples had the lowest SFE rates and Kaylashki Rubin samples had the highest. The experimental wines from V2 were characterized by a higher extract and better organoleptic taste. The highest SFE rates were found in 2015 harvest samples.
- The amount of TPC and anthocyanins in the experimental wines increased in the order Pamid 5/76 < Trapezitsa < Gamza 52-9-4 < Gamza 52-9-5 < Kaylashki Rubin. The 2013 vintage samples had the highest rates. The wines from V2 of the studied varieties contained more TPC, anthocyanins and colour intensity, but not always better tasting qualities.

- The positive effect of the aroma-releasing enzyme applied on the content of esters in wines and their aromatic characteristics was confirmed. The 2015 samples had the highest ratio of total esters. It was also observed an increase in their amount in V2 compared to the control (V1 <V2).
- No correlation was found on the effect of the applied enzyme on the total aldehydes amount. The lowest concentrations were analyzed in Trapezitsa wines. In all varieties, 2013 vintage samples contained the most aldehydes, with the highest rates found in Pamid 5/76 variants.
- The samples from the studied vintages were characterized by a high concentration of higher alcohols, the lowest being in wines, 2014 harvest, and the highest in the samples, 2015 harvest. No correlation was found between the tested components of the aromatic composition of the wines and their tasting evaluation.
- No strict correlation was observed between the investigated components of the aromatic composition of the experimental wines and their organoleptic profile, respectively, their tasting evaluation.

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# TECHNOLOGICAL AND SENSORY PROPERTIES OF SPONGE CAKES CONTAINING CRICKET FLOUR (*ACHETA DOMESTICUS*)

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**ABSTRACT**

Insects are exotic alternative protein source with huge potential for food industry by high nutritional value and limited environmental footprint. The aim of this study is to explore the colour characteristics, technological and sensory properties of sponge cakes enriched with cricket 5% and 10% cricket flour ($CF_5C$ and $CF_{10}C$), used as substitute of the wheat flour. Moisture, pH, $a_w$, springiness and specific volume decrease with increasing CF in a dose depend manner. Replacement of 10% cricket flour showed negative effect on the colour characteristics, shrinkage, specific volume, springiness and texture of sponge cakes. In $CF_{10}C$ a* shows highest values, while L* and b* present the lowest indications in comparison to other samples measured. The sensory panel found nonspecific taste and off-flavour in 10% CF based sponge cakes. Replacement of wheat flour with up to 5 % CF has slight effect on the sensory properties (appearance, cell siz uniformity, crumb tenderness, odour and taste) of sponge cakes and can successfully be used as innovative ingredient to enhance the protein content in bakery products.

## 1.Introduction

Pastries are most often made from wheat flour and are characterized by small amount of natural bioactive components. This leads to trade-offs in the quality of the finished product in terms of nutrition and health. The use of dietary fibres from various sources partially replaces wheat flour and contributes to the added value to those products (Anonymous, 2001).

Nowadays, food industry pays increasing attention to insects and insect flours as an alternative protein source with high nutritional value. Some beneficial aspects of insect usage are the significant content of protein, vitamins, and minerals and limited environmental footprint (Smarzyński *et al.* 2019). Insect consumption in Africa, Australia and Latin America has a long history (Melgar - Lalanne *et al*., 2019). Cricket flour (*Acheta domesticus*) is a good source of protein (da Rosa and Thys, 2019). It has good water- and fat- retaining properties and at the same time is a rich source of micro- and macro- elements (Biró *et al.*, 2020).

FAO report recommends the use of insect products as they are a rich source of protein and an alternative to tackling the growing population, respectively the growing hunger.

For the European market, this type of product is exotic and in recent years has been a subject of great interest from consumers (van Huis, 2013). From 1 January 2018, in some European countries, insects, as well as parts of them, are officially authorized for production and sale, such as the so-called "novel foods" (C/2017/8878).

Replacing part of the wheat flour with crickets flour helps to reduce the total gluten content and at the same time improves the amino acid and fatty acid composition of the product (Mishyna *et al.*, 2020). The success of the introduction of exotic foods in the menu of Europeans is associated with the added value and the shown interest that the product brings. However, the region-specific preferences, such as taste and texture, must also be taken into account (González *et al.,* 2019). The amount of the additive should be correctly determined so that the technological and organoleptic characteristics of insect-based food are not impaired (Biró *et al*., 2020).

Therefore, the present study aims to determine the technological, colour and sensory characteristics of sponge cakes processed by 5 and 10% replacing wheat flour with cricket flour (*Acheta domesticus*) in order to improve its nutritional value.

## 2. Materials and methods

### 2.1. *Ingredients*

Wheat flour of type 500 (WF), granulated sugar and chicken eggs were purchased from local market. Cricket flour (*Acheta domesticus*) (CF) is provided by Ento sinergy Ltd. The physicochemical parameters of the flours are presented in Table 1.

**Table 1.** Chemical composition and mineral content of wheat flour (WF) and cricket flour (CF).

| Parameters | WF | CF |
|---|---|---|
| Dry matter, % | 85.70 | 85.53 |
| Proteins, % | 11.57 | 63.64 |
| Fats, % | 1.00 | 14.43 |
| Carbohydrates, % | 88.76 | 12.90 |
| Energy value, kcal/100g | 410.72 | 436.03 |
| pH | 5.20 | 6.83 |
| Moisture,% | 14.30 | 14.47 |
| Zink, mg/kg | 7.40 | 160.00 |
| Manganese, mg/kg | 4.30 | 36.00 |
| Iron, mg/kg | 7.10 | 60.00 |
| Calcium, mg/kg | 0.19 | 650.00 |
| Sodium, % | 0.34 | 0.43 |
| Magnesium, % | 0.02 | 0.53 |

#### *2.2.1. Sponge cake preparation*

The cake batter was prepared (Table 2) by double mixing procedure with partitioning whipping of whites and yolks of egg (Stankov *et al.*, 2018). The control sponge cake (WFB) formulation contained only wheat flour. In $CF_5B$ and $CF_{10}B$ batter the wheat flour was partially replaced with 5% and 10% CF, resp. After mixing of batter was placed in metallic pans and baked in an electric oven for 30 min at 180 °C.

**Table 2.** Sponge cake batters formulations

| Ingredients | Amount based on wheat flour, % | | |
|---|---|---|---|
| | Control sample (WFB) | with 5% $CF_5B$ | with 10% $CF_{10}B$ |
| Yolk of eggs | 43.22 | 43.22 | 43.22 |
| White of eggs | 96.77 | 96.77 | 96.77 |
| Granulated sugar | 83.77 | 83.77 | 83.77 |
| Wheat flour type 500 | 100.00 | 95.00 | 90.00 |
| Cricket flour | - | 5.00 | 10.00 |

## 2.2. Methods

***2.2.1. Specific gravity*** (batters cakes)

The specific gravity of the sponge cake batter was calculated by dividing the weight of a batter cup to the weight of an equal volume of distilled water (AACC 10-95, 1983).

***2.2.2. Colour characteristics***

A Konica Minolta colorimeter CR-410 (Konica Minolta Holding, Inc., Ewing, New Jersey, USA) was used to evaluate the brightness (L* value), the red component (a* value) and the yellow component (b* value) (Hunt *et al.* 2012). Total colour difference (ΔE) is calculated:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}, \quad (1)$$

where ΔL*, Δa* and Δb* are the differences in the values of L*, a*, b* between those of the control and the corresponding experimental sample.

***2.2.3. pH value determination***

The pH value of the samples was measured electropotentiometrically (Osimani et al., 2018) with pH meter "Microsyst MS 2004" (Microsyst, Plovdiv), equipped with temperature and combined pH electrode. The pH electrode is of the Sensorex Combination Recorder S 450 CD type (Sensorex pH Electrode Station, Garden Grove, CA, USA).

***2.2.4. World photographic analysis***

Sample analysis was performed using a microscope (Olympus BX41TF, Japan) at a magnification of 100x.

***2.2.5. Determination of volume of the product***

Volume was measured by the small, homogeneous grain displacement method with a volume meter, [$cm^3$] (Ngo et al., 1986; AACC 10-05, 2000);

***2.2.6. Determination of specific volume***

It is calculated by the ratio of the volume of the cake to its mass, [$cm^3$ / g];

***2.2.7. Structural and mechanical properties of cakes***

Shrinkage [PU] and springness (relaxation) [PU] are determined with an automatic penetrometer (model DS VEB Feinmess, Dresden, Germany) (Stankov *et al., 2018*).

***2.2.8. Water activity ($a_w$)***

The water activity of the batter and the crumb samples was determined using a Novasina AG CH-8853 water activity meter (Zurich, Switzerland) at 20 °C.

***2.2.9. Moisture content***

The moisture content is determined after drying the sample at 104 - 105 ° C till reaching a constant weight AACC 44-15.02. (AACC, 1999).

***2.2.10. Water absorption capacity***

The water absorption capacity of the cake was measured by the method for determining of biscuits' swelling according to BDS 15221: 1981.

***2.2.11. Sensory evaluation***

Five member panel group with proven abilities was used to determine the sensory parameters (appearance, cell siz uniformity, crumb tenderness, odour and taste) of the cakes (Meilgaard et al. 1999) was used. The samples were scored using 1 to 5 scales.

***2.2.12. Photograph images***

For the determination of the sponge cake crumb structure, photographs were taken of the half-cut cake.

***2.2.13. Statistical analysis***

Statistical analysis of the average values of five time reps was made. All statistical procedures for the data of different samples were analyzed by SAS software (SAS Institute, Inc. 1990). The Student-Newman-Keuls multiple range test was used to compare differences among means. The results were expressed as mean values and standard errors of the mean. A p-value less than 0.05 ($p < 0.05$) was considered as significant.

## 3. Results and discussions

### 3.1. Physicochemical characteristics

As a result the water activity of the sponge system correlates to the forms of the bound water. By the slightly acidic reaction of CF, pH in batter decrease with the increase in CF concentration (10%, $CF_{10}B$) (Table 3). According to Osimani *et al*., 2018 the higher ash content of CF influences the buffering capacity in the matrix and decrease the pH of batter (Mariotti *et al.,* 2014; Taccari *et al*., 2016).

**Table 3.** Physicochemical and textural characteristics of sponge batter and cakes

| Physicochemical and textural characteristics | Sponge cake type | | |
|---|---|---|---|
| | Control sample (WFB) | with 5% $CF_5B$ | with 10% $CF_{10}B$ |
| Specific gravity (for batter) | $0.54^{a}$±0.02 | $0.66^{b}$ ±0.03 | $0.74^{c}$ ±0.02 |
| Volume, $cm^3$ | $210.00^{b}$±3.00 | $210.00^{b}$±2.00 | $200.00^{a}$±1.50 |
| Specific volume, $cm^3/g$ | $3.44^{c}$±0.06 | $3.28^{b}$±0.05 | $3.18^{a}$±0.06 |
| pH (for batter) | $7.60^{b}$±0.08 | $7.24^{a}$ ±0.02 | $7.17^{a}$ ±0.05 |
| Moisture, % | $43.37^{a}$±0.55 | $42.13^{a}$±2.09 | $41.56^{a}$±1.34 |
| $a_w$, crumb cake | $0.920^{b}$±0.004 | $0.905^{a}$ ±0.005 | $0.900^{a}$±0.002 |
| Springiness, [PU[1]] | $118.00^{c,}$±2.00 | $97.00^{b}$ ±1.00 | $74.00^{a}$ ±1.00 |
| Shrinkage [PU[1]] | $24.00^{b,z}$ ±1.00 | $25.00^{b,y}$ ±0.50 | $14.00^{a,z}$±0.50 |
| Water-absorbing capacity,% | $388.00^{b}$±3.00 | $310.00^{a}$ ±2.50 | $306.00^{a}$ ±3.00 |

WFB, cake batter containing 100% wheat flour; $CF_5B$ – cake batter with blend containing 5% cricket flour and 95% wheat flour; $CF_{10}B$ – cake batter with blend containing 10% cricket flour and 90% wheat flour; WFC – sponge cake containing 100% wheat flour; $CF_5C$, sponge cake with blend containing 5% cricket flour and 95% wheat flour; $CF_{10}C$- sponge cake with blend containing 10% cricket flour and 90% wheat flour.

Results are presented as Means ± Standard error of the means (SEM)

[1]PU - Penetrometer Units.

$^{a,b,c}$ Means with different superscripts in each column differ significantly ($p \leq 0.05$) of one parameter

With increasing the amount of CF, the moisture content (Table 3) decreases both in the batter and the cakes ($p \leq 0.05$). At the same time, the highest value ($p \leq 0.05$) for water activity ($a_w$) was found in the WFB control samples. Water activity ($a_w$) of control batter (WFB) was 1.63 ($p \leq 0.05$) and 2.17% ($p \leq 0.05$) higher than the samples with 5 and 10% addition of cricket flour ($CF_5B$ and $CF_{10}B$), respectively. The data confirmed previous researches proving that lower water activity result in higher moisture loss (Indriani, 2020). Another possible reason for the established decrease of $a_w$ is the lower moisture content of blended with 5% and 10% CF flour used for preparing the $CF_5B$ and $CF_{10}B$ sponge cakes.

### 3.2. Textural parameters

The quality of sponge cakes is determined by their baking properties (Table 3). An increase in the relative mass by decrease in the degree of aeration was found in CF enriched cakes ($p \leq 0.05$). The highest degree of shrinkage and springiness characterized the control (WFB) with the highest degree of softness. The $CF_5C$ specific volume, shrinkage

and springiness are lower ($p \leq 0.05$), but close to those obtained for WFC. Decreased springiness and more difficult chewing have also been reported by Pauter *et al*., 2018 in sponge cakes with CF. The mass and bulk between controls (WFB) and $CF_5B$ do not differ significant ($p \leq 0.05$). The data obtained for specific volume (Table 3) did not show a statistically significant ($p \leq 0.05$) difference between the two CF based sponge cakes ($CF_5C$ and $CF_{10}C$). Compared to WFC, the specific volume in $CF_5C$ and $CF_{10}C$ is about 5% ($p \leq 0.05$) lower, which is most likely due to the higher protein content and the lower degree of aeration of the batter. In contrast to our results, González *et al.,* (2019) replaced 5% WF with grounded *Hermetia illucens, Acheta domesitca, Tenebrio molitor* protein and fibre and did not report changes in specific volume in bread. Perhaps the presence of fibre in recipe composition helps to preserve the specific volume and texture of the product.

The deterioration of the parameters relative mass, springiness, shrinkage and specific volume increases with higher amount of CF ($p \geq 0.05$). The shrinkage determines the internal stability of the structure, as it is directly related to the ingredients used in making the batter. The exact amount of insect floor is very important. According to Indriani *et al.* (2020) the starch content is lower in the cakes after wheat replacement. On the other hand, cricket exoskeleton contained 8-9% non-soluble chitin, which is a source of insoluble fibre. Chitin makes foam bubbles unstable and decrease film layer stability. Poor foam properties of CF (*Acheta domesticus*) were reported by Yi *et al.,* (2013). A linear correlation between volume, hardness and insect flour used for processing of cinereous cockroach (*Nauphoeta cinerea*) enriched breads was prooven by de Oliveira *et al.* (2017). The results obtained show that the replacement of WF with CF in an amount of up to 5% has a lower impact on the technological characteristics of sponge cakes.

### 3.3. Colour characteristics

Blending the wheat flour with cricket flour affected the cake lightness ($p \leq 0.05$). The batter colour lightness (L*) (WFB) was 7.11% and 10.51% higher than that measured after the partial replacement with CP ($p \leq 0.05$). The same trend was established after baking. The L* value of WFC was 8.24 and 12.56% higher ($p \leq 0.05$), compared to the cakes with 5 and 10% cricket flour, respectively (Table 4).

**Table 4.** Colour indicators of the sponge batters and cakes

| | Cake batter | | | Sponge cakes | | |
|---|---|---|---|---|---|---|
| Sample | WFB | $CF_5B$ | $CF_{10}B$ | WFC | $CF_5C$ | $CF_{10}C$ |
| L*( C ) | $83.80^{c}\pm0.06$ | $77.84^{b}\pm0.06$ | $74.99^{a}\pm0.01$ | $77.83^{c}\pm0.12$ | $71.41^{b}\pm0.06$ | $68.05^{a}\pm0.13$ |
| a* ( C ) | $0.91^{a}\pm0.18$ | $1.30^{b}\pm0.01$ | $1.47^{c}\pm0.01$ | $0.91^{a}\pm0.03$ | $2.17^{b}\pm0.04$ | $3.08^{c}\pm0.05$ |
| b* ( C ) | $27.91^{c}\pm0.58$ | $23.54^{b}\pm0.02$ | $21.06^{a}\pm0.04$ | $26.10^{c}\pm0.08$ | $23.79^{b}\pm0.11$ | $22.34^{a}\pm0.16$ |
| ΔE | - | 7.46 | 11.17 | - | 6.42 | 10.70 |

WFB, cake batter containing 100% wheat flour; $CF_5B$ – cake batter with blend containing 5% cricket flour and 95% wheat flour; $CF_{10}B$ – cake batter with blend containing 10% cricket flour and 90% wheat flour; WFC – sponge cake containing 100% wheat flour; $CF_5C$, sponge cake with blend containing 5% cricket flour and 95% wheat flour; $CF_{10}C$- sponge cake with blend containing 10% cricket flour and 90% wheat flour.
Results are presented as Means ± Standard error of the means (SEM)
$^{a,b,c}$ Means with different superscripts in each column differ significantly ($p \leq 0.05$) of one parameter.

The control sponge cakes (WFC) was lowest in colour redness (a*) ($p \leq 0.05$) and highest in yellow colour component (b*) ($p \leq 0.05$). Both in the batter and in the baked cakes a* increases and b* decreases with higher CF replacement ($p \leq 0.05$). In a similar study of muffins with 5 and 10% CF a* and b* values shifted to the green and blue areas, respectively (Pauter *et al.*, 2018).

When using ingredient for wheat replacement, it is desirable for products to retain their colour characteristics in order to be accepted by the consumers (Biró *et al*., 2020). The darker colour in CF based cakes is expected, as the cricket flour is darker in colour, too. Decreases in L * values were also obtained in other studies after partial replacement of WF with CF in muffins (Pauter *et al.,* 2018), cricket-Enriched Oat Biscuit (Biró *et al.,* 2020) and in other cricket-based foods (Mishyna *et al.,* 2020). We can conclude that blending the WP with CF should not exceed 5% in order to avoid the colour deterioration of the final product.

It`s reported that deviation in ΔE above 3.5 is a proof for significant difference between two samples (Tkacz et al., 2020). Total colour difference (ΔE) of $CF_{10}C$ is 33% higher compared to ΔE of $CF_5C$. We can conclude that the replacement of WP with CF significantly changes the total colour difference (ΔE) in a dose-dependent manner ($p \leq 0.05$).

### 3.4. Sensory analysis

The results of the sensory analysis confirm the data for the relative mass of the batter. The sensory panel also confirms the data on the specific volume of cricket-based cakes, which decreases with increasing amount of CF. It is known that usage of edible insects with hard exoskeletons (crickets, grasshoppers) containing chitin, result in insect based products with soft texture. Even more, the legs and wings of grasshoppers and locusts should be removed before consumption (van Huis et al., 2013). Microscopic photographs of the batter clearly show the presence of solid particles from the chitin shells (Fig.1).

The high dry matter content and the correspondingly lower humidity of CF cakes are another reason for their drier texture.

The best evaluated in terms of appearance, cell siz uniformity, crumb tenderness, odour and taste is the control sample (WFC) followed by 5% cricket-enriched cakes (Fig. 2). Sensory analysis showed that the colour of the outer surface of WFC, $CF_5C$ and $CF_{10}C$ after baking did not differ statistically significantly, but the cell siz uniformity darkened with increasing % of CF replacement. These results are in sync with the colour lightness data (L *) (Table 4).


WFC – sponge cake containing 100% wheat flour; $CF_5C$, sponge cake with blend containing 5% cricket flour and 95% wheat flour; $CF_{10}C$- sponge cake with blend containing 10% cricket flour and 90% wheat flour.

**Figure.1.** Photographs of cross sections of the sponge cakes, and micro photograms of batters

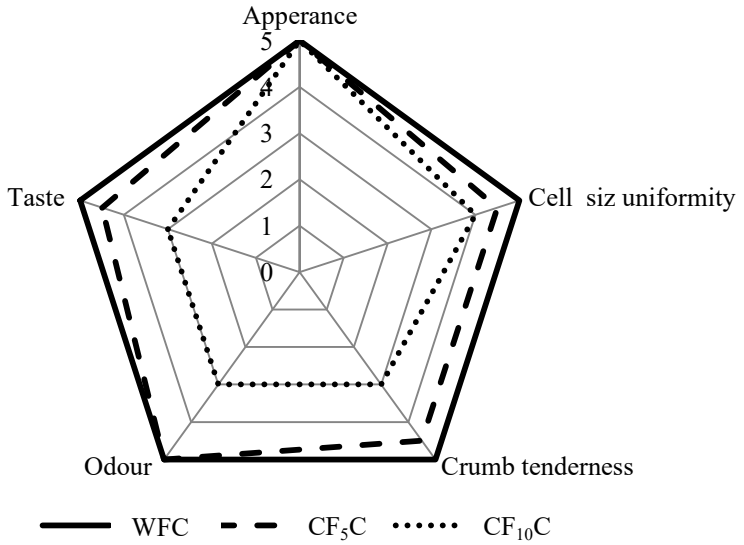


**Figure 2.** Sensory properties of sponge cakes

After partial replacement of WP with 10% CF, the cakes ($CF_{10}C$) have a visibly smaller volume (Table 3).

The most significant changes were found in the texture of $CF_{10}C$ blended sponge cakes. The results of the sensory analysis were in sync with the data obtained for shrinkage and springiness of the cakes (Table 3). The texture of cricket-based cakes was denser, with an increase in chewing hardness correlated with the amount of CF used. The sensory panel rated $CF_{10}C$ as too dry. A drier consistency has also been reported by Pauter et al., 2018 when adding CF to cakes. According to Kinyuru, Kenji, & Njoroge (2009), replacing WP with ground termite up to 5% does not affect the texture of the products. Apart from the amount, probably the type of insect flour used is another factor determining the maximum percentage used in the product. Although cricket flour does not have a strong specific aroma, $CF_{10}C$ sponge cakes have an uncharacteristic aftertaste, which is the reason for the low taste and smell grades given to $CF_{10}C$.

## 4. Conclusions

The high nutritional value of edible insects and the interest to that exotic alternative protein source played the attention of food industry as potential food ingredient. Quality characteristics of cricket flour labelled sponge cakes were strongly influenced by CF concentration. Replacement of wheat flour with 5% cricket flour had slight effect on the sensory properties of sponge cakes. Replacement with 10% cricket flour showed negative effect on colour characteristics with highest decrease in L* and a* value, as well as increase in b* value. The shrinkage, springiness and texture characteristics of cakes correlate with CF concentration and decreased significantly in 10% CF based sponge cakes. pH, moisture and $a_w$ decreased in CF blended cakes in a dose-dependent manner. In accordance to the sensory and textural characteristics of CF labelled sponge cakes the cricket flour can successfully be used up to 5% as WF substitute as innovative ingredient to enhance the nutritional value in baked products.

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# DETERMINATION OF PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITIES OF INFUSIONS PREPARED FROM LEMONGRASS (*Melissa officinalis*)

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Lemongrass (*Melissa officinalis*) contains high amount of phenolic acids, specifically rosmarinic acid. In this study, dried *Melissa officinalis* teas were prepared at different water temperatures (65, 80, 95 °C) and infusion times (60, 120, 240 s) to determine the total amounts of phenolic compounds, antioxidant activities, and the physical properties of the prepared infusions. From the result the highest total phenolics content (TPC) in ground samples was recorded in infusions prepared at 95 °C at 240 s, but no statistically significant difference ($p > 0.05$) was found between the TPC of infusions prepared at lower temperatures in the same period. It was determined that the effect of time was not significant ($p > 0.05$) during each heat application in both ground and non-ground samples except for the ground lemongrass tea samples at 80 °C. Rosmarinic acid content in the ground samples increased significantly ($p < 0.05$) due to the increase in water temperature and achieved the highest value of 19.04±0.21 mg/g when a temperature of 95 °C was applied. As the water temperature of each treatment increased, the pH values of the ground infusions decreased significantly ($p < 0.001$), with the lowest pH value of 5.51±0.01 at 95°C water temperature and 240 s infusion time samples. The effect of water temperature and infusion time on the soluble solid content of the samples was not significant ($p > 0.05$) (except non-ground sample at 120 s infusion time). The results will help future research on factors such as water temperature and infusion time, as well as grinding, to ensure that bioactive components are transferred to antioxidant-rich infusions.

## 1. Introduction

Lemongrass (*Melissa officinalis* L.) is a perennial medicinal herb from the Lamiaceae family, native to the Mediterranean. It is grown in Europe, North America, and Asia. In traditional medicine, lemongrass is widely used as a tea infusion in the treatment of gastrointestinal complaints, headaches, and fever (Shakeri, Sahebkar and Javadi, 2016). Like other Lamiaceae member plants, lemongrass contains high amount of phenolic compounds, especially rosmarinic acid, which was the predominant hydroxycinnamic acid group (Shanaida et al., 2018). These phenolic compounds contribute to the medicinal properties of lemongrass and are associated with the plant's high antioxidant capacity (Mabrouki, Duarte and Akretche, 2018). It exhibits therapeutic properties such as sedative, carminative, and anti-spasmodic effects, which is also used in the treatment of headache, rheumatism, indigestion, and hypersensitivity (Barros et al., 2013). Melissa officinalis is most consumed in the form of infusion and decoction.

During these processes, hydrophilic compounds, including flavonoids and phenolic acids, diffuse into the water and becomes lemongrass tea (Sentkowska, Biesaga and Pyrzynska, 2015). It has been reported that lemongrass teas have a significant antioxidant effect, and this is related to the amount of phenolic substances (Jiménez-Zamora, Delgado-Andrade and Rufián-Henares, 2016).

In recent years, it has been observed that the demand for medicinal and aromatic plants is increasing and preferred for human consumption to improve health status. There is a wide variety of herbal teas or tea blends that can be used for this purpose. Different techniques can be used in their preparation (such as infusion, decoction). It is known that factors such as the temperature of the water used, the particle size of the material used in tea making, the brewing time will affect the type and amount of the components that pass into the water (Castiglioni et al., 2015). Therefore, there will be differences in the bioactive properties of the tea obtained.

In this study, teas prepared at different temperatures and infusion times from dried Melissa officinalis was used. This study aimed to determine the total amounts of phenolic compounds, antioxidant activities, and the physical properties of the prepared infusions. Due to the many different applications of water temperature and infusion time used in preparing infusions such as lemongrass that people consider to be healthy, this study is important to determine the optimum water temperature and infusion time.

## 2. Materials and methods

### 2.1. Materials

Lemongrass used in the study was obtained from Afyonkarahisar Medicinal and Aromatic Plants Center which is the Department of the Turkish Ministry of Agriculture and Forest, grown and dried drog was used. Folin-Ciocalteu reagent, 1,1-dipheny l-2-picrylhydrazyl (DPPH), formic acid, gallic acid, chlorogenic acid, rosmarinic acid, transcinnamic acid, sodium carbonate, and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Preparation of Infusions

The packaged drogs (80 g) obtained from the center were first divided into two groups. Lemongrass samples in the first group were completely ground using a household grinder (Scm-2934, Sinbo). The other group was brewed without grinding. In the preparation of teas, modified methods of Golukcu et al. (2014) and Palamutoglu et al. (2018)'s have been used. Nine groups of infusions were prepared by 2 g of lemongrass samples in 200 ml distilled water for three different infusion times (60, 120, 240 s) at three different infusion temperatures (65, 80, 95 °C). At the end of the specified periods, the samples taken from the water bath and then were cooled to room temperature, and the liquid part collected by filtering through the filter paper were analyzed.

### 2.3. Soluble solid content

Soluble solid content of the infusions was determined using a hand-held refractometer (N2E/Atago, Tokyo, Japan).

### 2.4. pH

The pH of the infusion samples was determined using a pH meter (SevenGo/Metller-Toledo, OH, USA). The pH meter was calibrated before used.

### 2.5. Color

The color of the infusion was determined by measuring the CIE L *, a *, b * values using a colorimeter (Ci6X, X-Rite). The color analyzer was calibrated with white and black plates before used.

### 2.6. Determination of Total Phenolic Content

Determination of total phenolic content in methanolic extracts (1:10, v:v) obtained from lemongrass infusions was determined according to Kaur and Kapoor, (2002). Methanolic extract (0.5 ml), distilled water (7 ml), and Folin-Ciocalteu reagent (0.5 ml) were transferred to the test tube and mixed using a vortex for 3

minutes. Then, 20% sodium carbonate (2 ml) was added and mixed again. After the test tubes were kept in a water bath at 25 °C for 1 hour, the absorbances of the solutions were determined at 765 nm using a UV/Vis spectrophotometer (Optizen Pop/ Mecasys, Daejeon, Korea). Results are expressed as mg gallic acid equivalence (GAE)/100 ml tea determined by calibration curve (r2> 0.99) obtained from gallic acid (10, 20, 25, 50, 75, and 100 µg/ml).

### 2.7. Antioxidant Activity

The method of Choi et al. (2016) using DPPH radical was used in the determination of antioxidant activity. DPPH solution (0.4 ml; 96 mg/L, methanolic) was added to the methanolic extracts of teas (1.6 ml) and kept in the dark for 30 minutes. Then, the antioxidant activities (AA) were determined according to the formula below by reading the absorbances at 517 nm.

$$AA\ (\%) = [(Acontrol - Asample) / Acontrol] \times 100 \quad (1)$$

Acontrol: Absorbance of the solution using methanol instead of sample
Asample: Absorbance of the solution containing the sample

### 2.8. Determination of phenolic compounds

Phenolic compounds were determined by using High-Pressure Liquid Chromatography (HPLC) with a photodiode array detector (DAD). The modified method of Albishi et al. (2013) was used. HPLC (Ultimate 3000/ Thermo Fisher, Waltham, MA, USA) equipped with a quadruple gradient pump, degasser, autosampler, and DAD detector, 20 µl of the sample is automatically passed through the Hypersil ™ ODS-2 C18 column (250×4.6 mm, 5µm) at 30 °C and phenolic compounds determined at 300 nm. Elution solutions are A: 1% formic acid and B: 100% methanol. The samples were eluted according to the following flow pattern: up to 10 minutes 5% B, 10-15 minutes 50% B, 15-20 minutes 70% B, 20 minutes 80%, and 25 minutes 100% B. Flow rate 1.5 ml/min was applied. Standard curves (r2> 0.99) were prepared by defining the retention times of phenolic compounds (chlorogenic acid, rosmarinic acid, and trans-cinnamic acid).

### 2.9. Statistical Analysis

The analyzes were carried out in duplicate. The results were evaluated using Statistical Package for the Social Sciences (SPSS) software version SPSS 24. The effects of temperature and time on the ground and non-ground lemongrass infusions were separately determined using ANOVA. Differences between means were determined using Duncan Multiple Comparison Test.

## 3.Results and discussions

### 3.1. Soluble solid content

The soluble solid content and pH values of the infusions in which Melissa officinalis was used with and without grinding are given in Table 1. There was no statistically significant effect on soluble solid content values of teas prepared using ground lemongrass, neither the application of temperature nor the duration. It was observed that the time was not effective for each heat treatment in the unground samples. It has been determined that the non-ground infusions have more soluble substances in the samples at 95 °C in 120 s infussion time compared to the others, and the difference between 65 and 80 °C applications is not significant. In the infusions prepared with ground lemongrass, the highest brix value (3.15 ± 0.07 °Brix) was found in samples prepared at 65 °C for 120 s, and in the non-milled samples (2.55 ± 0.07 °Brix) prepared at 95 °C for 240 s. Palamutoglu et al. (2018) stated that the soluble solid content of elderflower infusions increased significantly depending on the increase in infusion time and water temperature. Dincer et al. (2008) reported that they obtained the highest soluble solid content values at 75 and 80 °C at the temperatures they used for instant mountain tea production.

### 3.2. pH

From Table 1, it was found that the pH values of the ground infusions decreased

significantly ($p<0.001$) as the water temperature of each treatment increased. Likewise, it was determined that the 240 s infussion time was very important in the samples prepared with ground lemongrass ($p<0.001$), while it was important in the other two infussion times ($p<0.01$). When the effect of time on the pH of the ground lemongrass infusions was examined, the result showed that there was a very significant difference in the 65 °C group ($p<0.01$) and significant differences ($p<0.05$) in the other groups. It has been determined that the time does not affect the 65 and 95 °C applications in the unground samples, however, there is a significant difference in the samples prepared at 80 °C. The highest and lowest pH values of teas prepared with the ground and non-ground lemongrass were determined as 6.02 ± 0.01 (65 °C, 60 s) 5.20 ± 0.01 (95 °C, 120 s), respectively. Palamutoglu et al. (2018) reported that the pH values of the samples decreased significantly due to the increase in temperature and time in the preparation of elderflower infusions.

**Table 1**. Soluble solid content and pH values of lemongrass infusions

| | | | Time (s) | | | Sig.[1] |
|---|---|---|---|---|---|---|
| | | Temperature (°C) | 60 | 120 | 240 | |
| Ground | Soluble solid content (°Brix) | 65 | 2.90±0.14 | 3.15±0.07 | 3.00±0.00 | ns |
| | | 80 | 2.95±0.07 | 3.05±0.07 | 3.00±0.00 | ns |
| | | 95 | 3.00±0.00 | 3.00±0.14 | 3.10±0.00 | ns |
| | | Sig.[2] | ns | ns | ns | |
| | pH | 65 | 6.02±0.01$^{aA}$ | 5.98±0.01$^{bA}$ | 5.88±0.01$^{cA}$ | ** |
| | | 80 | 5.66±0.02$^{bB}$ | 5.72±0.00$^{aB}$ | 5.63±0.02$^{bB}$ | * |
| | | 95 | 5.58±0.01$^{aC}$ | 5.53±0.01$^{bC}$ | 5.51±0.01$^{bC}$ | * |
| | | Sig.[2] | *** | *** | *** | |
| Non-Ground | Soluble solid content (°Brix) | 65 | 2.25±0.07 | 2.20±0.00$^{B}$ | 2.35±0.07 | ns |
| | | 80 | 2.15±0.07 | 2.15±0.07$^{B}$ | 2.35±0.07 | ns |
| | | 95 | 2.35±0.07 | 2.45±0.07$^{A}$ | 2.55±0.07 | ns |
| | | Sig.[2] | ns | * | ns | |
| | pH | 65 | 5.75±0.01$^{A}$ | 5.69±0.06$^{A}$ | 5.82±0.01$^{A}$ | ns |
| | | 80 | 5.64±0.01$^{aB}$ | 5.59±0.00$^{bA}$ | 5.64±0.01$^{aB}$ | * |
| | | 95 | 5.29±0.04$^{C}$ | 5.20±0.01$^{B}$ | 5.26±0.01$^{C}$ | ns |
| | | Sig.[2] | ** | ** | *** | |

Sig.[1]: Statistical significance of the effect of time, Sig.[2]: Statistical significance of the effect of temperature
[a-c]: The difference between the averages given in different letters in the same row is statistically significant.
[A-C]: The difference between the averages given with different letters in the same column is statistically significant.
ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

### 3.3. Color

The effects of different water temperature and time applications on the color values of lemongrass infusions are given in Table 2.

Table 2 shows that infusion time at 65 °C had a significant ($p<0.05$) effect on L*, but infusion times at higher infusion temperatures had no such effect. For non-ground samples, the effect of time on samples infused at 80 °C was statistically significant ($p<0.05$), and it was more significant ($p<0.01$) at the other two infusion temperatures. The temperature of the infusion had no effect on the treatment of ground samples for 240 s, whereas samples infused for

60 and 120 s were significantly ($p<0.05$ and $p<0.001$ respectively) affected by temperature. In non-ground samples, the effect of infusion temperatures on L* value at different infusion times was found to be significantly different.

**Table 2.** Color values of lemongrass infusions

| | | | Time (s) | | | Sig.[1] |
|---|---|---|---|---|---|---|
| | | Temperature (°C) | 60 | 120 | 240 | |
| Ground | *L** | 65 | 20.09±0.55$^{bB}$ | 18.63±0.04$^{bC}$ | 23.12±1.01$^{a}$ | * |
| | | 80 | 25.05±0.37$^{A}$ | 23.77±0.51$^{B}$ | 25.01±0.75 | ns |
| | | 95 | 24.92±1.11$^{A}$ | 25.64±0.18$^{A}$ | 23.53±0.00 | ns |
| | | Sig.[1] | * | *** | ns | |
| | *a** | 65 | 5.41±0.15$^{b}$ | 5.14±0.21$^{b}$ | 8.40±0.28$^{aA}$ | ** |
| | | 80 | 4.92±0.41 | 5.41±0.47 | 6.53±0.61$^{B}$ | ns |
| | | 95 | 3.95±0.93 | 3.82±0.91 | 2.97±0.18$^{C}$ | ns |
| | | Sig.[1] | ns | ns | ** | |
| | *b** | 65 | 12.22±0.25$^{b}$ | 9.43±0.95$^{cB}$ | 15.19±0.34$^{aA}$ | ** |
| | | 80 | 16.52±0.05$^{a}$ | 14.56±0.51$^{bA}$ | 15.23±0.28$^{bA}$ | * |
| | | 95 | 13.93±1.94 | 14.71±0.91$^{A}$ | 11.33±0.20$^{B}$ | ns |
| | | Sig.[1] | ns | * | ** | |
| Non-Ground | *L** | 65 | 35.54±0.53$^{aA}$ | 32.11±0.42$^{bA}$ | 31.70±0.26$^{bA}$ | ** |
| | | 80 | 32.24±0.18$^{aB}$ | 28.99±0.14$^{bC}$ | 28.87±0.82$^{bB}$ | * |
| | | 95 | 27.94±0.07$^{bC}$ | 30.27±0.37$^{aB}$ | 26.07±0.23$^{cC}$ | ** |
| | | Sig.[1] | *** | ** | ** | |
| | *a** | 65 | -0.16±0.06$^{AB}$ | -0.09±0.09 | -0.08±0.06$^{B}$ | ns |
| | | 80 | -0.34±0.16$^{B}$ | -0.23±0.03 | -0.22±0.12$^{B}$ | ns |
| | | 95 | 0.14±0.00$^{A}$ | 0.31±0.35 | 0.97±0.19$^{A}$ | ns |
| | | Sig.[1] | * | ns | ** | |
| | *b** | 65 | 7.84±0.37$^{A}$ | 8.05±0.20$^{B}$ | 9.75±1.21$^{AB}$ | ns |
| | | 80 | 4.83±0.42$^{bB}$ | 4.77±0.21$^{bC}$ | 6.66±0.40$^{aB}$ | * |
| | | 95 | 7.77±0.18$^{bA}$ | 12.35±0.96$^{aA}$ | 11.67±1.22$^{aA}$ | * |
| | | Sig.[1] | ** | ** | * | |

Sig.[1]: Statistical significance of the effect of time, Sig.[2]: Statistical significance of the effect of temperature
$^{a-c}$: The difference between the averages given in different letters in the same row is statistically significant.
$^{A-C}$: The difference between the averages given with different letters in the same column is statistically significant.
ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

### 3.4. Total Phenolic Content

Total phenolic content of ground lemongrass teas are given in Figure 1 and antioxidant activity values are given in Figure 2. The highest total amount of phenolic matter in ground samples was determined in teas prepared at 95 °C at 240 s, but no statistically significant difference was found between the phenolic content of teas prepared at lower temperatures in the same period. It was determined that it was significantly lower in samples prepared in the same period but at lower temperatures. Shanaida et al. (2018) determined the total amount of phenolic substance as 29.37-68.35 mg gallic acid equivalent /g dry sample in their infusion studies with 3 species from the Lamiaceae family. Except for the samples infused at 80 and 95 °C, the results of the total phenolic content analysis reported in Shanaida et al. (2018) research were similar in our research. It's possible that the variation is related to the herbal material used, as well as the infusion treatment method (such as time, temperature, grinding degree).

Mabrouki et al. (2018) found the highest amount of phenolic matter in ethanol extract in their studies on the effects of samples extracted using different solvents(ethanol, acetone,

hexane) from lemongrass on the total amount of phenolic matter and antioxidant capacity.

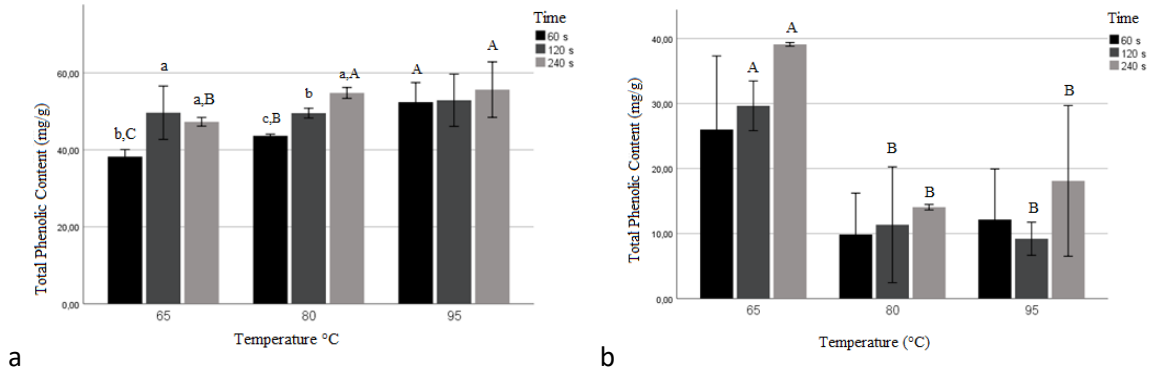


**Figure 1**. Total phenolic amount of *M. officinalis* infusions a. ground and b. non-ground
[a-c]: The difference between the averages given in different letters at the effect of time is statistically significant.
[A-C]: The difference between the averages given with different letters at the effect of temperature is statistically significant.

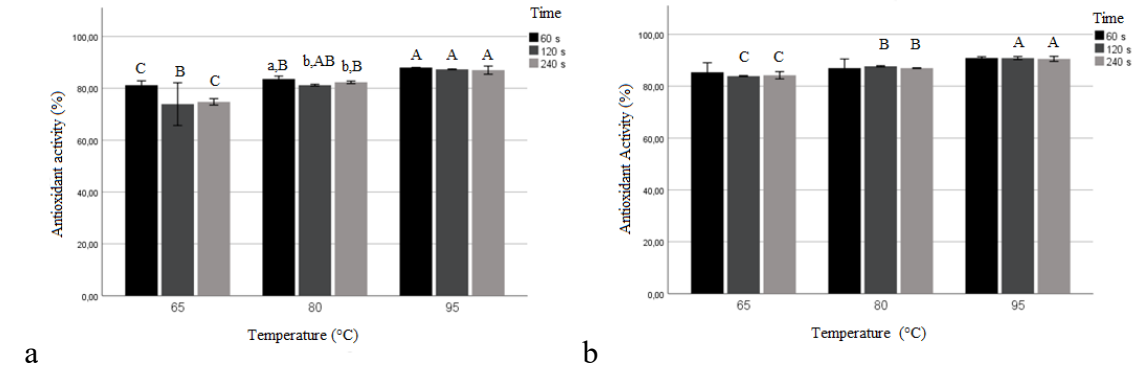


**Figure 2.** Antioxidant activities of unmilled *M. officinalis* infusions a. ground and b. non-ground
[a-c]: The difference between the averages given in different letters at the effect of time is statistically significant.[A-C]: The difference between the averages given with different letters at the effect of temperature is statistically significant.

When compared to ethanolic extracts, Papoti et al. (2019) found that aqueous lemongrass extracts showed the highest total phenolic content value and antioxidant activity. In addition, Papoti et al. (2019) found that lemongrass infusions had more phenolic compounds than chamomile and olive leaf infusions, and that 2% of infusions could be preferred in terms of sensory characteristics and nutritional qualities.

### 3.5. Antioxidant Activity

From Figure 2, it was determined that the differences between the mean levels of the antioxidant activities of the ground and non-ground lemongrass teas increased due to the increase in the temperature during each infusion time were statistically significant, except for the group prepared from unground lemongrass for 60 s. Except for the ground lemongrass tea samples at 80 ° C, it was determined that the effect of time was not significant during each water temperature in both ground and non-ground samples.

Barros et al. (2013) reported based on the literature that the antioxidant activity of lemongrass is mainly due to rosmarinic acid. However, they stated that the synergistic interaction between other antioxidant active compounds in the samples should not be neglected.

**Table 3.** Chlorogenic, rosmarinic and transcinnamic acid values of lemongrass infusions

| | | | Time (s) | | | Sig.[1] |
|---|---|---|---|---|---|---|
| | | Temperature (°C) | 60 | 120 | 240 | |
| Ground | Chlorogenic acid (mg/g) | 65 | 7.31±0.73$^{B}$ | 12.15±3.49 | 10.75±0.47$^{B}$ | ns |
| | | 80 | 12.58±0.90$^{A}$ | 14.06±0.67 | 13.02±1.07$^{B}$ | ns |
| | | 95 | 13.78±0.03$^{A}$ | 16.46±1.48 | 16.98±0.94$^{A}$ | ns |
| | | Sig.[1] | ** | ns | * | |
| | Rosmarinic acid (mg/g) | 65 | 6.80±0.44$^{C}$ | 8.77±1.80$^{B}$ | 8.73±0.48$^{B}$ | ns |
| | | 80 | 11.44±0.68$^{bB}$ | 11.73±0.84$^{bB}$ | 14.58±0.55$^{aA}$ | * |
| | | 95 | 16.61±0.62$^{bA}$ | 19.04±0.21$^{aA}$ | 16.36±0.74$^{bA}$ | * |
| | | Sig.[1] | ** | ** | ** | |
| | Trans-cinnamic acid (mg/g) | 65 | 0.08±0.00$^{B}$ | 0.10±0.03 | 0.12±0.00$^{A}$ | ns |
| | | 80 | 0.14±0.02$^{aA}$ | 0.01±0.01$^{b}$ | 0.18±0.01$^{aA}$ | ** |
| | | 95 | 0.05±0.00$^{B}$ | 0.09±0.11 | 0.04±0.03$^{B}$ | ns |
| | | Sig.[1] | ** | ns | * | |
| Non-Ground | Chlorogenic acid (mg/g) | 65 | 3.31±1.05$^{B}$ | 3.87±0.76$^{B}$ | 4.28±0.25$^{B}$ | ns |
| | | 80 | 2.30±0.17$^{bB}$ | 2.53±0.02$^{bB}$ | 4.20±0.10$^{aB}$ | ** |
| | | 95 | 6.74±0.75$^{bA}$ | 9.17±1.12$^{abA}$ | 10.62±0.56$^{aA}$ | * |
| | | Sig.[1] | * | ** | ** | |
| | Rosmarinic acid (mg/g) | 65 | 3.24±0,52 | 3.56±0.85 | 3.98±0.27 | ns |
| | | 80 | 2.08±0.06$^{b}$ | 2.21±0.09$^{b}$ | 3.95±0.05$^{a}$ | *** |
| | | 95 | ND | ND | ND | |
| | | Sig.[1] | ns | ns | ns | |
| | Trans-cinnamic acid | 65 | 0.02±0.01$^{A}$ | 0.02±0.01 | 0.04±0.00 | ns |
| | | 80 | 0.001±0.00$^{Bb}$ | 0.001±0.00$^{b}$ | 0.03±0.00$^{a}$ | *** |
| | | 95 | 0.03±0.00$^{A}$ | 0.02±0.03 | 0.02±0.03 | ns |
| | | Sig.[1] | * | ns | ns | |

Sig.[1]: Statistical significance of the effect of time, Sig.2: Statistical significance of the effect of temperature
a-c: The difference between the averages given in different letters in the same row is statistically significant.A-C: The difference between the averages given with different letters in the same column is statistically significant.
ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ND: not detected.

Akowuah and Zhari (2010) determined the antioxidant activities of the main polyphenols and extracts from the leaves (dried, ground) of the Lamiaceae family. According to their study the levels of these polyphenolic compounds at the extraction temperature of 40 °C were statistically significantly higher than those obtained at 60 °C and above extraction temperatures. So the antioxidant activities of the extracts had significantly higher radical scavenging activity (DPPH) at low temperatures. Researchers have reported that polyphenolic compounds are not stable compounds, but degradation reactions occur at high temperatures. They reported that when the temperature is 60 °C and above, polyphenol oxidase can be activated and rosmarinic acid and sinensetin may be degraded (Akowuah and Zhari, 2010).

Rosmarinic acid was not detected at 95 °C infusions of nonground samples in our study, which is similar to the findings of others. From this perspective, it is possible to conclude that the cells in the ground samples have physically disintegrated, the polyphenol oxidase enzyme has been considered unusable by the diffusion, and rosmarinic acid has not been broken down.

Mabrouki et al. (2018) stated that DPPH radical scavenging activity increased due to the increase in the concentration of extracts.

From Figure 2, the samples infused at 95 °C in both groups had the highest antioxidant activity. Although rosmarinic acid has a significant effect on the antioxidant activities of infusions, other components can also be considered effective, according to Mabrouki et al. (2018) and Barros et al. (2013).

### 3.6. Phenolic compounds

From Table 3, it was observed that the amounts of chlorogenic, rosmarinic, and trans-cinnamic acid in lemongrass teas prepared by grinding were higher than the teas prepared without grinding, as in the results of total phenolic content.

The highest amount of chlorogenic acid was determined in teas prepared from ground lemongrass in samples prepared at 95 °C for 240 s. However, the effect of time on this temperature value was found to be statistically insignificant. A statistically significant increase was observed in teas infused for 60 and 240 s with the increase in temperature. Likewise, they reported that antioxidant activities had significantly higher radical scavenging activity (DPPH) at low temperatures. Researchers have reported that polyphenolic compounds are not stable compounds, but degradation reactions occur at high temperatures. They reported that when the temperature is 60 °C and above, polyphenol oxidase can be activated and rosmarinic acid and senisteine may be degraded.

Barros et al., (2013) reported based on the literature that the antioxidant activity of lemongrass is mainly due to rosmarinic acid. However, they stated that the synergistic interaction between other antioxidant active compounds in the samples should not be neglected.

It was determined that the differences between the mean levels of the antioxidant activities of the ground and non-ground lemongrass teas increased due to the increase in the temperature during each holding period were statistically significant, except for the group prepared from unground lemongrass for 60 s. Except for the ground lemongrass tea samples at 80 ° C, it was determined that the effect of time was not significant during each heat application in both ground and non-ground samples. Mabrouki, Duarte and Akretche, (2018) stated that DPPH radical scavenging activity increased due to the increase in the concentration of extracts.

When Table 3 was examined, it was observed that the amounts of lemongrass teas prepared by grinding the chlorogenic, rosmarinic, and trans-cinnamic acid contents were higher than the teas prepared without milling, as in the results of total phenolic content.

The highest amount of chlorogenic acid was determined in teas prepared from ground lemongrass in samples prepared at 95 ° C for 240 h. However, the effect of time on this temperature value was found to be statistically insignificant. A statistically significant increase

was observed in teas infused for 60 and 240 s with the increase in temperature. It was determined that the chlorogenic amount decreases when the temperature rises from 65 ° C to 80 ° C in the unground samples and increases when it is increased to 95 ° C. The amount of chlorogenic acid increased due to the increase in time in teas brewed at 80 and 95 ° C. The difference between the averages was found to be insignificant in the samples applied at 65 ° C.

When the rosmarinic acid content was examined, the effects of the time and temperature applications were statistically seen at different degrees of significance, while rosmarinic acid was not detected in any of the samples when a temperature of 95 ° C was applied to the non-ground samples.

Shanaida et al., (2018) reported that the most common phenolic compound in samples of the Lamiaceae family is rosmarinic acid and its amount is at the level of 3.64-5.28 mg / g dry weight.

According to the results of our study, the amount of rosmarinic acid in the ground samples increased significantly due to the increase in temperature at each time application. When 95 °C temperature is applied, it is seen that the highest value is obtained for 120 s. It should be considered that deterioration may occur due to the high temperature due to the extension of the time. In addition, the effect of the polyphenol oxidase enzyme may have lost its effect due to direct exposure to heat due to the cell structure that is broken down due to grinding. Therefore, an increase was observed in the amount of total phenolic substance and rosmarinic acid due to the increase in temperature. The total phenolic substance and rosmarinic acid amounts of the teas obtained from the ground lemongrass vary. The total phenolic amount decreases when the temperature rises from 65 °C to 80 °C. No significant difference was found between teas brewed at 95 °C and 80 °C. These differences in teas prepared from unground lemon herbs may be primarily due to the inhomogeneity of the samples. Because of the negative changes in the permeability of the cell wall due to the increase in temperature in these samples and the low solvent contact surface, the extraction of phenolic compounds decreases. At the same time, it is thought that with the slow conduction of temperature, the temperature in the plant tissue may stay longer in the optimum temperature range for the polyphenol oxidase enzyme and decompose rosmarinic acid.

**Table 4.** Correlations between some parameters of ground lemongrass infusions

| | Brix | pH | TPM | DPPH | Chlorogenic acid | Rosmarinik acid | Trans-cinnamic acid |
|---|---|---|---|---|---|---|---|
| Temperature | 0.076 | -0.943** | 0.635** | 0.893** | 0.791** | 0.942** | -0.293 |
| Brix | | -0.046 | 0.501* | -0.147 | 0.424 | 0.094 | -0.355 |
| pH | | | -0.687** | -0.800** | -0.812** | -0.924** | 0.097 |
| TPM | | | | 0.303 | 0.824** | 0.753** | -0.147 |
| AA | | | | | 0.471* | 0.764** | -0.275 |
| Chlorogenic acid | | | | | | 0.835** | -0.249 |
| Rosmarinic acid | | | | | | | -0.108 |

*: $p<0.05$, **: $p<0.01$

Therefore, the time we used is thought to be very low compared to the infusion times in our study. For this reason, it is thought that the total amount of phenolic substance that can be extracted may have been low. Likewise, Sentkowska, Biesaga and Pyrzynska (2015) reported that the phenolic content and antioxidant capacities of teas prepared with the decoction method were higher than those obtained by the infusion method in their study comparing lemongrass infusions and decoctions. In their study, they applied the infusion and decoction times as 10, 15, and 20 minutes. However, they reported that phenolic compounds such as rutin, quercetin and myricetin were in higher amounts in the infused samples.

**Table 5.** Correlations between some parameters of non-ground lemongrass infusuions

| | Brix | pH | TPM | DPPH | Chlorogenic acid | Rosmarinik acid | Trans-cinnamic acid |
|---|---|---|---|---|---|---|---|
| Temperature | 0.546* | -0.945** | -0.738** | 0.955** | 0.724** | -0.514 | -0.043 |
| Brix | | -0.608** | -0.050 | 0.487* | 0.866** | 0.797** | 0.321 |
| pH | | | 0.612** | -0.930** | -0.828** | 0.653* | -0.030 |
| TPM | | | | -0.664** | -0.181 | 0.623* | 0.436 |
| AA | | | | | 0.698** | -0.604* | -0.035 |
| Chlorogenic acid | | | | | | 0.961** | 0.308 |
| Rosmarinic acid | | | | | | | 0.961** |

*: $p<0.05$, **: $p<0.01$

## 4. Conclusions

In this research, the effects of temperature and time conditions on lemongrass infusions were determined. Although the effect of the grinding degree was not the subject of this study, the results give an idea that the composition of the infused components of the grinding degree may change. Results showed that ground lemongrass was infused at different temperatures for a different infusion time, more rosmarinic acid passed into the water. The amount of rosmarinic acid passed into the water was lower in non-ground samples and could not be detected at the infusions of the highest temperature treated samples. According to the antioxidant activity data, the effects of varied infusion times on the antioxidant activity of samples infused at different temperatures were not significant in general. Therefore, in the future, studies on the effect of grinding degree on infusion can be conducted taking this situation into consideration. The results showed that the amount of rosmarinic acid decrease due to the increase in the infusion temperature in the ground samples. For this reason, studies can be conducted to determine the effect of optimum grinding degree, infusion temperature, and infusion time for the optimum activities of bioactive components that are infused.

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# ANTIOXIDANT AND A-AMYLASE INHIBITION ACTIVITY OF *RUTA CHALEPENSIS* L EXTRACTS

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**ABSTRACT**

In the present study total phenolic compounds (TPC), Total flavonoids content (TFC), α-amylase inhibitory activity, and antioxidant activity were measured by the DPPH test of methanol and ethyl acetate extracts of the leaves and flowers of *Ruta chalepensis* L were evaluated. The extraction yield using methanol for the flower and leaves were about 25%, while those for ethyl acetate were about 3.4%. TPC of the methanol extracts for the flowers and leaves of the Ruta was around 1150 mg GAE /100 g dried Ruta, while TPC of ethyl acetate extract of the Ruta leaves and flowers were 760 and 290 mg GAE /100 g dried Ruta. The methanolic extracts of Ruta leaves and flowers exhibited the strongest DPPH radical scavenging activity. The IC50 for both extracts were about 12 mg TPC/mL). However, the ethyl acetate extract of flowers showed the lowest DPPH radical scavenging activity (IC50 = 96.7 mg TPC/ ml) and it was significantly different than that of leaves (IC50 = 62 mg TPC/ml). The inhibitory effect of methanolic extracts of leaves on the α-amylase was the lowest (42.2%) followed by ethyl acetate of flowers (53.9%). Whereas, the ethyl acetate extract of leaves showed the highest inhibitory effect against α- amylase (63.7%) followed by methanolic extract of flowers (57.9%). The results obtained in this study clearly indicate that R. *chalepensis* L has a significant potential to use as a natural antioxidant as well as an antidiabetic agent.

## 1. Introduction

The use of different types of synthetic antioxidants such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxy Toluene (BHT) in the food industry has raised many potential risks, and recent studies have shown the susceptibility to different types of cancer when using these antioxidants, thus recent research is moving towards the use of natural sources of antioxidants (Li et al., 2014). Moreover, there is a global interest in the use of herbs and aromatic plants in food preservation and in folk medicine (Christaki et al., 2012). Recent research has focused on the properties and characteristics of the extracts of the aromatic plants as well as their essential oils, as they resemble and acquire antimicrobial in addition to antioxidant activities (Chouhan et al., 2017). Antioxidant and antimicrobial activities of aromatic plants are attributed to many potent compounds, including flavonoids, eugenol, coumarins, carvacrol, and cinnamaldehyde (Khameneh et al., 2019). Active research has grown rapidly to look for more effective and safer plant-based hypoglycemic compounds (Saleh et al., 2013), despite the fact that these plants have been used since ancient times (Subbulakshmi and Naik, 2011). Many drugs used nowadays are from plant sources, as they

are considered as their primary sources, for example, the plant Galega officinalis is the primary source of glucophage (metformin), which is a hypoglycemic drug (Kumar and Nandi, 2017).

*Ruta graveolens* L, *Ruta chalepensis* L, and *Ruta montana* L are species from Ruta, which is a genus of the Rutaceae family (Pollio et al., 2008). *Ruta graveolens* L (also known as garden Rue)is a dicot herb grown mainly in many parts of the world, native to the Mediterranean region(including Jordan), it is also grown in southern Europe, northern Africa, India, and other tropical regions (Asgarpanah and Khoshkam, 2012), it's used by different populations (including the Jordanian population) for many beneficial purposes, especially in traditional medicine for its antirheumatic activity, analgesic and antispasmodic effects (Soare et al., 1997), it is also used as anticancer, anti colic, antiseptic, abortifacient, anthelmintic and antihypertensive (Ahmad et al., 2010).

Its aerial parts are mainly used in Jordan for locally produced ghee to enhance its color and flavor (Ahmad et al., 2010).

This study aimed at investigating the total phenolic content of methanol and ethyl acetate extracts of the leaves and flowers of *Ruta chalepensis* L., their antioxidant activity, and their effect on the activity of α-amylase used in the hydrolysis of starch. health has been determined. Yoghurt, which is suitable for lactose intolerant individuals, is also easy to digest (Dewit, 2010; Pochart and Desjeux, 1988).

## 2. Materials and methods

### 2.1. Materials

The plant material of the present study *Ruta chalepensis* L. was collected from the north region of Jordan (Bani Kenana district) and purchased in the spring season of 2018. Folin-Ciocalteu Reagent and sodium was from AppliChem, GmbH (Darmstadt, Germany). 2-chloro-p-nitrophenyl-α-D-maltotrioside, Quercetin, Gallic acid, α-Amylase, and Aluminum Trichloride were from Sigma-Aldrich (Steinheim, Germany), 2,2-Diphenyl-1-picrrylhydazyl was from (ICN, Biomedical INC, USA). Sodium carbonate was from Merck (Darmstadt, Germany). The used solvents were of HPLC grade.

#### *2.2.1. Preparation of methanol and ethyl acetate extracts of leaves and flowers of Ruta chalepensis L.*

The fresh leaves and flowers of *Ruta chalepensis* L were dried in an electrical oven at 40ºC. The dried samples were grinded using a domestic coffee grinder. Four extracts were prepared by boiling 10 g of the grinded leaves and flowers in 100 ml of ethanol or ethyl acetate. The methanol and ethyl acetate extracts were filtered in a 250 and 100 ml volumetric flask, respectively and the volume was made to the mark with the corresponding solvent.

#### *2.2.2. Determination of the yield of extracts*

To determine the yield of extraction, 20 ml (in duplicates) from each extract was placed in a previously weighed Petri dish, and the extract was evaporated at 80ºC in an oven for 3 hrs.

#### *2.2.3 Determination of Antioxidants*

*2.2.3.1. Determination of total phenolic compounds (TPC)*

The phenolic compounds in the 4 extracts of the ground flowers and leaves of *Ruta chalepensis* L were determined by the Folin-Ciocalteu reagent (FCR) according to the method of Al-Ismail *et al* (2006). Briefly, 0.1 ml of methanol extracts (10 mg/ml), ethyl acetate extracts (3.3 mg /ml), and standard solution (gallic acid) were mixed with 0.5 ml of Folin-Ciocalteau reagent. After 3 min, 2 ml of 10% (w/v) of sodium carbonate solution was added. The final mixture was shaken and then incubated for 1 h in dark at room temperature. The absorbance of all samples was measured at 650 nm using a spectrophotometer (Labomed spectrophotometer, model UVD-2900, Labomed, USA). and the results are expressed in mg gallic acid equivalents (GEA) per 100g dry weight of plant material

#### *2.2.3.2. Determination of total flavonoid content (TFC)*

The content of flavonoids in the 4 extracts of the ground flowers and leaves of *Ruta*

*chalepensis* L were determined according to the method reported by Miliuskas *et al*., (2004). 0.5 ml from each extract or standard (quercetin) was mixed with 1 ml of 2 % aluminum trichloride in ethanol solution; the mixture was diluted with water into a 25-mL volumetric flask and allowed to stand for 40 min at room temperature. The absorbance of the sample was then measured at 415 nm using a spectrophotometer (Labomed spectrophotometer, model UVD-2900, Labomed, USA). Flavonoids content was expressed in mg of quercetin equivalent (QE) per 100g dry weight of plant material

***2.2.3.3. Determination of Diphenyl-1-picrryylhydazyl (DPPH) free radical scavenging activity of the extracts.***

DPPH radical scavenging effect was determined according to the method of Al-Ismail *et al* (2006). A 0.2 ml of ethanol solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl) (50 mg/ 100 ml) was mixed with different levels of each extract, and the mixture was brought to a total volume of 4.0 ml with the corresponding extracting solvent. The mixture was mixed thoroughly and was allowed to stand for 45 min in a dark place. The absorbance was then measured at 515 nm and the radical scavenging activity of the tested samples was expressed as %inhibition according to the following formula (Brand-Williams *et al*., 1995),

Inhibition (%) = [(Abs. control –Abs. sample)/ Abs. control] x 100

$IC_{50}$ is the concentration of extract in mg/ml needed to scavenge 50% of the DPPH radical, which was calculated from their concentration-response curves.

***2.2.3.4. Determination of α-amylase inhibitory activity by CNP-G3 Assay of the extracts.***

According to the method of Suganuma, et al. (1997), the release of 2-chloro-4-nitrophenol (CNP) from CNP-G3 by porcine pancreas α-amylase was determined, where a 450 µl reaction mixture containing a solution of 0.2 M potassium thiocyanate and 0.15 mM CNP-G3 dissolved in 0.05 M phosphate buffer solution with a pH of 7.0 and an aqueous solution of the extract at a concentration of 3.3 mg/ml was pre-incubated for 5 minutes at 25ºC, which was followed by the addition of a volume of 20 µl of a freshly prepared α-amylase solution (1 mg/ml) in a phosphate buffer with a pH of 7.0.

During the reaction, the absorbance of the mixture was read at 405nm, and the increase in the absorbance determined the release of CNP from CNP-G3as follows,

The inhibitory activity% = [(A-B)/A] x 100

Where A is the increase in absorbance during the reaction when the extract is absent, and B is the increase in absorbance during the reaction when the extract is present.

## 2.3. Statistical Analysis

Statistical analysis was performed using (SPSS for Windows, Rel. 22.0, 2013, Chicago, SPrSS Inc.). Data were presented as mean ± SD, Significance was tested by ANOVA at *P*-value ≤0.05, and mean differences were determined by Duncan's multiple range test.

## 3. Results and discussions

### 3.1. Extraction yield

There are many steps to obtain phytochemicals from plants such as milling, grinding, homogenization, and extraction. Among these steps, extraction is the main step for recovering and isolating phytochemicals from plant materials (DO et al. 2014). In this study flowers and leaves of *Ruta graveolens* extracts were obtained by using methanol and ethyl acetate. Extraction yields of leaves and flowers using methanol (ca 25% w/w) were significantly greater than the corresponding extract using ethyl acetate (ca 3.4% w/w) (Table 1). It can be concluded that the more polar solvent will produce more extraction yield. The higher yield of methanol extract could be due to compounds other than phenolic, such as pigments, carbohydrates, and proteins, that may have been extracted by methanol (Zieli_nski and Kozłowska, 2000). The results of this study agree with the extraction yields of *Limnophila aromatic* (Do, 2014) and some medicinal plants (Sultana et. al. 2009).

### 3.2. *Total polyphenolic compounds (TPC) and flavonoids contents (TFC)*

Table 1 shows the TPC of the extracts measured using Folin Ciocalteu method. TPC of the methanol extracts of the flowers and leaves of the *Ruta* were 1175 and 1131 mg Gallic acid equivalent/100 g dried sample and they were not significantly different (P> 0.05). Whereas TPC of the ethyl acetate extract of *Ruta* leaves was significantly ($p \leq 0.05$) greater than that of flowers. The TPC of ethyl acetate extract of the leaves was 2.5 times greater than those of the ethyl acetate extract of flowers. The methanol extract of leaves and flowers contains more phenolic content as compared to the corresponding ethyl acetate extracts. The TPC of methanol extracts (Leaves or flowers) were about 4 and 1.6 times greater than those of ethyl extracts of leaves and flowers, respectively. It was observed that the effect of solvents on TFC is similar to that of TPC (Table 1). The highest TFC was obtained in the methanol extracts, while ethyl acetate showed lower efficiency in extracting flavonoids. Furthermore, the amount of TFC of the methanol extract of flower (409 mg/100g) was slightly but significantly greater than that of leaves (383 mg/100g. However, no significant difference in TFC between *Ruta* flowers or leaves was found. The TFC in the ethyl extracts was low when compared to TPC. The results indicated that solvent polarity plays a vital role in increasing phenol solubility (Sultan et al. 2009). These results indicate that the polarity of the solvent used in the extraction influences extracting of different phenolic compounds from the leaves and flowers of *Ruta.* The TPC of the methanolic extract of *Ruta chalepensis* leaves in this study was comparable to those reported by Ouerghemmi et al (2017) (1190.6 mg GAE/100 g), while those of the flowers were slightly greater (1688 GAE mg/ 100g. Mohammad et al. (2015) reported that there was no effect of methanol and ethyl acetate on the extraction level of polyphenols from *Ruta chalepensis* grown in Tunisia. On contrary to that, Athmouni *et al*. (2015) reported that the methanolic extract of *Scorzonera undulata* (*Asteraceae*) exhibited higher TPC than its ethyl acetate extract.

**Table 1.** The yield of extraction, total phenolic content (TPC), and total flavonoids (TFC) of the methanolic and ethyl acetate extracts of leaves and flowers of *Ruta chalepensis L*

| Extract | Yield% | TPC (mg GAE /100 g dried Rutta) | (TFC) (mg /100 g dried Rutta) |
|---|---|---|---|
| Ethyl acetate flowers | $3.2 \pm 0.1^{b}$ | $290 \pm 5^{c}$ | $22.6 \pm 1.1^{c}$ |
| Ethyl acetate leaves | $3.4 \pm 0.2^{b}$ | $726 \pm 13^{b}$ | $29.6 \pm 1.4^{c}$ |
| Methanol flowers | $25.8 \pm 0.6^{a}$ | $1175 \pm 46^{a}$ | $409 \pm 12^{a}$ |
| Methanol leaves | $25.4 \pm 0.8^{a}$ | $1131 \pm 17^{a}$ | $393 \pm 13^{b}$ |
| • Different superscript within the same column are significantly different ($P \leq 0.05$) | | | |

### 3.3. *Antioxidant activity*

Due to the presence of different antioxidant components in the crude extract and the complexity of the oxidation–antioxidation processes, no single testing method can provide a comprehensive picture of the antioxidant profile of a given sample. Several assay methods have been developed and applied to screen and evaluate the total antioxidant activity of plant extracts (Prabhakar et al., 2006). In this study, the antioxidant activity of *R. chalepensis* L. extracts have been determined by α-diphenyl-β-picrylhydrazyl (DPPH). DPPH radical is a stable organic free radical with an absorption band at 517 nm. It loses this absorption when accepting an electron or a free radical species, which results in a visually noticeable discoloration from purple to yellow. The DPPH activity was expressed by $IC_{50}$. The $IC_{50}$ of a compound is inversely related to its antioxidant activity, as it expresses the amount of antioxidant required to decrease the DPPH concentration by 50%, which is obtained by 50%, which is obtained by interpolation from linear regression analysis (Liu et al. 2009). A lower $IC_{50}$ indicates a higher antioxidant activity of a compound. Table 2 shows the $IC_{50}$ values of the DPPH radical scavenging activity assay of the extracts. It was found that the methanolic extracts of *Ruta* leave and flowers possess the strongest DPPH radical activity ($IC_{50}$ = around 0.0125 mg TPC/mL) and no significant difference between them. However, the ethyl acetate extract of flowers showed the lowest DPPH radical activity (IC50 = 0.242mg TPC/ ml) and it was significantly different than that of leaves ($IC_{50}$ = 0.155 mg TPC/ml). Mohammad et al (2015) reported that methanolic extract *of R.* graveolens *L* showed higher DPPH activity than that of ethyl acetate extract which goes parallel with the results of this study. Generally, extracts that contain a high value of polyphenols exhibit high antioxidant activity (Srivastav *et al*., 2015).

**Table 2.** DPPH scavenging activity evaluated by IC50 and inhibition% of α-Amylase activity of the methanolic and ethyl acetate extracts of leaves and flowers of *Ruta chalepensis* L

| Extract | $IC_{50}$ | | Inhibition% of α-Amylase activity |
|---|---|---|---|
| | mg TPC /ml | µl extract solution | |
| Ethyl acetate flowers | 0.242 ±0.011 | 832 ± 6.4[a] | 53.9 ± 1.3[c] |
| Ethyl acetate leaves | 0.155 ± 0.013 | 213 ±4.9[b] | 63.7± 1.2[a] |
| Methanol flowers | 0.013± 0.006 | 27.1 ± 2.0[c] | 57.9 ±0.6[b] |
| Methanol leaves | 0.012± 0.006 | 25.7 ± 1.0[c] | 42.2 ± 1.9[d] |
| *Different superscript within the same column is significantly different (P≤0 .05) | | | |

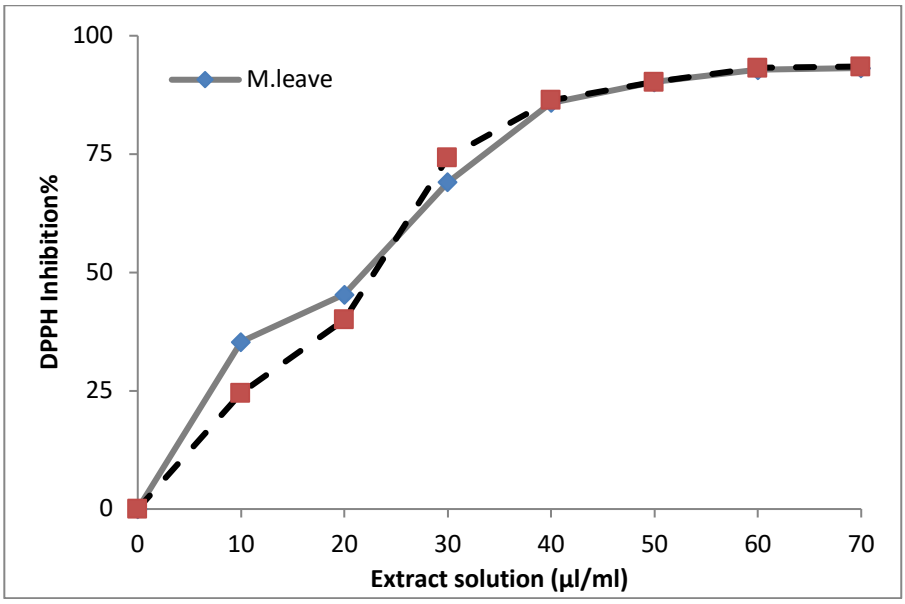


**Figure 1.** DPPH scavenging activity of methanol extracts of *Ruta* leaves and flowers

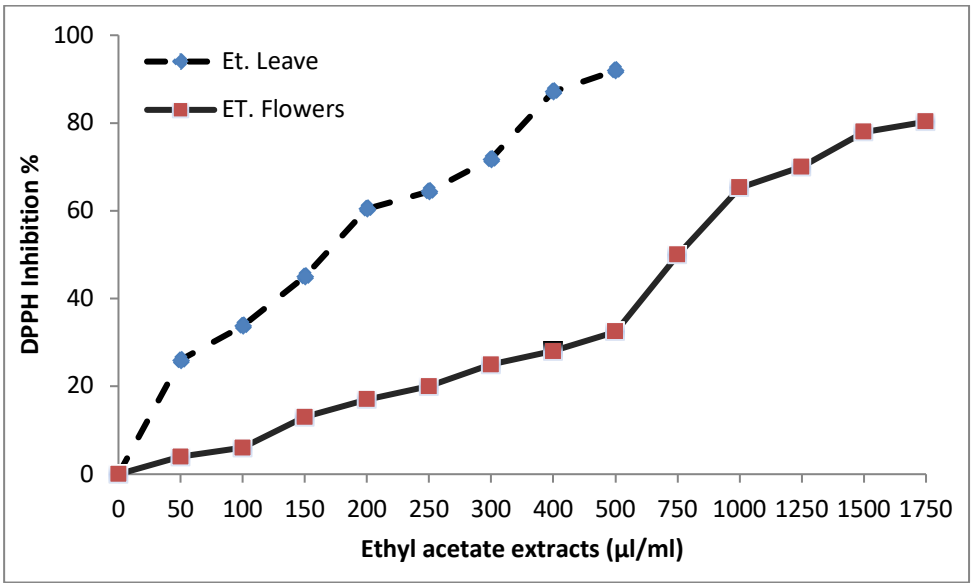


**Figure 2.** DPPH scavenging activity of Ethyl acetate extracts of *Ruta* leaves and flowers

### 3.4. *α- Amylase Inhibitory Activity of Rut chalepensis L. Extracts*

Alpha-amylase is a membrane-bound enzyme that is located on the brush border of the small intestine and is required for the breakdown of carbohydrates into monosaccharides (Lebovitz, 1997). Theα-amylase inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates and are potentially useful for the control of obesity and diabetes (Adesegun et al., 2013). The inhibitory effect of methanolic and ethyl acetate extracts on the α-amylase activity is reported in Table 2. The results indicated the inhibitory effect did not go parallel with those of antioxidant activity. The methanolic extracts of leaves with the highest

antioxidant and TPC showed the lowest inhibitory effect against α- amylase activity (42.2%) followed by ethyl acetate of flowers (53.9%) which had the lowest TPC and antioxidant activity. Whereas the ethyl acetate extract of leaves showed the highest inhibitory effect against α- amylase (63.7%) followed by the methanolic extract of flowers (57.9%). The results indicate that the inhibition of α- amylase activity might be due to other compounds rather than phenolic compounds.

## 4. Conclusions

The results of the present study revealed that the higher polarity of the solvent, the higher extraction yields, and the higher TPC and flavonoids. In the methanolic extracts, the distribution of TPC between flowers and leaves was equal. While it was not in the case of ethyl acetate extracts, since the TPC in leave extract was greater than that in flowers. Also, the results revealed that the higher TPC the higher antioxidant activity measured by the DPPH test. The inhibition effects the extract of α-amylase activity based on other factors than the amount of TPC, since the ethyl acetate extract of leaves showed the highest inhibitory effect followed by the methanolic extract of flowers. The positive results of this study may enhance the probability of using plant extracts to prolong the shelf life of many fat-based or oil-based products and the possible effect of such extracts as hypoglycemic agents and antidiabetic agents with consequent health benefits.

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# AMORPHOPHALLUS PAEONIIFOLIUS (ARACEAE): A NUTRACEUTICAL FOR FOOD DISORDERS, NOVEL BACTERIAL & VIRAL INFECTIONS

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**ABSTRACT**

*Amorphophallus paeoniifolius* is a very common tuber plant having diverse secondary metabolites and palatability levels. Keeping the problems of food shortage & novel infectious diseases throughout the world, an attempt has been made through fieldworks during 2009 to 2020 to gather the information on its ethnobotany, bioactive compounds from lab work and pharmacological properties from secondary sources to make it future nutraceutical against food disorders & novel microbial diseases. The tuber is used as food and medicines, it is rich with primary & secondary metabolites and its extracts are used to treat various infectious diseases. The compounds present in the species have potential to make novel drugs against present health problems throughout the world. The present study highlights the importance of wild tuberous plants in mitigation of food shortage, food disorders, anti-microbial resistance, novel bacterial & viral diseases like MDR-TB and COVID-19.

## 1.Introduction

Foods and medicines are the prime needs of human beings since primitive. The primitive human get these needs from wild. Land is limited and the population of world is increasing at alarming rate with modern life styles and anthropogenic activities leading food problems, disorders, anti-microbial resistance & novel microbial diseases. Hence, now we need nutraceuical from wild. Among the wild sources and contemporary health issues throughout the world, *Amorphophallus paeoniifolius* (Dennst.) Nicolson is right choice to study for getting future nutraceutical and pharmaceuticals against food problems & novel infectious diseases like COVID-19.

The genus *Amorphophallus* is among the most striking of all plants including the largest flower structures of the entire world flora. It is a perennial underground corm which grows mostly in semi-shady areas (Anuradha & Neeraj, 2014). *Amorphophallus paeoniifolius* stands out in the genera by their importance on local die in many regions of Asia and also their common use in medicine (Dey et al., 2016a). It was originated in India and later distributed to other regions of the world by human. The local short-distance dispersal is mostly by birds by feeding on its berries and dropping the seeds. Beetles are also known for cross-pollination in this species. The highly nutritive values as food and efficient neutraceutical for various ailments and other uses have placed the species in a special position for researchers to explore more and more. It is known to be frequently traded commercially in India (Santosa et al., 2017).

It is therefore cultivated not only as food but also as feed for animals. It prefers shady areas and hence makes a potential crop for intercropping which can be grown under the tree canopy. Such measures will encourage feed for animals such as cows and pigs and overcome feed shortage (Santosa et al., 2017; Koni et al., 2017).

Farmers have harvested the leaf petiole for use as a disinfectant for fish ponds. *Amorphophallus paeoniifolius* stands in a perfect position for an alternative source of peroxidase with excellent stability. This is because peroxidase has been using for various purposes such as wastewater treatment, biotechnological, biomedical and other applications but the high cost of commercially available horseradish peroxidase (HRP) is restricting its application (Singh et al., 2017).

Keeping the nutraceutical & pharmaceutical potentials (Table 1) and emerging of novel viral diseases in last two decades, some research has been done at selected regions of India (Sikkim Himalayas, Indo-Burma Biodiversity Hotspots, Eastern Ghats, Western Ghats, Coastal parts of India and Chotanagpur plateau) for collection of food & medicinal values followed by collection of corm (Coastal areas of Puri district of Odisha, India) for estimation of primary and secondary metabolites using standard methods (Kumar et al., 2017; Sadashivam & Manickam, 2010) and collection of pharmacological values from secondary sources.

**Table 1.** Etnomedicinal use(s) of *Amorphophallus paeoniifolius* in study areas

| Parts used | Mode(s) | Target ailment | Collection site(s) |
|---|---|---|---|
| **Corm** | Dried powdered of corm with warm water | Jaundice | Jharkhand state |
| **Corm** | Boiled | Dysentery & Body pain | Odisha state |
| **Shoots** | Juice | Sinusitis | Odisha state |
| **Corm** | As vegetables | Gastritis & to Purifies the blood | Manipur state |
| **Corm** | Dried roots | Piles & Dysentery | Odisha state |
| **Corm** | Fresh roots | Stimulant & Expectorant | Odisha state |
| **Leaves & Stem** | Juice | Ulcers | Kerala state |

## 2.Methodology

The study was developed in six locations from different parts of India (Sikkim Himalayas, Indo-Burma Biodiversity hotspots, Eastern Ghats, Western Ghats, Coastal part of India & Chotanagpur). The plant experiment was identified by usual methodology in taxonomy and using morphological characteristics (Haines 1925). The methodological framework for the ethno-botanical study were as per the standard techniques of exploration and germplasm collection (Hawkes 1980; Christan and Brigitte 2004), qualitative and quantitative ethno-biological approaches in the field, interviews, elicitation methods, data collection and further authentication (Martin 1995; Cotton 1996). The standard participatory rural appraisal method (Cunningham 2001; Gerique 2006) was adopted for sampling and data collection to incorporate the indigenous knowledge.

## 3.Results and discussions

### 3.1.Botanical description & distribution of family Araceae

Araceae is one of the most diverse family of monocots, has a cosmopolitan distribution and is represented by 125 genera and around 3750 species (Nauheimer et al., 2012). The species can be found in a wide variety of habitats and has many kinds of life forms from geophytes, climbing, epiphytes, terrestrial to rare aquatics (Mayo et al., 1998). The stems are usually glabrous and slightly succulent; its tissues often form latex tubes or raphides. The leaf ranges from simple and entire to compound and highly divided and may be basal or form an aerial stem. The Araceae family has a unique form of inflorescence forming a spadix with bisexual or unisexual or sometimes sterile, which then gets subtended by a spathe. Membranous sheaths are usually present at the base of the petiole or peduncle. Flowers vary from small to minute, crowded on a simple fleshy spadix with a green or colored spathe. Spadix often produced beyond the flowers. Fruits are usually baccate, free, or confluent. Seeds usually embedded in mucilaginous pulp. In recent times this family is one of the horticulturally important plants (Saxena & Brahman, 1996).

### 3.2.Chacracterization and description of the genus *Amorphophallus*

*Amorphophallus* is a diverse genus of Araceae family, with approximately 200 species (Jaleel et al., 2011). The maturation period of different species of *Amorphophallus* sometimes varies in terms of years. Most species are seasonal and show a period of activity and dormancy sometimes for decades and in few cases even centuries (Stewart & Wilbert, 2011). The plant produces a single inflorescence followed by a solitary leaf. The inflorescence consists of a bract known as spathe which envelops the spike-like organ known as a spadix. The flowers are highly reduced and found at the base of the spadix. After the growing season, the plant dies back to a large underground corm. On successful pollination, the flower can be as tall as 2 m. Some species emit an odour of rotting flesh. Moreover, some people regard its inflorescence as bizarre. The solitary leaf resembles a small tree with hundreds of leaflets from its leaf blade (Stewart & Wilbert, 2011; Dey et al. 2012).

Very stout herb; tuber dark brown, depressed hemispherical, rough with several nodes with seasonal rhizomatous buds; Leaf broad, 3-partite, the lateral segments bifurcate, pinnatifid with oblong lobes lobes or leaflets, acuminate, rachises winged, leaflets ovate to lanceolate; peduncle much shorter than spathe, elongating when fruiting, peduncle surface is similar with petiole both in wild and cultivated species, turns brownish green-brown when fruit is ripening; spathe with a campanulate tube, suddenly widening into an irregular spreading and strongly undulate, pale green to brown with pale green-whitish green spot outside, glossy dark brown to dark red-purple inside; spadix stout, longer than spathe; inflorescence produces very unpleasant odour; infructecens cylindric (Saxena & Brahman, 1996).

### 3.3.Diversity and distribution of *Amorphophallus*

Hetterscheid & Ittenbach (1996) reported 200 species of *Amorphophallus* distributed all over the world. Ittenbach & Lobin (1997) reported six new species and two new subspecies of the same genus. Although Boyce & Croat (2011) anticipated a total species of 219 of the same genus. It is distribution range from areas near the coastal line to an altitude of 900 m above sea level. They are more adapted to areas with shady and low light intensities which suit best in humid tropical areas. Such adaptability is quite suited to cultivate it under a forest tree canopy. This can enhance the availability of feed for animals (Santosa et al., 2017).

The genus is distributed in the paleotropics mainly confined to the Tropical and Sub-tropical regions of Asia and Africa with maximum diversity found in the Southeast Asia with about 70% of the total estimated species. *Amorphophallus* also shows maximum morphological diversity out of the total aroid

genera (Hetterscheid & Ittenbach, 1996). The genetic diversity is relatively high among the Indian, Thai, and Indonesian species (Santosa et al., 2017).

*Amorphophallus paeoniifolius* is widely found cultivated in Indonesia and other Asian countries (Santosa et al. 2017). This species is also widely cultivated in India, Sri Lanka, China, Malaysia, Thailand, Philippines and Africa (Behera et al., 2014). Reports on the *A. paeoniifolius* documented that it was originated in India and later extended its distribution to other parts of the world (Hetterscheid & Ittenbach, 1996; Devi et al., 2013).

### 3.4.Ethnobotanical values of *A. paeoniifolius*

#### *3.4.1.Traditional food systems*

During 2009 to 2020, the third author (Dr. Sanjeet Kumar) have visited different regions of India for documentation of ethnobotanical values and documentation of floral wealth of India and found that all parts of *A. paeoniifolius* is used as food through different traditional food practices by different tribal & rural communities. It was observed that tuber or corm of *A. paeoniifolius* is soaked overnight in water and used as vegetables by the Santhal tribal community of Giridih district of and Kuswaha community of Hazaribagh district of Jharkhand state whereas the Ho community of Mayurbhanj district, Juang community of Kendujhar district, Santhal community of Dhenkanal district and fishing community of Mahanadi river areas of Odisha state use the tubers after boiling. The local community of Manipur state use the leaves as vegetables and tribal community of Kerala state use the plant parts as vegetables after sundried. *Amorphophallus paeoniifolius* is known for its starchy nutritive food and is widely used in many countries including India, Malaysia, Indonesia, Philipines, etc. It is gaining importance as a cash crop with high export potential. It is a delicacy in many parts of India (Dey et al., 2012). It is widely used in ayurvedic preparations and pickles (Das et al., 2009). It is one of the traditional recipes in Bohag Bihu in Assam (Barnali and Zaman, 2013). It is rich in vitamin A, Vitamin B-6, fiber, and certain key minerals which makes the right choice as a vegetable with high nutritive value. The high fibre content makes it a good ingredient to promote weight loss and lower cholesterol levels (Rajlakshmi et al., 2001; Singh et al., 2016).

#### *3.4.2.Folk medicines*

*A. paeoniifolius* are also used to cure many diseases and disorders. It was noted that tribal communities of Giridih district of Jharkhand state use the corms against jaundice whereas dried corms are used against stomach problems by the tribal communities of Odisha state (Table 2).

**Table 2.**Medicinal properties in correlation to the phytochemical compounds

| Bioactive compounds | Group of Compound | Solvent of Extraction | Target activity | Sources |
|---|---|---|---|---|
| **Tetradecene, 1-pentadecanol, hexadecenoic acid** | | Methanol | Anti-oxidant activity | Basu et al. (2013) |
| **Ambylone** | Triterpenoid | Petroleum ether | Antibacterial activity | Khan et al. (2008) |
| **3,5-diacetyltambulin** | Flavonoid | Chloroform | Antifungal activity | Khan et al. (2007); Khan et al. (2008) |
| **Quercetin** | Flavonoid | Ethanol | Antitumour activity | Ansil et al. (2014) |
| **β - sitosterol** | Betulinic acid | Methanol | Against inflammation | Dey et al. (2016) |

| | | | | |
|---|---|---|---|---|
| **Quercetin** | Flavonoid | Methanol | Hepatoprotective activity | Sharstry et al. (2010) |
| **β - sitosterol** | Betulinic acid | Petroleum ether | Antiosteoporetic activity | Sanaye et al. (2018) |
| **Lupeol, Quercetin & Glucomannan** | Not Clear | Not Clear | Prevents ulcerative colitis and Inflammatory bowel disease | Lee et al. (2012); Lee at al. (2016); Suwannaporn et al. (2013) |
| **Flavonoids** | | Acetone and Phenol | Antidiabetic activity | Arva et al. (2013) |
| **Flavonoids, Alkaloids & Steroids** | | Methanol | Antihelmenthic activity | Dey et al. (2017b) |
| **Tannins, Flavonoids, Saponins & Polyphenol** | | Ethanol | Antidiarrheal activity | Wright et al. (2005); Polambo et al. (2006); Perez et al. (2005) |
| **Ethanol** | | | Anti-oxidant activity | Basu et al. (2013) |
| **Petroleum ether & Ethanol** | | | Cytotoxicity | Behera et al. (2014); Dey et al. (2016) |
| **Methanol & Chloroform** | | | Anticancer | Ansil et al. (2014); Jagathese et al. (2010) |
| **Aqueous** | | | Analgesic | Hemalatha et al. (2019) |
| **Petroleum ether** | | | Anticonvulsant activity | De et al. (2012) |

Traditionally tuber roots are considered to be carminative, restorative and possess blood purifier properties and have been using for treatment of abdominal disorders, tumours, enlargement of spleen and asthma. It is also reported to possess tonic and appetizer properties (Dey et al., 2012). The dried roots are used for the treatment of piles and dysentery while the fresh roots act as stimulant and expectorant (Singh et al., 2016). The leaf and stem juice are used to get relief from ulcers. Tribal people like Kurichia, Adiya, Kuruma at Wayand of Kerala state uses the concoction of the dried powder of the corm and curd to treat jaundice and piles (Devi et al., 2013). The boiled corm is used in the treatment of dysentery and rheumatism, and its apical shoots are used to cure sinusitis (Husain, 1992; Barnali and Zaman, 2013). It is also reported to be used for the treatment of elephantiasis, inflammations, haemorrhoids, bronchitis, anorexia, antihelminthic, CNS depressant, hepatopathy, spleenopathy, fatigue and anemia (Nair, 1993; Dey et al., 2012). Apart from its uses to serve the needs of human consumption and medicines, it is used to feed cows or pigs

during the dry season. Besides, it is also used as a disinfectant in fishponds (Khan et al., 2009). Kurichia and Adiya tribes in Kerala used the dried, powdered corm with curd and hot water to treat jaundice (Prasad et al., 2013).

### 3.5.Nutraceutical values of *A. paeoniifolius*

#### *3.5.1.Nutraceutical potential*

The corm of *A. paenoiifolius* is collected from Bhubaneswar-Konark road of Puri district of Odisha state. After collection the corm was washed and kept for estimation of primary & secondary metabolites. Carbohydrate, starch, fibber, protein and lipid was estimated using standard methods of Sadashivum & Manickam (2018), Total phenol (Ainsworth & Gillespic, 2007), tannin (Kumar et al., 2017) & total oxalate (Nguyen & Savage, 2013) is estimated. The results revealed that corm has highest content of starch followed by carbohydrate, fiber, protein, lipid, total phenol, total tannin and total oxalate (Figure 1). The richness of primary metabolites indicates that it will be good future food and from it, food derivatives might be manufactured using value addition of *A. paenoiifolius*. It will be helpful to reduce food problems worldwide. The presence of phenolic compounds indicates that it might be used as an option food to improve immunity and act as an antioxidant agents (Lin et al., 2016; Ding et al., 2018) which will be helpful as a preventive food against infectious diseases.

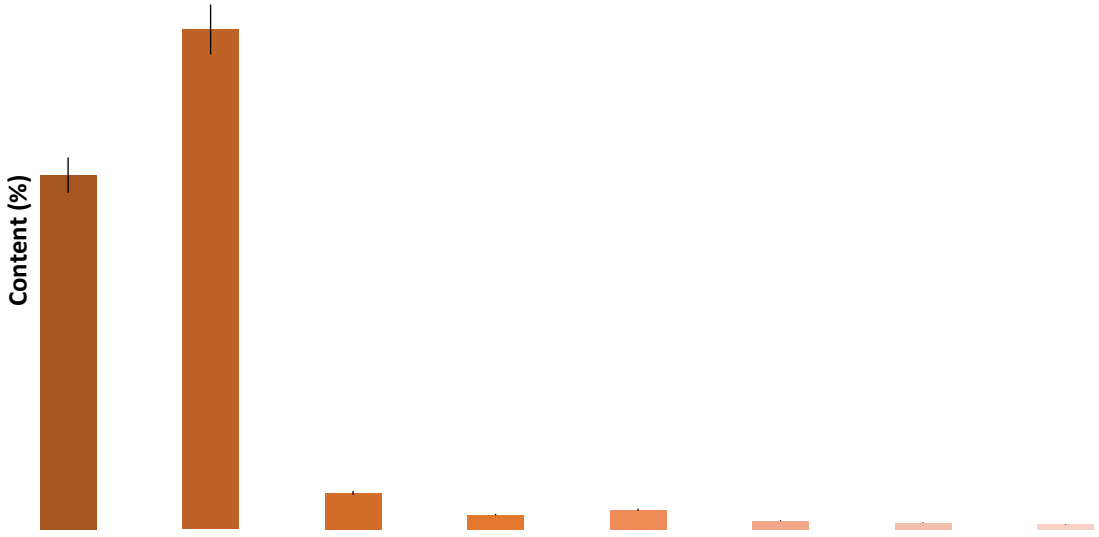



**Figure 1.** Nutraceutical potential of *A. paeoniifolius*

#### *3.5.2.Anti-nutritional properties*

In addition to the beneficial nutritive and medicinal values, *A. paeoniifolius* has anti nutritional or toxic properties which make it less commonly used as a vegetable. The freshly cut form of it shows the presence of oxalates owing to the acrid property. Acridity is the itching or burning sensation in the skin, followed by swelling (Kumar et al., 2017). Oxalates are reported to chelate minerals such as Iron, Zinc, Calcium, and Magnesium, making it unavailable to the body. Higher consumption of oxalate could also be fatal. A minimum amount of oxalate ingestion to make it fatal is 40-50 mg in an adult human. However, experimental data have shown that this toxic property can be negated by boiling for 10 minutes. Reports have been found that sun drying can also reduce the oxalate content. This process can reduce the oxalate at a safe level and also can restore other nutritional properties (Iwuoha & Kalu, 1995; Kumar et al., 2017). Tannins can form complexes with metal ions, proteins, and polysaccharides. In

*Amorphophallus*, tannins are reported to form complexes with proteins, and so it subsequently effect the growth rate of animals. It is reported to inhibit the growth of fibre degrading bacteria in the digestive tract of ruminants. These lead to less efficiency as feed. However, it can be overcome by fermentation as fermentation can decrease the tannin content to a safer level. Hydrogen cyanide is present in the form of cyanogen glucosides. The concentration of it varies depending upon the variety and its environmental conditions. A high level of Hydrogen cyanide can damage the central nervous system in animals, including humans. The content of hydrogen cyanide can be reduced by drying, soaking, and fermentation. Another such compound is phytate present in the same. Phytic acid affects the metabolic processes of the intestine in animals as it chelates metal ions such as phosphorous and zinc ions making it unavailable to the body. The phytate content can, however, be reduced by increasing the heating or by sun-drying (Koni et al., 2017). Oxidative browning occurs when the tissue is damaged in the corms of *A. paeoniifolius.* Tissue damaged during food processing has resulted in the loss of nutritional properties as well as commercial and economic values. Such oxidative browning is caused by a polyphenol oxidase, a copper-containing compound. This enzyme is reported to be not sensitive to temperature, and high temperature is required to inactivate the said enzyme. However, it can be inactivated with the combination of pH below 5 or above 7, high temperature, L ascorbic acid, and chloride effectively.

### *3.5.3.Effect on cholesterol*

*Amorphophallus paeoniifolius,* in combination with *Vigna radiata*, has shown to maintain the cholesterol levels. Elevation of Low-Density Lipoprotein (LDL), also known as bad cholesterol, has contributed to the risk of Coronary heart diseases. Benil and his co-workers have shown a good synergistic effect of combination of *V.radiata* and *A. paeoniifolius* in lowering the LDL *i.e.,* bad cholesterol and increase in the HDL (High-density lipoprotein) *i.e.,* good cholesterol levels by providing omega-3 fatty acids. This was compared to a standard drug Cholestyramine which makes a very efficient nutraceutical for treating such diseases (Singh et al., 2016; Benil et al., 2017).

## 3.6.Bioactive compounds & Pharmacological values of *A. paeoniifolius*

### *3.6.1.Secondary metabolites*

*Amorphophallus paeoniifolius* is also known as Elephant foot yam and popular for its rich content of secondary metabolites like such as glycosides, flavonoids, alkaloids, phenolic compounds, tannins and minerals like potassium, phosphorous, calcium, iron, ascorbic acid, and β-carotene. Such a rich source of metabolites could meet the daily leading requirement of the body (Misra et al., 2001; Singh et al., 2016; Dey et al., 2017b). Studies with regard to extraction with different solvents, phytochemical test along with Thin Layer Chromatography (TLC), Column Chromatography and High Performance Thin Layer Chromatography (HPTLC) have shown the presence of various bioactive compounds including as mentioned alone (De et al., 2010; Firdouse & Alam, 2011; Natraj et al., 2011; Jayaraman et al., 2010). Significant reports are based on the methanolic extract of *A. paeoniifolius.* The methanolic extract of it showed a significant content of flavonoid, alkaloids, steroids, and phenolic compounds. TLC of the methanol extract of it showed 7 bands with different $R_f$ values which indicate the diversity of bioactive compounds in it (Ferdouse et al. 2011). It gives the better understanding for the related reports on gastroprotective activity in albino rats, antihelminthic activity against *Pheretima posthuma*, analgesic activity, antioxidant activity, anti-inflammatory activity and antimicrobial activity using methanol extracts of *A. paeoniifolius* (Nataraj et al., 2009a; Natraj et al., 2009b; Nataraj et al., 2011; Dey et al., 2010; Ansil et al., 2011; Das et al., 2009). The ethanol extract also showed anti-diarrheal activity; petroleum ether extract showed CNS

depressant activity which could be responsible for the analgesic activity. The methanol extract and chloroform extract both showed the antibacterial activity which reflects the presence of phenolic compounds in plant parts of *A. paeoniifolius* (Chibane et al., 2019).

***3.6.2.Antimicrobial activity***

There are lots of work has done on the antimicrobial activity of corm. The documentation revealed its anti-microbial potential. The ethanol and methanol extract of the tuber of *A. peoniifolius* shows antibacterial activity against gram positive bacteria like *Bacilus subtilis, B. cereus, B. thuringiensis, Staphylococcus aureus, Streptococcus β-haemolyticus,* and gram negative bacteria like *Escherichia coli, Shigella dysenteriae, S. sonnei, S. flexneri, Pseudomonas aeruginosa* and *Salmonella typhi* using disc diffusion method. It also shows good antifungal activity against *Candida albicans* but least on *Aspergillus niger, A. flavus* and *Rhizopus aryzae*. Reports have also been demonstrated that chloroform and petroleum ether show more effective antimicrobial activity. In general methanol extract of the said tuber showed maximum inhibition for most of the bacterial strains. However, ethyl acetate extract of the said tuber has also been reported to inhibit *B. subtilis* as well as *S. aureus*. The triterpenoid compound ambylone extracted from petroleum ether and flavonoid compound 3,5-diacetyltambulin from chloroform have also been suggested to be responsible for its antibacterial and antifungal property (Khan et al., 2007; Khan et al., 2008; Dey et al., 2016a; Dey et al., 2017b; Kadali et al., 2016; Muthukumaran et al., 2016). The above mentioned activity show the broad spectrum on its anti-microbial potential against future novel microbial diseases.

***3.6.3.Antioxidant activity***

Oxidative stress in the body leads to an increase in the level of enzymes that generate and release free radicals and disruption of electron transport chain leading to complications such as neurodegenerative diseases, damage in biomolecule such as lipids, proteins, and DNA, cancer, vision loss, etc. Polyunsaturated fatty acids of the cell membrane are prone to react with the Reactive Oxygen Species (ROS), causing damage to the cell (Schieber & Chandel, 2014). The antioxidant activity of tuber of *A. paeoniifolius* in different solvents such as aqueous, methanol, ethanol, ethyl acetate all elucidated and noticeable positive activity. A defence mechanism to relief the oxidative stress include SOD (superoxide dismutase), CAT (catalase), $GP_x$ (glutathione peroxidase) and other phytochemicals such as phenolic acids, flavonoids, ascorbic acid, tocopherols, uric acid, tannins, lycopene, glutathione, etc. The enzymes present in *A. paeoniifolius* have been reported to serve as potential antioxidants (Hamid et al., 2010; Sanjay et al., 2009). Reports showed that DPPH (Diphenyl picryl hydrazyl) radical scavenging activity, Hydroxyl radical scavenging activity depicted maximum solvent extract activity in ethanol extract while phosphor molybdenum assay had shown maximum antioxidant activity with methanol extract of the said tuber. The methanol extract of *A. paeoniifolius* has been known to prevent the elevation of serum AST (serum glutamic-oxaloacetic transaminase), ALT (serum glutamic pyruvic transaminase), LDH (lactate dehydrogenase) enzyme levels that are responsible for releasing free radicals in the body (Singh & Wadhwa, 2014). Analysis of methanol extract of the said tuber demonstrated the presence of tetradecene, 1-pentadecanol, hexadecanoic acid, which showed a relative antioxidant activity (Basu et al., 2013). The above reports revealed the antioxidant potentials of the corm of *A. paeoniifolius*.

***3.6.7.Antitumor activity***

In the proliferation of uncontrolled growth of cells, the mechanism of apoptosis is generally targeted. The decrease in ($GP_x$), superoxide dismutase and catalase enzymes as a form of oxidative stress created by a carcinogen in liver and kidney. The ethanol extract of *A. paeoniifolius* tuber showed significant antitumor activity against DMBA (7,12-dimethyl benz anthracene) induced

mammary tumour rats. Flavonoids are known to possess antitumor activity in cell proliferation and angiogenesis. The flavonoid in ethanol extract of the said tuber was found to be 8.8g/100g and was reported to be equivalent to quercetin. Treatment with the said tuber extract could increase the mentioned enzyme levels towards normal. On the other hand, methanol extract and sub-methanol extract of chloroform of *A. paeoniifolius* tuber have also been demonstrated inhibition of growth against human liver cancer cell lines, PLC/PRF/5 in a dose-dependent manner. This apoptosis-inducing potential was determined by DAPI staining, annexin V-FITC staining, and JC-1 staining. Cytotoxicity assay method in-vitro has been used to check the chemotherapeutic activities. Antioxidant activity and cell toxicity can be contributed to the antitumor activity of the plant (Ansil et al., 2014; Jagathese et al., 2010; Florento et al., 2012).

***3.6.8.Analgesic activity***

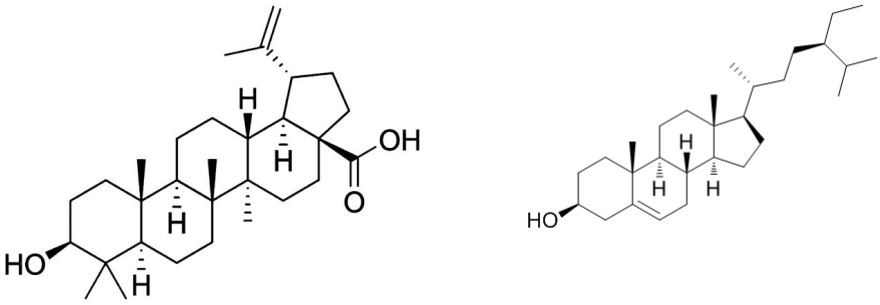
Analgesics are common pain killers given to relieve pain in the body. They include non-prescribed drugs such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDS) like aspirin and opoid drugs such as morphine. NSAIDS inhibit cyclooxygenases COX-1 and COX-2 enzyme and thereby disrupting the production of prostaglandins to decrease pain, lower fever, and reduce inflammation (Ricciotti & Geraid, 2011). Antidepressant work by blocking the monoamine uptake while anticonvulsants act by blocking the sodium channels or by increasing the extracellular levels of inhibitory transmitter GABA (Gamma-Aminobutyric acid). Several important centrally acting drugs target the GABA, a receptors like benzodiazepines. The methanol extract of tuber of *A. paeoniifolius* showed good analgesic activity given in a dose-dependent manner with a maximum 500 mg/kg of body weight shown in experiments by Dey and his co-workers (Dey et al., 2016b). The experiment had been carried out in two different methods viz. acetic acid writhing response method and tail flick method in mice. The extract of *A. paeoniifolius* was compared to the diclofenac sodium as standard to observe the increasing analgesic activity. In addition to this, the aqueous extract of the said tuber extract also exhibited analgesic activity due to peripheral and central inhibition of prostaglandin synthesis. Earlier studies revealed that petroleum extract of the above said tuber showed central depressant as well as a muscle relaxant (Angayarkanni et al., 2007; Dey et al., 2016b; Hemalatha & Sathiya, 2019).

***3.6.9.Anti-diabetic activity***

Although Sugars such as glucose, galactose and rhamnose, and carbohydrates are identified from the elephant foot yam tuber, members of *Amorphophallus* genus have been traditionally used to control diabetes. Acetone extract of the tuber given at 0.1% and 0.25% in the diet of steptozotocin induced diabetic rats showed an improved condition in urine output, urine sugar, fasting blood sugar, and glomerular filtration rate. Reports have shown that phenols and flavonoids play a major role in the anti-diabetic activity. They scavenge free radicals and prevent the secondary complications of diabetes. Flavonoids are also reportedly known to generate beta cells of pancrease and to stimulate insulin secretion (Arva et al., 2013; Soares et al., 2017). Now-a-days diabetic is very common and people face to select the food. In this case, it might be an optional food for them.

***3.6.10.Gastroprotective activity***

Methanol and aqueous extract of the tuber of *A. paeoniifolius* have been demonstrated to prevent or control inflammation and damage in colon of induced ulcer in rats.




Betulinic acid β-sitosterol

**Figure 2.** Bioactive compounds available in *A. paeoniifolius*

Reports demonstrated the pre-treatment with extracts of the said tuber for ulcerative colitis in acetic acid-induced ulcerative colitis in Wistar rats turned out to be quite beneficial for mucosal damage, inflammation and oxidative damage (Dey et al., 2017a). The tuber extracts were also shown to exhibit gastrokinetic activity which correlates with the correction of gastrointestinal disturbances (Dey et al., 2017a). Earlier studies found out that β-sitosterol and betulinic acid (Figure 2) are key constituents for the anticolitic activity. It has also been demonstrated that betulinic acid exhibited anticolitic activity on 2,4,6-trinitrobenzenesulfonic acid-induced ulcerative colitis in rats through inhibitory influence on inflammatory mediators and antioxidant activity (Sener et al., 2013). β-sitosterol, on the other hand, exhibited anticolitic activity on the above mention rat through inhibition of proinflammatory cytokines and cyclooxygenase (COX-2). Other compounds like lupeol, quercetin, and glucomannan in the tuber extract also showed preventive effect on ulcerative colitis and inflammatory bowel diseases (Lee et al., 2012; Lee et al., 2016; Suwannaporn et al., 2013).

***3.6.11.Hepatoprotective activity***

Methanol, ethyl alcohol and aqueous extracts of the tuber of *A. paeoniifolius* have shown to exhibit hepatoprotective activity against induced liver damage in rats through paracetamol and carbon tetrachloride. Liver damage is characterised by the increase in the serum hepatic enzymes levels of sGOT, sGPT, sALP, and sB. Pre-treatment with the above extracts considerably reduced the said hepatic enzymes levels comparable to the commercial drugs such as Silymarin and Liv 52. Methanol extract demonstrated a better activity as compared to the aqueous extract. Reports also suggested that flavonoids and steroids may play a key role in the hepatoprotective activity. Quercetin, a flavonoid compound has reportedly screened for the same activity. This study supports the previous findings, but more bioactive compounds need to clarify the exact mechanism of its activity (Hurkadale et al., 2012; Singh & Wadhwa, 2014; Benil et al., 2017; Dey et al., 2016a; Sharstry et al., 2010; Sanjay et al., 2009).

***3.6.12.Anti-osteoporetic activity***

Osteoporosis, a common health problem is more prone to women after menopause. The reason for it is the decreased level of estrogen and bone density with aging in the body. The petroleum ether extract of *A. paeoniifolius* has been shown to treat the osteoporetic activity. The petroleum extract of the said tuber showed the presence of β-Sitosterol, which shows a similar structure as that of estrogen. Since the structure of β-Sitosterol mimics estrogen, the petroleum ether extract can effectively be an alternative to hormone replacement therapy. The experiment on ovariectomised rats has shown positive results. This phytoestrogen has been proven scientifically and can be an excellent way to include in the diet to reduce such health issues (Sanaye & Bohra, 2018; Joy et al., 2016; Li et al., 2013).

### *3.6.13.Anti-helmintic activity*

Parasitic worm infection has affected over 2 billion all over the world infecting mostly the gastrointestinal tract in animals including man causing deprivation of food, injury to organs and secreting toxins in the body. The principle treatment of parasitic worms involves the disturbance in the integrity of the parasites, co-ordination of the neuromuscles and protective mechanism against host immunity leading to starvation, expulsion, or digestion of the parasite. In the search for reverse pharmacology in controlling helminths, *A. paeoniifolius* has been one of the sources to control such parasites. An experiment illustrated that the methanol extract of the above said tuber showed significant antihelmintic activity against *Pheretima posthuma* collected from soil and *Tubifex tubifex* collected from aquarium in a dose-dependent manner comparable to the standard drug piperazine citrate. As for the mechanism of piperazine is to paralysed the parasite by blocking the neuromuscular transmission and later expel the paralysed parasites by peristalsis but the exact bioactive compound responsible the antihelmintic activity is yet to discover. Moreover, methanol extracts of the said tuber have been demonstrated many times to the presence of flavonoids, alkaloids, and steroids (Dey et al., 2017b).

### *3.6.14.Anti-diarrheal activity*

Diarrhoea, characterised by the excessive loss of fluids and electrolytes from the body leading to dehydration, abdominal cramps, frequent loose, watery stools, fever, etc. is common in man and other animals due to various infections (Anigilaje, 2018). Active phytochemical components of various parts such as flavonoids, terpenoids, steroids, alkaloids and phenolic compounds that are demonstrated to have antibacterial activity are correlated to the antidiarrheal activity (Wright et al., 2005). The ethanol extract of leaves of *A. paeoniifolius* was reportedly exhibited to reduce the severity of diarrhoea in castor oil induced diarrhoea in swiss albino rats. The above said extract when given in 100,200 and 400 mg/Kg significantly reduced the frequency of diarrhoeic faeces in a statistically significant manner ($p<0.05$) (Purwal et al., 2011). Prostaglandins are known to induce intestinal mucosal secretion accompanied by stomach cramp thereby enhancing diarrhoea (Hawkey and Rampton, 1985). On the other hand tannins and flavonoids are suggested to have antidiarrheal activity causing to retain colonic water and electrolytic re-absorption (Palombo, 2006). Flavonoids in association with saponins are reported to inhibit the prostaglandins, motility and hydrolytic secretions (Perez et al., 2005). It has also been illustrated that the combined effect of polyphenols and tannins work in a synergistic way to decrease the intestinal secretion and promote water balance in the body (Dubreuil et al., 2013).

### *3.6.15.Anti-convulsant activity*

Epilepsy is a neurological disorder which is associated with signs of abnormal brain activity, periods or seizures of unusual behaviour and loss of awareness. The main cause of seizure development is caused by the imbalance between the excitory and inhibitory neurotransmission in the brain. Drugs for epilepsy are also known as anticonvulsant drugs. Plant-based anticonvulsant activity has also been reported from petroleum ether extract of *A. paeoniifolius.* The said extract demonstrated an effective anticonvulsant activity in doses of 200, 300 and 400 mg/Kg on isoniazid induced mice which are comparable to the standard drug diazepam (De et al., 2012). It was also put forth that the petroleum ether extract of the said tuber also exhibited central nervous system depressants in a statistically significant way ($P<0.05$). It induced sedation and a decrease in the locomotor activity in mice. The 1500 mg/Kg doses have also been illustrated as a safety dose (Das et al., 2009).

### *3.6.16.Cytotoxicity*

To analyse the cytotoxicity test is very important for a wild species to make them a strong pharmaceutical agent. Many reports are documented regarding the cytotoxicity of A. paeoniifolius. Petroleum ether and ethanol extracts of *A. paeoniifolius* tuber showed

significant antiproliferative activity against Hep-2 cells. Although methanol extract of the said tuber is also reportedly found to reduce the growth rate of MCF-7 cell lines, which is associated with the antiproliferative properties. $IC_{50}$ of the extract of *A. paeoniifolius* in <100µg/ml on the cell line is potentially cytotoxic. This confirmation of cell cytotoxicity is supported by another cytotoxicity experiment that was determined against brine shrimp nauplii which showed a positive result (Behera et al., 2014; Dey et al., 2016a). The above experimental reports make *A. paeoniifolius* a preventive food against cancer.

***3.6.17.Antiviral agents against coronavirus***

Severe acute respiratory syndrome coronavirus emerged in early 2003 to cause a very severe respiratory syndrome caused by corona virus and in 2019, a type of corona virus again come from Bat in China and within some days became pandemic known as COVID-19 (Li et al., 2020). Wholeworld fighting with COVID-19 and searching new medication to mitigate the infections and pre & post preventive drugs from plant wealth. Luo et al. (2007) documented that plant species of family Araceae have anti-viral activity whereas Rajbhandari et al. (2009) reported the anti-viral activity of plant (*Arisaema flavum*) belongs to Araceae family against Human influenza virus (A/WSN33; H1N1) & herpes Simplex virus type 1 (HSV-1). Indrasetiwan et al. (2019) showed the antiviral activity of Araceae (*Anthurium plowmanii*) against Hepatitis B virus. Hence, all the pervious works indicate that *A. paeoniifolius* might be useful against COVID-19 as a nutraceutical. Another evident is presence of lectin in *A. paeoniifolius.* Lectin, a carbohydrate-binding protein used to regulate virus receptor binding activity. Keyaerts et al. (2007) reported that plant lectins are potent inhibitors of coronavirus by interfering with two targets in the viral replica cycle, and Fei et al. (2003) and Mondal et al. (2012) reported presence of lectins in *A. konjac* and *A. paeoniifolius*. Hence, *A. paeoniifolius* might be used as preventive food against COVID-19 (Figure 3) and their isolated bioactive compounds might be playing a vital role to mitigate the infections of this pandemic viral disease as a wild nutraceutical.

## 4.Conclusions

Considering the valuable properties in terms of health and nutrition, feed, enzymes for industrial purpose and medicines contributed by the secondary metabolites of *A. paeoniifolius,* we should rethink going back to plant-based nutraceutical or reverse pharmacology in other term. With the increasing use of chemicals as food, food additives, disinfectants, medicine, processed products and lifestyle that are endlessly depending on chemicals has become a threat not only to man but also to other living beings on earth. The question that is arising with the safety of GMO (Genetically Modified Food) food products or unknown side effects with the altered genes for mass production, or the attempts with chemical-based medicines to control diseases are working in a different way arising multi drug resistance bacteria or new diseases. Therefore phytochemical compounds that are already available and not been commercially available in plants need to be explored more and make commercially available throughout the world. This practice will not only prevent many of the diseases without side effects but also create a sense to conserve them. *A. paeoniifolius* being rich in nutrients and secondary metabolites that can treat for various ailments (Figure 4). As from literature and information collected during the fieldworks, we conclude that the mentioned plant as nutraceutical to mitigate the food problems & food disorders. There is need for advance scientific research in gene level on removing factors responsible for acrid properties of plant parts to make it more palatable keeping remain the other metabolites. Therefore, priority might be given for attaining food self-sufficiency through proper utilization of plant parts of *A. paeoniifolius.* For developing nutraceutical and isolation of active compound(s) against microbial infections & coronavirus, rapid bioactivity should do against pathogens. The number of techniques might be

used to regulate the production of desired active metabolites like lectin via gene expression, over expression, addition of precursors,elicitation, bioreactor scaling and metabolic engineering etc against novel dirorders and infectious diseases like COVID-19.

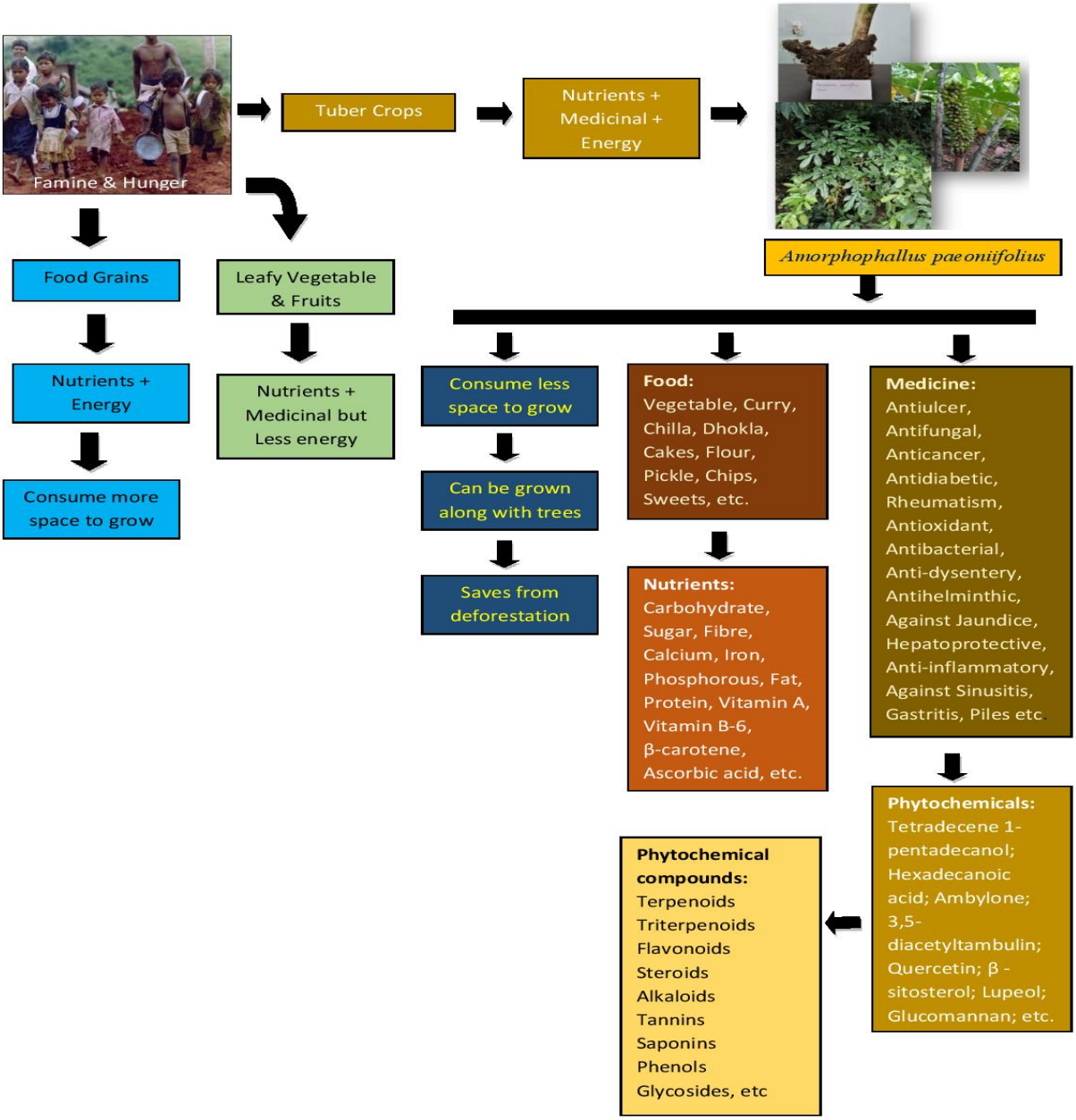



**Figure 3.** Nutraceutical & pharmaceutical importance of *A. paeoniifolius*

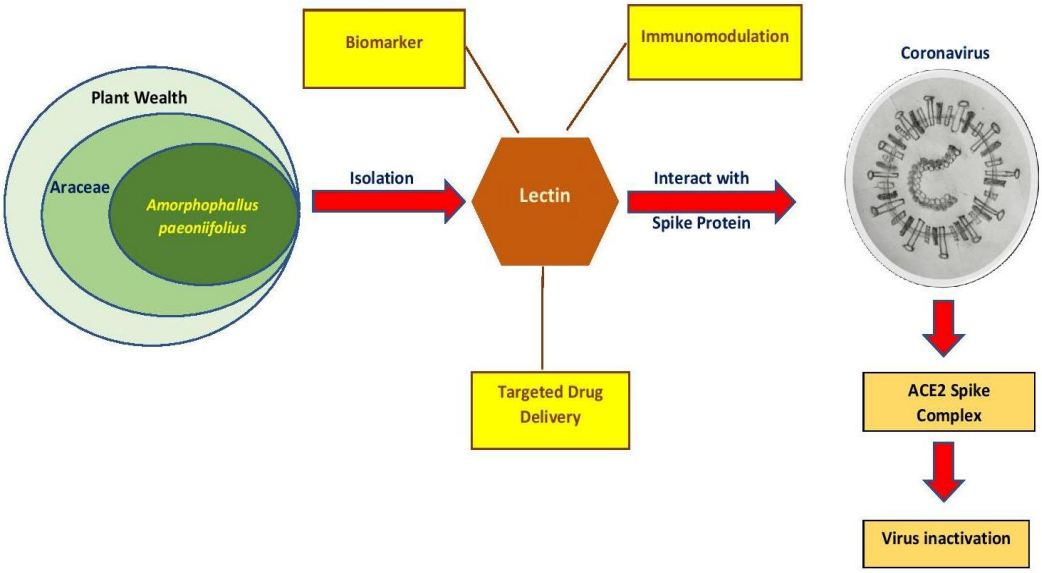



**Figure 4.** Future aspects of Araceae family including *A. paeoniifolius* against COVID-19 as a nutraceutical agent

## 5.References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# HISTAMINE LEVELS AND HISTAMINE PRODUCING BACTERIA IN FOUR SELECTED FISH SPECIES DISPLAYED IN THREE FISH MARKETS WITHIN TRIPOLI-CITY LIBYA

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**ABSTRACT**

The study aimed to assess temperature, histamine level and histamine producing bacteria (HPB) in four species of fresh fish samples i.e, *Sardinellaaurita*, *Boopsboops*, *Trachurusmediterraneus* and *Scomberscombrus* that are collected from three markets (A, B, C) within Tripoli city Libya. The results revealed that 95% of the fish samples had a temperature range between 5-22$^0$C, while 5% had a temperature < 5$^0$ C. Histamine was recorded in 43% of the samples. The ranges of histamine in sardine, bouge, saury, and mackerel samples were 1.29-5.74; 1.34-29.74;1.31-7.57 and 1.39-2.49 mg/100 g meat, respectively. These levels did not exceed the maximum limit (10 mg/100) adopted by the Libyan authority, except one sample (29.74 mg/100 g meat). A significant difference (P<0.05) in histamine levels was observed among the three markets. However, a non-significant difference (P>0.05) was observed between the fish species. The range for the means of HPBC in sardine, bouge, saury, and mackerel samples were $1.8\times10^4$-$5.4\times10^4$; $6.4\times10^4$-$2.0\times10^5$;$6.4\times10^4$-$6.910^5$; $1.6\times10^4$-$4.1\times10^5$cfu/g fish meat, respectively. Most of the HPB isolates were belonged to the family *Enterobacteriaceae* and some belong to the family *Vibrionaceae*. *Vibrio fluvialis* recorded the highest prevalence percentage (18%) followed by *Erwiniaspp*, *S. putrrefacienes,* and *K. planticola*, i.e.,12.2, 11.9 and 10.0%, respectively. The results of this study reflect the poor cooling conditions of the samples and poor cooling techniques practiced in these markets. Therefore, the hygienic practices in these markets have to be improved, and preferably the HACCP system has to be implemented.

## 1.Introduction

Histamine fish poisoning (known as scombroid fish poisoning) is the most common form of fish intoxication caused by seafood products and usually presents an allergic reaction. Many reports of histamine poisoning outbreaks were associated with the consumption of raw, cooked, frozen, salted and canned fish (Feldman et al. 2005; Becker et al. 2001; Lehan and Olley, 2000; Etkind et al. 1987; and Merson et al. 1974) which reflects the fact that histamine is heat stable and is not affected by freezing, salting and/ or drying. In Europe, 56 out of the 71 food borne diseases and outbreaks (78.9%) reported during 2011, were attributed to histamine fish poisoning (EFSA,2013).Under improper refrigeration or icing conditions, certain species of fish are sensitive to histamine formation. Tuna, Sardine, Bouge, Saury, and Mackerel are examples of such species (Alhalluge, 2012). The flesh of these species of fish contains the amino acid histidine, which is the precursor for histamine formation by decarboxylase enzymes via bacterial action, under improper icing or cooling conditions (FDA, 2001). The rate of histamine formation

and accumulation to a toxic level is so fast that it occurs before fish spoilage indices can be detected by sensory evaluation (Kim et al. 2004).

Ayesh et al. (2012) mentioned that the United State, Food and Drug Administration (FDA) had stated that histamine levels must be used as a guideline in Hazard Analysis and Critical Control Point (HACCP) programs for fish and has set the maximum action level of 50 ppm. It has also been reported that the family Enterobacteriaceae is the most important histamine forming bacteria in fish. *Morganellamorganii, Klebsiella pneumonia, Proteus vulgaris, and Hfniaalevi*are known to originate from fish implicated incidents of histamine poisoning (Huss et al. 2000; Lehan and Olley, 2000; and Frank, 1985).

Little is known about the epidemiology of histamine fish poisoning in Libya, particularly regarding the overall risk in regularly consumed fish species sensitive to histamine formation. Therefore, this study aimed to evaluate display conditions in terms of temperature, histamine producing bacteria count, and histamine levels in Sardine, Bouge, Saury, and Mackerel fish species displayed for sale in three main fresh fish markets located within Tripoli city, Libya.

## 2.Material and methods

### 2.1.Sample collection

Fresh fish samples (113 samples) of sardine, Bouge, Saury, and mackerel were collected directly from fish quantities displayed for sale in three main fish markets A, B, and C in Tripoli City -Libya. The samples were collected at 7,00 – 8,00 A.M. and at 12 – 1 P.M., during the period from July to December of their fishing season. The samples were kept in sterile polyethylene bags and transferred in icebox within 15 minutes to the Microbiology and fish disease laboratory at the marine research center in Tajoura, Libya. Meanwhile, the temperature of the displayed fish was measured at the time of sample collection.

### 2.2.Samples preparation for chemical and microbiological analysis.

A sample consists of 5 – 6 pieces from each sample of fish species were randomly withdrawn. Meat muscles with skin were cut from the back and sides of each fish body with a sterile knife and homogenized in a sterile blender, and then the meat homogenate was divided into two 25 grams' parts. One part was used for histamine determination while the other part of the homogenate was used for bacteriological analysis.

### 2.3.Histamine determination.

#### *2.3.1.Fish samples extract preparation.*

Five grams of fish meat homogenate were homogenized with 20 ml of 6% trichloroacetic acid solution (TCA) previously cooled to $4^0$C. in electric blender for 3 min. The homogenate was filtered through Whatman No. 2 filter paper. The filtrate was placed in a 50 ml volumetric flask and the volume was completed to the mark with distilled water (Hwang et al, 1997).

#### *2.3.2. Preparation of stock Standard histamine solution.*

A stock solution of histamine was prepared by dissolving 0.0828 grams of histamine dichloride (C2H9N3.2HCL) (Acros Organics New Jersey USA) in a small volume of 0.1 M HCl solution in 50 ml volumetric flask and the volume was completed to the mark with 0.1 M HCL. This gives a 1 mg/ml histamine stock solution. Then 1 ml of this stock solution was transferred to a 10 ml volumetric flask and the volume was completed to the mark by 0.1 M HCl solution. This gives 0.1 mg histamine/ml stock solution. Then working standard solutions 0.02 – 4 ug histamine were prepared from the 0.1 mg/ml histamine stock solution (Hwang et al, 1997).

#### *2.3.3. Histamine separation and determination.*

The benzyl derivatives of the standard histamine solutions and the fish samples extracts were prepared according to Anderson (2008) and Hwang et al (1997). One ml of 2 M sodium hydroxide (NaOH) solution and 10 ul of Benzyl chloride were added sequentially to 2 ml of standard histamine solution or the fish sample extracts. The resulting solutions were vortex

mixed and allowed to stand at 30° C for 40 min. Then Benzoylation process was stopped by adding 2 ml of saturated sodium chloride solution (NaCl) and the mixed solution was extracted with 3 ml of Diethyl ether to separate the histamine. The solution was then centrifuged at 10,000 g for 10 min at 40° C, and the separated upper organic layer was transferred to a dry clean test tube and evaporated to dryness by purified Nitrogen gas. The dried residue was dissolved in one ml Acetonitrile. Aliquots of 20 ul from the residue acetonitrile solution were injected into the HPLC unit (Perkin Elmer 200 equipped with UV detector) at the Food and Drug Control Center Tripoli branch according to the operation conditions showed in table (1). The gradient elution program began with 50,50 (v/v) acetonitrile, water at a flow rate of 1 ml/min for 19 min, followed by a linear increase to 90,10 acetonitrile, water (1 ml/min) during the next 1.0 min. The acetonitrile, water mix decreased to 50, 50 (1.0 ml/min) for 10 min.

**Table 1.** Operation conditions for the high-performance liquid chromatography used for Histamine determination in fish samples extracts.

| Category | Operation condition |
|---|---|
| Column type used | C 18 – reversed-phase column |
| Length and diameter of column | 125 X 2.5 mm |
| Mobile phase | Acetonitrile , water ( 50 , 50 ) |
| Detector | UV |
| Wavelength | 254 nm |

**2.4.Determination of histamine producing bacterial count (HPBC).**

Twenty-five grams of minced homogenized fish meat was mixed with 225 ml of 0.1 % sterile peptone water in sterile electric blender for 1 minute. Then, serial decimaldilutions of $10^{-2}$, $10^{-4}$ , and $10^{-5}$ were prepared from the homogenate and were used for HPBC determination on duplicate plates containing Niven's medium agar according to Swanson et al. (2001). All plates were incubated inverted at 25° C for 48 $\pm$ 2 hours. Plates were incubated at 25° C as recommended by Nickelson et al (2001) for routinequalityassessment for fresh fish and frozen seafood products.

Colonies with purple halo grown on Niven's medium were counted, aseptically isolated and then purified by streaking technique on trypticase soy agar plates. The plates were incubated at 25° C for 24 hours. Pure isolates were Gram-stained and microscopically examined under oil immersion, before identification using API 20 E kits according to Korashy et al. (2005).

**3. Results and discussions**

**3.1.Fish samples Temperature and their Histamine contents.**

The temperature of the fish samples included in this study ranged between < 5 – $22^0$ C. Percentage of samples that had temperatures < 5, 5 – 14 and 15 – $22^0$ C were 5, 52, and 43% respectively of the total samples examined in this study (Table 2).

**Table 2.** The temperature ranges of Fish samples collected from the three fish markets in Tripoli city, Libya, and the percentage of each range.

| Temperature range ($^0$C ) | Numbers of samples | The percentage of each range |
|---|---|---|
| ‹ 5 | 6 | 5.31 % |
| 5 – 14 | 59 | 52.20% |
| 15 – 22 | 48 | 42.50 % |

Histamine wasrecorded in 43% of the fish samples. The histamine content of these samples ranged between 1.29 – 29.74 mg/100 g of meat with an average of 2.9 ± 4.15 mg/ 100 g of meat. The temperature of these samples ranged between< 5 to 22$^0$C, and 53% of them had a temperature range of 5 – 14$^0$C (Table 3). The results also showed that 41 and 52% of the samples collected during the morning and noontime respectively contained histamine (Table 4).

**Table 3.** The percentages (%) of fish samples containing histamine, the ranges and means of their histamine levels in reference to their temperatures.

| **Fish temperature ($^0$C)** | **Total fish samples** | **Samples containing histamine (%)** | **Histamine Level (mg %)** | | |
|---|---|---|---|---|---|
| | | | **Min** | **Max** | **Mean** |
| < 5 | 5 | 10 | 1.29 | 2.35 | 1.82 ± 0.38 |
| 5 – 14 | 26 | 53.06 | 1.30 | 7.57 | 2.49 ± 1.47 |
| 15 – 22 | 18 | 36.73 | 1.31 | 29.74 | 3.84 ± 6.64 |
| Total | 49 | 43.36 | 1.29 | 29.74 | 2.91 ± 4.15 |

**Table 4.** The percentages of fish samples containing histamine in reference to their Sampling time.

| **Sampling time** | **Total number of samples** | **Number of samples containing histamine** | **% of samples containing histamine** |
|---|---|---|---|
| Morning | 88 | 36 | 41 |
| Noon | 25 | 13 | 52 |

The percentages of sardine, bouge, saury, and mackerel samples that contained histamine were 31, 46, 53, and 30 % respectively. Their histamine content ranged between 1.29 – 5.74, 1.34 – 29.74, 1.31 – 7.57 and 1.39 – 2.49 mg / 100 g meat respectively (Table5). Statistical analysis of histamine levels according to the types of fish included in this study did not show significant differences ($P > 0.05$) (table 5). Meanwhile, the histamine contents of the fish samples collected from the fish market A were significantly different ($P < 0.05$) from the histamine contents of the fish samples collected from the other two fish markets B and C (table 6). Hence, the highest histamine level (29.74 mg/100 g) was recorded in this market in bouge fish samples.

**Table 5.** The percentages (%) of fish samples containing histamine, the ranges and means of their histamine levels in reference to the Fish Types studied.

| **Fish type** | **Total samples** | **No. samples containing histamine** | **Samples containing histamine (%)** | **Histamine Level (mg %)** | | |
|---|---|---|---|---|---|---|
| | | | | Min | Max | Mean |
| Sardine | 32 | 10 | 31.25 | 1.29 | 5.74 | 2.02 ± 1.35 |
| Bouge | 28 | 13 | 46.42 | 1.34 | 29.74 | 4.51 ± 7.72 |
| Saury | 23 | 17 | 53.13 | 1.31 | 7.57 | 2.78 ± 1.65 |
| Mackerel | 30 | 9 | 30.30 | 1.39 | 2.49 | 1.85 ± 0.45 |

No significant differences between the means at P = 0.05.

**Table 6.** Percentages of fish samples containing histamine in reference to the fish markets examined

| Fish market code | Total samples | No. samples containing histamine | Samples containing histamine (%) | Histamine Level (mg %) | | |
|---|---|---|---|---|---|---|
| | | | | Min | Max | Mean |
| (A) | 26 | 8 | 30.77 | 1.39 | 29.74 | $7.14^{b}\pm9.45$ |
| (B) | 50 | 23 | 46.00 | 1.29 | 4.15 | $2.13^{ad}\pm0.83$ |
| (C ) | 37 | 18 | 48.65 | 1.31 | 5.52 | $2.03^{a}\pm1.01$ |
| Total | 113 | 49 | 43.36 | 1.29 | 29.74 | $2.91\pm 4.15$ |

Means having same superscript letters aren't significant at P = 0.05

### 3.2.Histamine producing bacterial count (HPBC).

The HPBC of Sardine, Bouge, Saury and Mackerel collected from the three markets ranged between $5 \times 10^2 – 1.4 \times 10^6$ , $2 \times 10^3 – 2.6 \times 10^6$, $2 \times 10^3 – 2.6 \times 10^6$ and $5 \times 10^2 – 2.7 \times 10^6$cfu / g meat respectively (table 7). The lowest value for HPBC $5 \times 10^2$cfu / g fish meat was observed in muscle tissues of Sardine and Mackerel samples collected from the fish market B While, the highest HPBC value $2.7 \times 10^6$cfu / g fish meat was recorded in the muscle tissues of mackerel samples collected from the fish market C. The results of the statistical analysis for the HPBC in Sardine, Bouge, Saury, and Mackerel among the three fish markets were not significantly different (P>0.05).

**Table 7.** The counts (cfu/gm) of histamine producing bacteria (HPBC) in Sardine, Bouge, Saury and Mackerel collected from the three markets examined

| Fish type | | Sardine | Bouge | Saury | Mackerel |
|---|---|---|---|---|---|
| Fish Market (A) | Min | $3.0\times10^3$ | $6.0\times10^2$ | $1.0\times10^4$ | $5.0\times10^2$ |
| | Max | $1.4\times10^6$ | $1.1\times10^6$ | $1.5\times10^6$ | $5.7\times10^4$ |
| | Mean | $2.8\times10^5$ | $2.0\times10^5$ | $2.6\times10^5$ | $1.6\times10^4$ |
| Fish Market (B) | Min | $5.0\times10^2$ | $2.0\times10^3$ | $2.0\times10^3$ | $5.0\times10^2$ |
| | Max | $8.2\times10^4$ | $2.8\times10^5$ | $2.8\times10^5$ | $2.6\times10^5$ |
| | Mean | $1.8\times10^4$ | $6.4\times10^4$ | $6.4\times10^4$ | $4.2\times10^4$ |
| Fish Market (C) | Min | $1.0\times10^3$ | $8.0\times10^3$ | $1.3\times10^4$ | $5.0\times10^2$ |
| | Max | $2.1\times10^5$ | $2.6\times10^6$ | $2.6\times10^6$ | $2.7\times10^6$ |
| | Mean | $5.4\times10^4$ | $5.7\times10^5$ | $6.9\times10^5$ | $4.1\times10^5$ |

*Means of HPBC in each fish types are not significantly different between the three markets (P > 0.05).

### 3.3.Identification of HPB isolated from fish samples

According to the identification tests for the isolates from the fish samples, twenty-six (26) bacterial types were identified as HPB. Most of these isolates belong to the family *Enterobacteriaceae*, which are not indigenous to the marine environment, and some belong to *Vibrionaceae*. The results presented in table 8 indicated that the prevalence percentages of *V. fluvialis, Erwiniaspp, S. putrefaciens,* and *K. planticola* were 18.3, 13.2, 11.9, and 10.0 % respectively, while the prevalence percentages of *M. morganii, P. aeruginosa* and *A. baumaii* were almost equal i.e.6.40, 5.90, and 5.50 %respectively. The prevalence percentages of

other isolates were lower and ranged between 0.45–3.20 %.

Variations were observed in the prevalence percentages of most types of HPB isolates during the period of the study and even for the same type of bacteria, since the prevalence percentages of *V. fluvialis* during the months of July, August, September, October, November, and December were 21.0, 39.0, 30.0, 42.0, 30.0 and 25.0 % respectively, while those of *Erwiniaspp* were 29.0, 8.0, 21.0, and 9.0 respectively. However, the occurrence of *S. putrefaciens* and *P. aeruginosa* was only recorded in samples collected during November and December with the percentages 12.0 and 13.0 %, respectively (figure 1).

**Table 8.** The prevalence percentages (%) of histamine producing bacteria in fish samples collected from three fish markets located within Tripoli city, Libya.

| Type of bacteria | fish market (A) | fish market (B) | fish market (C ) | % of total isolates |
|---|---|---|---|---|
| *V. fluvialis* | 23.70 | 17.70 | 24.30 | 18.30 |
| *Erwiniaspp* | 13.40 | 9.70 | 17.40 | 13.20 |
| *S. putrefaciens* | - | 14.20 | 14.50 | 11.90 |
| *K. planticola* | 9.80 | 7.08 | 17.40 | 10.00 |
| *M. morganii* | 4.90 | 5.30 | 8.70 | 6.40 |
| *P. aeruginosa* | - | 7.96 | 5.79 | 5.90 |
| *A.baumanii* | 9.80 | 4.40 | 5.80 | 5.50 |
| P. flouorscens | - | 5.31 | 1.45 | 3.20 |
| Pantoeaspp | - | 6.20 | - | 3.20 |
| *K. pneumonia* | 6.60 | 0.88 | 2.90 | 2.87 |
| *E. cloacae* | - | 1.80 | 5.80 | 2.58 |
| *A. hydrophila* | 3.30 | 3.50 | - | 2.18 |
| *P. mirabilis* | - | 2.70 | 1.50 | 1.78 |
| *O. anthropic* | - | 2.65 | 1.45 | 1.71 |
| *K. oxytoca* | - | 2.65 | - | 1.75 |
| *B. cepacia* | - | 2.65 | - | 1.30 |
| *S. plymuthica* | 8.10 | - | - | 0.90 |
| *Brucellaspp* | - | 0.90 | 1.50 | 0.86 |
| *P. vulgaris* | - | 1.77 | - | 0.86 |
| *S. liquefaciens* | 4.30 | - | - | 0.86 |
| *S. maltophilia* | - | 0.90 | 1.45 | 0.86 |
| *R. aquatilis* | - | 1.88 | - | 0.86 |
| *P. alcalifacients* | - | - | 5.41 | 0.86 |
| *P. rettgeri* | - | - | 3.31 | 0.45 |
| *E. coli* | - | - | 3.30 | 0.45 |
| *C. freundii* | 1.45 | - | - | 0.45 |

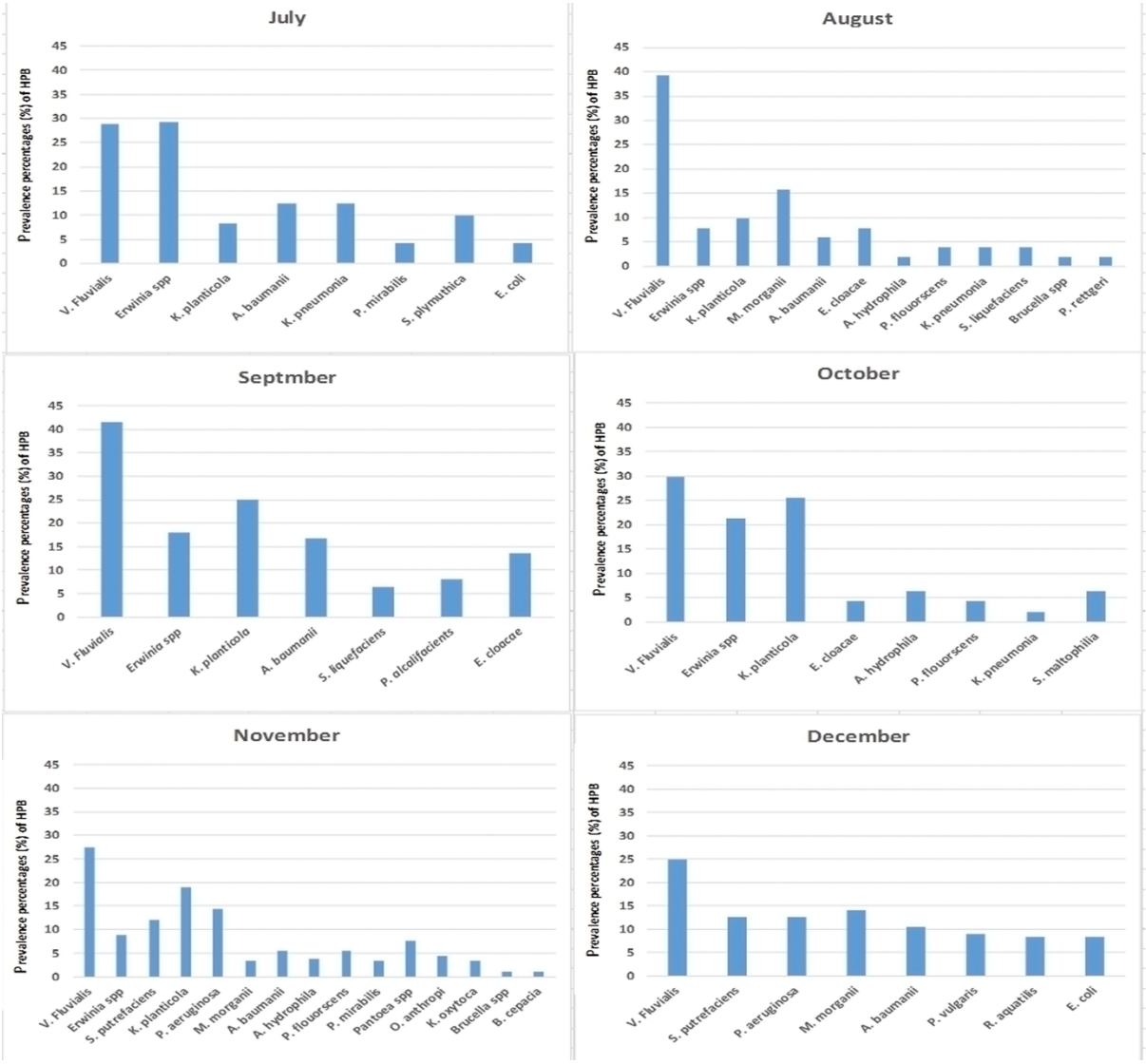


**Figure1.**Prevalence percentages (%) of HPB in fish samples collected during the period from July to December.

## 3.4.Discussions

### *3.4.1. Fish temperature and histamine contents.*

Fish is a perishable food, which needs immediate cooling from the moment of catching until received by the consumer (from boat to throat) to assure its safety. The fish types included in this study (Sardine, Bouge, Saury, and Mackerel) are the most popular fish consumed in Libya,because of their reasonable prices and availability during their fishing season, which lasts from May to December. These fish species are susceptible to histamine formation due to their high contents of the free amino acid histidine that is the precursor for histamine formation by the decarboxylase enzyme through bacterial action when exposed to temperature /time abuse (Kim et al. 2004; Moreno et al. 2001 andRawles, 1996).

The results from this study showed that 95% of the collected fish samples (107/113) had a temperature range between 5 to $22^0$ C,5% of the fish samples (6/113) had a temperature below $5^0$ C (table 2). These results reflect the poor cooling conditions of the fish samples, and the poor cooling techniques practiced in the three markets, especially for those who depend solely on ice because of melting by the end of the day. These conditions will render fish samples more susceptible to histamine formation. The proper

icing and /or cooling practices of these types of fish should be below $5^0$ C, and as close as possible to the melting point of ice to keep them in good quality and not suitable for histamine formation.

The results presented in Table 3 showed that 43% of the fish samples collected from the three markets contained histamine. The temperatures of these samples ranged between< 5 to $22^0$ C, and more than 50% of these samples had a temperature range between 5 to $14^0$ C. Even though histamine was recorded in 43% of all samples examined in this study, the levels did not exceed the maximum limit (10 mg %) adopted by the Libyan authority, except one sample. However, the presence of histamine in the samples indicates the fact that these samples were exposed to temperature-time abuse somewhere during handling. This is supported by the findings that 41 and 52% of the samples collected in the morning and noontime respectively contained histamine. In addition to that, the lowest percentages of samples contained histamine were recorded in fish samples that had a temperature < $5^0$ C, while the highest percentage was observed in samples that had temperature range between 5 – $14^0$ C, and the highest mean of histamine content($3.84 \pm 6.64$ mg %) was recorded in the samples that had temperature range between 15 to $22^0$ C. Kim et al.(2002) and Lehan et al. (2000) reported that histamine production in fish starts at $\geq 5^0$ C, and the optimum temperature for histamine formation range from 20 to $30^0$ C in the presence of HPB that belong to *Enterobacteriaceae.* However, Auerswald et al. (2006) pointed out that > $15^0$ C is the optimum temperature for HPB growth and production of decarboxylase enzyme. Meanwhile, Kim et al. (2003) reported that $25^0$ C is the optimum temperature for histamine production in fish.

Wide variations in the percentages of histamine levels in Sardine, Bouge, Saury, and Mackerel samples were observed as shown in table 5. However, a non-significant difference was recorded between them. These results reflect the randomness of these samples in terms of handling conditions to which they were exposed from catching until the time of sample collection. This is clear from the high value of standard deviations for the mean of histamine content ($2.9 \pm 4.15$ mg %) in these samples (table 3).

One-way analysis of variance for the histamine content in the fish samples among the three fish markets showed significant differences ($p < 0.05$) (table 6). The mean value of histamine content in the fish samples collected from the fish market A 7.14 mg % was significantly different from the means values for histamine contents in the fish samples collected from the other two markets (table 6). These differences could be related either to variations in icing or cooling practices applied during handling and/ or to the fact that one of the fish samples collected from the fish market showed a high histamine content 29.74 mg % and this might contribute to the overall mean value of these group of samples from the fish market A.

The range of histamine content found in this study for mackerel samples was 1.39 – 2.49 mg %, which is higher than that reported by Lokuruk et al. (2006), in samples of Mackerel (Scomber*scomber*) collected from Brooklyn port in New York city, USA where the range of histamine was 0.20 – 0.21 mg %, and with the study of Fletcher et al. (1995) where the histamine contents in Mackerel *(Scomberaustralasicus*) samples did not exceed 1 mg %. The results of the histamine contents in Mackerel samples in this study were lower than that reported by Okuzumi et al. (1982) in fresh Mackerel (Scomber japonicas) samples collected from markets in Tokyo- Japan where the histamine contents ranged 2 – 87.2 mg %, and by Joshi et al. (2011) 20 – 30 mg % in Indian Mackerel (*Rastrelliger Kamasutra*) samples collected from local markets in Kalyan city-India.

The range of histamine levels (1.31 – 7.57 mg %) reported in this study for Saury fish sample was lower than that recorded in Saury (*Saira cololabis*) fish samples collected from Korea and Japan markets where the histamine levels ranged between 0.01 – 31.43 and 2.0 – 144 mg %respectively (Kim et al. 2009 and Okuzumi et al. 1982).

The variations between the results of histamine contents obtained in fish samples collected from the three local markets and that recorded in fish samples of other regions could be related to the variations in handling practices (time /temperature) that these fish samples were exposed to from catching until sample collection.

### *3.4.2. Histamine producing bacterial count (HPBC).*

The range for the means of HPBC in Sardine and Mackerel samples in this study were 1.8 x $10^4$ – 5.4 x $10^4$ and 1.6 x $10^4$ – 4.1 x $10^5$cfu / g fish meat respectively, which are higher than those (2.5 x $10^3$, 2.1 x $10^3$ and 2.2 x $10^3$cfu /g fish meat) reported by Korashy et al. (2005) in samples of Sardine (Sardinella*gibbosa*), European Sardine (Sardinella*pilchardus*) and Atlantic Mackerel (Trachurustra*churus*) respectively. The results of this study were also higher than those reported by Okuzumi et al. (1982) where the range of HPBC for fresh Sardine (*Sardinellamelanostic*), Saury (*Coloabissaira*) and in Japan Mackerel (*Scomber japonicas*) was 1.1 x $10^4$ – 3.0 x $10^4$, 5.7 x $10^3$ – 2.1 x $10^5$ and 1.0 – 1.0 x $10^2$ (estimated) cfu / g respectively. Additionally, Lopez-Sabater et al. (1996) found that HPBC in Mackerel was 3.1 x $10^2$cfu / g fish in Spain, which is lower than the counts recorded in this study.

Statistical analysis of the results for HPBC in each fish species among the three markets (table 7) showed non-significant differences ($P > 0.05$). This might be related to the randomness of the collected samples and the unknown variations in handling conditions that these fish species were exposed to from fishing boats until collected from the three markets.

### *3.4.3 HPB isolates.*

The results from this study showed that most of the HPB isolates from the fish samples belong to the family *Enterobacteriaceae* that are not indigenous to the marine environment and some belong to*Vibrionacea* (table 8). These findings agree with the results of Economou et al. (2007), Emborg et al. (2005), Ababouch et al. (1991) and Taylor (1986), where they found that most of the histamine producing bacteria in fish belong to the family *Enterobacteriacea*e such as *Klebsiella pneumonia, E. coli, Morganellamorganii, Enterobacterclocacae, Serratia marcescens and Hafnia alvei.*

The prevalence percentages of the bacterial isolates presented in table (8) ranged between 3.20 and 18.30%. The highest percentage (18%) was recorded for *V. fluvialis* followed by *Erwiniaspp, S. putrefaciens,* and *K. planticola* where their prevalence percentages were 13.2, 11.9, and 10.0% respectively. The prevalence percentages of *S.putrefaciens* and *P.fluoroscens* in the Sardine samples were 11.9 and 3.2% out of the total isolates respectively. These percentages are resembling those (10 and 20%) reported by Ababouch et al. (1991) in Sardine (*Sardinella pilchardus) caught from Atlantic coast.*

When comparing the results of this study with that of Economou et al. (2007) where, 77 types of HPB were isolated, which account for 53% of the total number of bacteria in 30 samples of fresh and frozen Albacore tuna (*Thunnusalalongua*) collected from five countries. There was a similarity in types of bacteria isolated from the samples among which were*P.fluorescens, P. aeruginosa, E.coli, B. capacia.* The observed differences were in their prevalence percentages, which were higher in the tuna samples compared to the fish samples of this study.

The variations in the prevalence percentages of the most types of HPB isolates during the period of the study and even for the same type of bacteria illustrated in figure (1), reflect the important effect of the month of the year on the type of bacteria found on the fish samples. These findings are in good agreement with those of Yoshinga et al. (1982) and Kim et al. (2009). Furthermore, the results obtained from this study are in agreement with the results of Yagoub (2009) who found that 53.3% of isolated bacteria from fresh fish in Khartoum, Sudan belong to *Enterobacteriaceae* and the incidence percentage of species belong to this family during summer, autumn and winter were 60, 33 and 20% respectively.

## 4.Conclusions

The obtained results indicated that the fish species included in this study were exposed to time-temperature abuse during handling and display, since only 5% of the samples collected, had a temperature below $5^0$ C and 43% out of the total samples contained histamine. Most of the isolated HPB belong to the family *Enterobacteriaceae* and some to *Vibrionaceae*, and their prevalence depended on the month of the year. Therefore, it is strongly recommended to set up a plan to improve the handling practices and display conditions of such fish types to keep them safe from boat to the throat.

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# TOXIC RISKS ASSOCIATED WITH APITHERAPY PRODUCTS

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**ABSTRACT**

The history of honeybee products utilization for medicinal applications dates back thousands of years. Today, the benefits attributed to these products by traditional medicine are confirmed by scientists, research data revealing antibacterial, antifungal, anti-inflammatory, cytotoxic and antihepatotoxic activities for honey, propolis, royal jelly and bees' pollen. The paper addresses the problem of toxicological risk assessment of honey and related products. Although there is substantial scientific data to sustain the use of apitherapy as a prophylactic tool, as well as treatment for several medical conditions, the quality and safety of these products needs to be carefully assessed. Several contaminants and toxic compounds have been identified in honeybee products (phytotoxins, heavy metals, pesticides, antibiotics, 5-hydroxymethylfurfural, mycotoxins), and, in some cases, the presence of these compounds was associated with severe outcomes.

## 1. Introduction

Alternative medical therapies include a broad spectrum of practices and beliefs. Biologic-based practices (dietary therapy, herbal medicine, and dietary supplements – nutraceuticals) are one of the broadest categories, relying on the use of chemical substances or dietary alterations to promote healing. Apitherapy is a form of Complementary and Alternative Medicine that uses honeybee products, such as bees' pollen, honey, royal jelly, propolis, beeswax, and bee venom, for therapeutic purposes (Diehl and Eisenberg, 2000). The history of honeybee products use for medicinal applications dates back thousands of years. Today, honey is classified as a functional food. Scientific research demonstrated the antimicrobial, anti-inflammatory, and antioxidant potential of the product, investigating its efficiency in wound healing (Oryan *et al.,* 2016), hepatic and renal protection against different aggressive agents (eq. $CCl_4$) (El-Haskoury *et al.,* 2018), gastrointestinal protection (Mundo *et al.,* 2004; Nasuti *et al.,* 2006) and immunostimulatory effect (Ota *et al.,* 2019). Propolis is a complex resinous substance collected by bees, also containing salivary secretions and enzymes, and it is used for protection against invading insects and microorganisms and in beehives repair. It is a rich source of essential elements (Ca, Mg, Fe, Cu, Zn, Mn, Ni), vitamins, and phenolic compounds (caffeic acid phenethyl ester being one of the most studied compounds found in propolis), but the composition varies according to specific flora at the site of collection (Rufatto *et al.,* 2017). Scientific data revealed that propolis exhibits antibacterial, antifungal, anti-inflammatory, and cytotoxic activities (Rufatto *et al.,* 2017; Dobrowolski *et al.,* 1991). Bee pollen is a product that results from the agglutination of flower pollens with nectar, combined with salivary bees' secretions. It represents a good source of bioactive substances and energy and also possesses many therapeutic and protective effects: antimicrobial, antifungal, antioxidant, anti-inflammatory, anti-carcinogenic, anti-allergic, hepatoprotective,

improving the cardiovascular and digestive systems, immunity booster, and aging delaying (Pascoal *et al.,* 2014; Huang *et al.,* 2017; Li *et al.,* 2018). Royal jelly is a honeybee secretion product used to feed the larvae and adult queens. Studies have indicated antioxidant, anti-inflammatory, anti-hepatotoxic, and anticancer activities for royal jelly (Pasupuleti *et al.,* 2017). There is substantial scientific data to sustain the use of apitherapy as a prophylactic tool, as well as treatment for several medical conditions, but one very important aspect that needs to be assessed is the safety of these products. Quality parameters must be imposed for all nutraceuticals and dietary supplements.

## 2. Contaminants and toxic compounds in honeybee products

### 2.1. Phytotoxins

The composition of honeybee products varies greatly depending on the flora at the site of collection. Some secondary plant metabolites (pyrrolizidine alkaloids, grayanotoxins, hyoscyamine, hyoscine, saponin, strychnine, gelsemine, tutin, hyenanchin, oleandrin, and oleandrigenin) can be transferred to honey and related products, leading to toxic effects in humans (Figure 1) (Grigoryan, 2016).

Pyrrolizidine alkaloids are one of the most common natural toxins, being identified in over 6000 plants. They possess genotoxic and hepatotoxic effects. Contaminated honey is a possible source of intoxication. The ingested dose is usually not high enough to cause acute poisoning, but a long-time consumption of low doses of pyrrolizidine alkaloids can cause liver fibrosis, pulmonary arterial hypertension, somatic mutation, and liver cancer. Fetuses and neonates are more susceptible to pyrrolizidine alkaloids poisoning, even at extremely low levels (Zhu *et al.,* 2018).

Tutin is a plant-derived neurotoxin, responsible for many intoxication cases associated with honey consumption; clinical signs include nausea, headache, vomiting, dizziness, and in severe cases seizures and coma. These cases are common in New Zealand, where *Coriaria arborea* (tutu), which is known to contain tutin, grows (Fields *et al.,* 2014).

Grayanotoxins are often found in "mad honey", produced by bees from the nectar of *Rhododendron ponticum*, a member of the *Ericaceae* family, which is used in indigenous medicine, especially in the treatment of hypertension and sexual dysfunctions. This phenomenon was known from ancient times (Xenophon recorded the intoxication of Greek soldiers stationed on the Black Sea coast), but its prevalence is still high, especially in Turkey, where "mad honey" can be purchased in local markets. The common symptoms of intoxication are dizziness, bradycardia, nausea, vomiting, presyncope and syncope, blurred vision, hypotension, and fainting (Silici *et al.,* 2015; Demircan *et al.,* 2009). Besides "mad honey poisoning" described in Turkey, Nepal, and Korea, and "Tutin honey poisoning" described in New Zealand, there is another type of honey poisoning caused by *Tripterygium wilfordii* Hook F. It occurs mainly in southwestern China, and it is characterized by multiple-organ damage and high mortality. The toxic potential of the plant is mainly related to the presence of triptolide – a highly toxic substance – whose major target is the kidney, acute renal failure being the main cause of death (Zhang *et al.,* 2016; Zhang *et al.,* 2017).

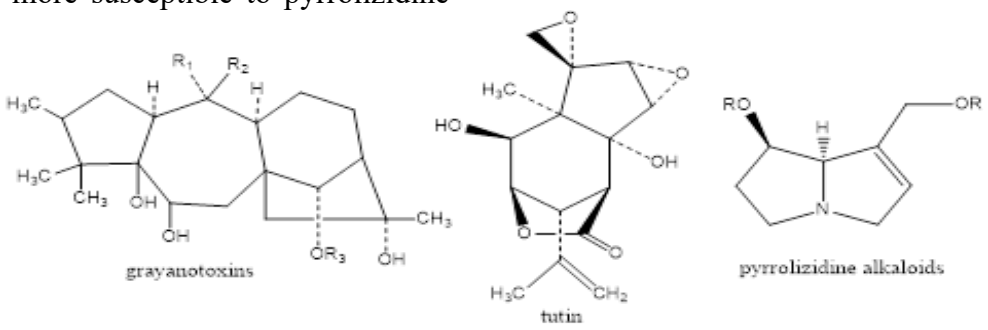


**Figure 1.** Phytotoxins commonly found in honeybee products

## 2.2. Toxic elements – Heavy metals and metalloids

Heavy metals pollution is a serious problem nowadays around the world, affecting the quality of the atmosphere and waters and also posing a threat to the health and life of human beings and animals via the food chain (Aghamirlou *et al*., 2015).

The content of toxic elements (heavy metals and metalloids – Table 1) in honeybee products depends on many factors: floral source (some plant species can selectively accumulate toxic elements), environmental contamination and conditions (geo-climatic conditions, geochemical characteristics of the soil, anthropogenic activities – eq. chemical industries in the vicinity) and production methods. Therefore, bees and their products can often serve as bio-indicators for heavy metal contamination. Many studies on this matter were conducted and the results showed significant differences in element concentrations between honey products of different botanical origins and from different geographic areas (Czipa *et al.,* 2015; Bilandžić *et al.,* 2017). Two of the most frequently studied metals are lead and cadmium. Lead originates mainly from motor traffic, contaminates the air, and then nectar, and honeydew. It is not generally transported by plants, unlike cadmium, which is transported from the soil to plants, also contaminating nectar and honeydew. Lead pollution is expected to diminish, due to the reduction of toxic car emissions worldwide, associated with the introduction of car-engine catalysts (Bogdanov, 2006).

The quantities of heavy metals accumulated in different apiculture products can vary. Honey usually has a lower degree of contamination than other products, probably due to bees' "filtering" capacity, but lead residues found in propolis are often high, indicating that the harvest area should not be placed near heavy traffic roads (Bogdanov, 2006).

**Table 1.** Toxic elements identified in honeybee products

| Toxic element | Toxic effects | References |
|---|---|---|
| Pb | • neurotoxicity<br>• gastrointestinal and renal dysfunctions | Aldgini *et al.,* 2019; Ru *et al.,* 2013; Aljedani, 2017; Talk Gajger *et al.,* 2016; Kieliszek *et al.,* 2018 |
| Cd | • gastrointestinal and renal dysfunctions<br>• osteomalacia and osteoporosis<br>• carcinogenic effects | Aldgini *et al.,* 2019; Ru *et al.,* 2013; Aljedani, 2017; Kieliszek *et al.,* 2018 |
| Hg | • gastrointestinal, hepatic and renal damage<br>• neurotoxicity | Aghamirlou *et al*., 2015; Ru *et al.*, 2013; Talk Gajger *et al.,* 2016; Kieliszek *et al.,* 2018 |
| Al | • toxic effects on the central nervous, skeletal and hematopoietic systems | Bilandžić *et al*., 2017 |
| Cr | • carcinogenic and teratogenic effects<br>• nephrotoxic and hepatotoxic effects | Harmanescu *et al.,* 2007 |
| Ni | • sensitization and allergic contact dermatitis<br>• hepatotoxic and nephrotoxic | Bilandžić et al., 2017; Aldgini *et al.,* 2019 |
| As | • carcinogen (skin, kidney, lung, bladder, and liver cancers) | Aldgini *et al.,* 2019; Talk Gajger *et al.,* 2016; Kieliszek *et al.,* 2018; Fiorentini *et al.,* 2019 |
| Ba | • acute toxicity: low potassium levels, cardiac arrhythmia, gastrointestinal dysfunction or paralysis, muscle twitching, and high blood pressure<br>• chronic exposure: kidney damage, respiratory failure, the development of neurodegenerative diseases, including multiple sclerosis | Bilandžić *et al*., 2017 |
| Be | • carcinogen to humans | Bilandžić *et al*., 2017 |
| Sb | • acute cardiac toxicity and myocarditis<br>• prolonged skin contact – dermatitis kidney and liver damage, vomiting | Bilandžić *et al*., 2017 |

### 2.3. Pesticides

Pesticides are indispensable for today's agriculture, increasing crop productivity and minimizing losses due to uncontrollable pests, but exposure to pesticides has several negative consequences for all living beings, including humans. Pesticide residues present in the environment, but mostly in food products, can cause skin rashes, respiratory disorders (asthma attacks); chronic exposure is often associated with cancer, neurological and reproductive dysfunctions, and birth defects (Gil *et al.,* 2016).

Bee products can also be a source of pesticide exposure, due to both environmental pollution (honeybees are exposed by consumption of contaminated pollen and water or by contact with plants and soil) and wrong beekeeping practices (administration of pesticides and antibiotics, in order to control hive infestations).

Organohalogens, organophosphates, organonitrogens, pyrethroids, carbamates are examples of pesticide residues most frequently reported in honey and related products (Figure 2), in countries like Brazil, Turkey, Spain, Colombia, China, France, Portugal, and India (López *et al.,* 2014). Organophosphates and organohalogens occupy the first positions in terms of detection frequency. Although organochlorine pesticides (eq. lindane) have been prohibited by law for decades in most countries, their residues are still present as pollutants in water, soil, air, and food products, due to their high persistence. Organochlorine pesticides are lipophilic substances, soluble and stable in beeswax, and an amount of these substances gradually migrates from wax into the stored honey (Gaweł *et al.,* 2019). Bee pollen samples are also a good indicator for environmental monitoring, many studies being focused on quantifying pesticide residues in pollen samples (de Oliveira *et al.,* 2016). However, the distribution of pesticides in different apiculture products is heterogeneous. There are studies indicating that, in some cases, pesticides, especially herbicides, can contaminate bees and pollen and rarely appear in honey. This effect probably appears due to the filtering capacity of bees (Fléché *et al.,* 1997). Similar results were obtained when pesticide levels in wax, pollen, and honey were compared. Beeswax was the most contaminated hive compartment regarding quantities of pesticides detected (Jan *et al.,* 1993; Calatayud-Vernich *et al.*, 2018). Some authors also argued whether honey consumption represents an important source of pesticide exposure and a threat to human health, contributing substantially to the daily intake of pesticides, based on the consumed quantities and the degree of contamination, which is generally very low. However, different national regulations have established maximum concentrations of pesticide residues permitted in honey and related products, but the lack of homogeneity causes problems in international trade. The maximum limits of pesticide residues in honey are not included in the Codex Alimentarius (Grigoryan, 2016; Al-Waili *et al.,* 2012). Maximum residue limits (MRL) were also established in the European Union for several pesticides used in agricultural and beekeeping practices (Table 2). In the EU, an action level of 0.01 mg/kg is often considered for pesticides with no fixed MRL (***, 2005).

It is important to control bees' exposure to pesticides because bees end up inhabiting a toxic hive, exposed to different pesticides cocktails, and their health is seriously affected. Another concern is related to the safety of bee products consumers. In this context, Yuan *et al.* investigated the photodegradation phenomenon of organophosphorus pesticides in honey medium, concluding that photodegradation could become an accepted method for organophosphorus pesticides removal from honey (Yuan *et al.,* 2014).

**Table 2.** Maximum levels of pesticide residues for honey and other apiculture products – Regulation (EC) No 396/2005

| No | Pesticide | MRL (mg/kg) | No | Pesticide | MRL (mg/kg) |
|---|---|---|---|---|---|
| 1 | Asulam | 0.05* | 20 | Fenpyroximate | 0.01* |
| 2 | Azoxystrobin | 0.05* | 21 | Fipronil | 0.01 |
| 3 | Bicyclopyrone | 0.05* | 22 | Fluopyram | 0.05* |
| 4 | Boscalid | 0.5 | 23 | Fluoxastrobin | 0.01* |
| 5 | Bromopropylate | 0.1 | 24 | Flutolamil | 0.02* |
| 6 | Cabendazin + Benonyl | 1 | 25 | Fosetyl | 0.5* |
| 7 | Chlordane | 0.01 | 26 | Fosetyl-Al | 0.5* |
| 8 | Chlormequat | 0.05* | 27 | Gibberellic acid | 0.1 |
| 9 | Cyprodinil | 0.05* | 28 | Haloxyfop | 0.05 |
| 10 | DDT | 0.05 | 29 | Heptachlor | 0.01 |
| 11 | Diclofop | 0.01* | 30 | Isoprothiolane | 0.05* |
| 12 | Difenoconazole | 0.05* | 31 | Isopyrazam | 0.05* |
| 13 | Endosulfan | 0.01* | 32 | Lindane | 0.01* |
| 14 | Endrin | 0.01 | 33 | Mepiquat | 0.05* |
| 15 | Epoxiconazole | 0.05 | 34 | Oxamyl | 0.05* |
| 16 | Etofenprox | 0.05 | 35 | Prothioconazole | 0.05* |
| 17 | Fenoxaprop-P | 0.05 | 36 | Spinetoram | 0.05* |
| 18 | Fenpropidin | 0.02* | 37 | Trifloxystrobin | 0.05* |
| 19 | Fenpropimorph | 0.05* | 38 | Triflumezopyrim | 0.05* |

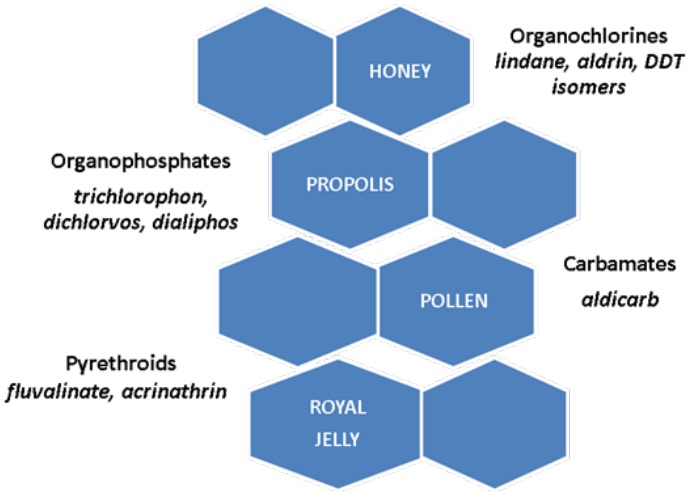


**Figure 2.** Common pesticides found in bee products

### 2.4. Residues of veterinary medicinal products – Antibiotic residues

Exposure of the general population to antibiotics can have negative effects, like allergenic reactions, or even contributing to the expansion of the increased bacterial resistance phenomenon. Therefore, the use of antibiotics is forbidden in beekeeping practice in the EU, due to the risk of antibiotic residues in honey. There are many studies regarding the monitoring of antibiotic residues in honey. Although EU directive 2377/90 states that honey should be free of antibiotic contamination, research in this domain revealed that many analyzed samples of honey contained traces of antibiotics, generally used against bacterial plant pests. Typically found antibiotics residues include streptomycin, sulphonamides, chloramphenicol, macrolides,

tetracycline, streptomycin, and nitrofurans (Grigoryan, 2016; Al-Waili *et al*., 2012).

### 2.5. 5-Hydroxymethylfurfural

5-Hydroxymethylfurfural is an indicator of the poor quality of food products, and it is usually formed during the heating or preservation of honey. There are several factors that favor the formation of 5-hydroxymethylfurfural: use of metallic containers for storage, physicochemical properties of honey (eq. acidity), humidity, and thermal and photochemical stress. Due to the mutagenic, carcinogenic, and cytotoxic potential, the amount of 5-hydroxymethylfurfural is limited by EU regulations to 40 mg/kg (exceptions: 80 mg/kg in honey from regions with tropical temperatures, and 15 mg/kg in honey with low enzyme levels) (***, 2001). Ample research was performed in this direction, investigating the level of 5-hydroxymethylfurfural in honey samples from different countries (like Spain or The Czech Republic), the results indicating that honey is unlikely to exceed the 40 mg/kg limit unless it is mishandled, commercial honey-processing methods (eq. heating above 15°C) being usually incriminated for the appearance of undesirable compounds, including 5-hydroxymethylfurfural (Grigoryan, 2016).

### 2.6. Genetically modified organisms (GMOs)

The appearance of genetically modified plants, grown in some countries, has raised new concerns about the impact of this phenomenon on apiculture, in terms of the marketability of bee products. In the European Union, it is compulsory to notify the consumers if the GMO content in food is above 1%. Pollen is the most affected bee product, because it contains genetic material, while honey contains only a small percentage of pollen (less than 0.1% pollen, if the honey is sieved) and probably will not require any specific appellations (Grigoryan, 2016; Bogdanov, 2006).

### 2.7. Microbiological contaminants and mycotoxins

In terms of microbiological contaminants, bacterial risks associated with honey consumption are usually low, but honey has been identified as a dietary risk factor for infant botulism. *Clostridium botulinum* is a Gram-positive, anaerobic, spore-forming bacterium that produces a neurotoxin. Honey samples can contain *Clostridium botulinum* spores, but botulinum toxin is not found in honey, because the natural antimicrobial potential and the high osmotic pressure prevent spores' germination. However, the spores can colonize the undeveloped gut of infants, leading to infant botulism, a rare neuroparalytic disease that can occur among babies under 1 year of age (Grigoryan, 2016).

Aside from *C. botulinum*, mold, yeasts, and other spore-forming bacteria can be found in honey. The main sources of contamination are the environment and not respecting good manufacturing practices, contamination with fungi and bacteria usually indicates inadequate hygiene conditions during collection, processing, and storage (Grigoryan, 2016; Bogdanov, 2006). Mycotoxins are toxic compounds produced by fungi that colonize different nutritional substrates. Bee products represent an adequate medium for the development of fungi, thus being subjected to the risk of accumulating mycotoxins. Among mycotoxins, aflatoxins and ochratoxin A are the most common, being involved in both acute and chronic intoxications (González *et al.,* 2005).

Aflatoxins, produced by species of *Aspergillus*, are hepatotoxic, teratogenic, mutagenic, and carcinogenic mycotoxins. Ochratoxin A, also produced by species of *Aspergillus* (eq. *Aspergillus ochraceus*), is nephrotoxic, hepatotoxic, teratogenic, and immunotoxic, being associated with fatal endemic human nephropathies (González *et al.,* 2005). Unprocessed honey is a poor medium for synthesis of mycotoxins, and it is relatively safe, from this point of view (Martins *et al.,* 2003;

Eissa *et al.*, 2014), but bee pollen was found to be a substrate that stimulates ochratoxin A production by *Aspergillus ochraceus*. The levels of mycotoxin found in the incubation medium containing bee pollen was significantly higher than in corn, wheat, and rice grains (Medina *et al.,* 2004). González G *et al*. analyzed the presence of mycotoxins and mycotoxin-producing fungi in bee pollen and stressed the importance of application of good manufacturing practices, sanitization procedures, and analysis of risks and critical control points to honeybee products (González *et al.,* 2005).

## 3. Conclusions

The benefits associated with the consumption of honey and other related bee products are unquestionable. As highlighted above, scientific research revealed antimicrobial, anti-inflammatory, antioxidant, immunostimulatory activities for these products, promoting general health and well-being. Despite these considerations, it is also important to point out the possibility of contamination with several toxic compounds, which can lead to grave outcomes in some cases. If for honey, the most popular bee product, intensely consumed around the world, quality criteria are usually clearly specified, when it comes to other related products, many quality regulation gaps can be found. Another problematic issue is represented by the existence of different national standards for honey produced and commercialized in different parts of the world (European countries, the United States, Canada, Australia, and India have separate standards), making difficult the elaboration of general guidelines for quality assessment.

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# COMPARATIVE THE ANTIOXIDANTS CHARACTERISTICS OF ORANGE AND POTATO PEELS EXTRACTS UNDER DIFFERENCES IN PRESSURE AND CONVENTIONAL EXTRACTIONS

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**ABSTRACT**

This investigation aimed to decrease the extraction time of natural antioxidants and add commercial dimension to plant extracts. Impact the difference in pressure (DE) on antioxidant properties was studied by estimating total phenolic and total flavonoid contents (TPC, and TFC), DPPH˙ scavenging radical activity ($IC_{50}$ ), inhibition lipids peroxidation by both TBARs and β-Carotene/Linoleic acid bleaching (βCB) assays, antimicrobial activities, and yield of extracts, comparison with the resulting by conventional extractions (SE). The results showed positive effects of OPE, and PPE on antioxidants and antimicrobial activities, and the extracts of DE were the highest value to both orange and potato peel extracts. However, increase the yield of extracts and TFC by the decrease of ethanolic concentration of both orange and potato peel extracts, TPC, DPPH˙ scavenging radical activity, TBARs, βCB, and antimicrobial activity was increased by the increase of ethanolic concentration, and the extraction by DE was the highest value. The absolute ethanolic potato peel extract by soak extraction method (SE) was the lowest value of yield of extract and TFC (21.38±1.08, and 29.73±1.03; respectively), while absolute ethanolic orange peel extract by extraction method by DE was the highest value of TPC, DPPH˙ scavenging radical activity ($IC_{50}$ ), TBARs, and βCB (262.19±1.19, 21.18±1.18, 78.82±0.85, and 83.15±1.15; respectively). Also, the effect of absolute ethanolic orange peel extract by the difference in pressure on antimicrobial activity was the highest.

## 1.Introduction

In recent years, due to the insufficiency of resources of food, it's become interesting to the utilization of food wastes resulting from several sources and how and/or what is the benefit of re-usage as the feedstock of many products. In general, agricultural and food factories waste used in animals' feeds, the results indicated to roughly one of third of food products lost or wasted globally, which amounts to about 1.3 billion tons per year, approximately, and of course, a huge amount of resources and emission of gas caused by food production are also emission in vain (FAO, 2018). The wastes result from agricultural and food factories considered raw material to many products for it has minerals, pigments, vitamins, other phytochemical compounds have antioxidants and antimicrobial activity, and enough amount of starch.

By-products of food manufactory, such as pomace and peels, represent an abundant source of bioactive compounds. In many cases, these by-products are not used to their potential Besides, the transaction with waste and by-products in a sustainable and environmentally friendly way is becoming a highly important issue in the food manufactory. Due to the European Landfill Directive, the food industry is

obliged to decrease the percentage of waste and by-products going to landfills by 2020 (Kosseva, 2009).

Antioxidants are utilized to elongate shelf-life and preserve the nutritional quality of lipid-containing foods as well as to modulate the consequences of oxidative damage in the human body (Halliwell et al., 1995). The use of synthetic antioxidants such as tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) in maintaining foods is now prohibited or under strict regulation in many countries because of their associated toxic and carcinogenic side effects (Buxiang and Fukuhara, 1997; Jo et al., 2006). The increased demand for natural foods nowadays has bound the food industry to include natural antioxidants in foods. Natural antioxidants have been used instead of synthetic ones to retardation lipid oxidation in foods to improve their quality and nutritional value. Retrogradation of meat lipids can directly affect the color, flavor, texture, nutritive value, and safety of food (Ruiz et al., 1999; Camo et al., 2008; Velasco and Williams, 2011; Mirzadeh, et al. 2020). Consequently, there is solicitude in using naturally occurring antioxidants as food additives. Several natural antioxidants have been added to food preparation and manufacturing and have increased the shelf life and oxidative stability of stored food products (Chen et al., 2008).

Citrus fruits received considerable attention in recent years to consume widely around the world and the potential curative benefits associated with high levels of flavonoids, and antioxidant, anticancer, and anti-inflammatory properties (Benavente-Garcia and Castillo, 2008). A large amount of consumption and processing of citrus fruit results in the generation of a huge quantity of citrus peels which is considered food industrial waste. The results showed Industrial processing increase the value of citrus fruits by producing a wide range of by-products such as pectin, pulp, and flavonoids, etc. (Fakhari et al., 2005).

Potatoes are generally peeled during processing. Potato peels had been proposed as dietary fiber (Arora and Camire, 1994), and a source of natural antioxidants. Polyphenols considered as an important group of antioxidants present in potatoes are largely concentrated in the peel, which has an important role in the defiance mechanism against phytopathogens (Friedman, 1997). Potato peels have therefore been the subject of study in many pharmacology and food industry studies.

The aims of the present investigation were carried out to study the effect of extraction methods by the difference in pressure for different ethanolic concentrations of orange and potato peel extracts on antioxidants activity and antimicrobial activity.

## 2. Materials and methods

### 2.1. Materials

#### *2.2.1.Samples*

Orange peels (*Citrus xsinensis*) were obtained from private workshops in Mahata Square, Zagazig town, El-Sharkia Governorate, Egypt on 12/2019.

Potato wastes (*Solanum tuberosum*) were obtained from Farm Frites factory on, 10th of Ramadan on 05/2019.

Absolute Ethanol Alcohol, citric acid, $Na_2CO_3$, sodium hydroxide, phenolphthalein, potassium iodide, sodium thiosulfate, starch, chloroform, acetic acid, and hydrochloric acid were purchased from El-Gomhoria Chemical Company, Zagazig, Egypt .

Folin-ciocalteu reagent, gallic acid, β-carotene, linoleic acid, tween 20, TBA (thiobarbituric acid), phosphatidyl-choline, potassium chloride, iron chloride, TCA (trichloroacetic acid), DPPH (2,2-diphenyl-1-picrylhdrazyl), and butylated hydroxyl anisole (BHA) were purchased from Sigma Chemical Company, Cairo, Egypt.

### 2.2.Methods

#### *2.2.1.Preparation of Samples*

The primary procedures were to separate the parts of the contents of potatoes factories wastes (Water, Starch, and peels). Then, Potato peels (PP) were transferred and washed well with water to urge obviate the remnants of starch

protruding it, then, stacked on trays for a half-hour to urge eliminate excess water before drying. The orange peels (OP) were examined to eliminate the damaged parts and stacked on trays. Both samples were dried in an oven-dryer at 37° C for 48 hr. The samples were flipping once every hour within the first four hours. Then, the dried samples were ground to a fine powder, place in plastic bags, and wrapped with foil, and stored at -20° C until the subsequent procedures.

### *2.2.2.Extraction of the Antioxidants Extracts*

Extraction of the antioxidants extracts of dried samples was conducted using two methods (Soak Extraction (SE), and Extraction by the difference in pressure (DE) in several concentrations of ethanolic Solvent (absolute, 70%, and 50% of ethanolic Solvent). 100 g of dried weight of skin and potato skin samples were soaked in 1000 ml of every one of the three concentrations of ethanolic Solvent, separately, in 2000 ml conical flask for 48 hr. on the stirring hotplate at 37° C (Fisher Scientific, Pittsburgh, PA) with a magnetic stirrer (1000 rpm) (the first method), and also the same procedure for less than 6 hr. with decrease the pressure to 0.6 Pa every 30 min within the sample flask during extraction (the second method). The obtained extracts were filtered using paper (Whatman No. 1, England), concentrated employing a rotary evaporator (EYELA, Japan), freeze-dried (Thermo-Electron Corporation-Hot power dry LL300 freeze dryer), and weighed to work out the yield of extracts. Then, stored at frozen temperature until used.

### *2.2.3.Determination of Total Phenolic Contents (TPC)*

The concentration of total phenol content of various antioxidant extracts was measured by a UV spectrophotometer (Jenway-UV–VIS Spectrophotometer), supported a colorimetric oxidation/reduction reaction, that described by Škerget et al. (2005). The oxidizing reagent usage was Folin–Ciocalteu reagent in step with AOAC (2005). 0.5 mL of diluted extract (10 mg in 10 mL solvent) 2.5 mL of Folin–Ciocalteu reagent (diluted 10 times with distilled water) and a couple of mL of $Na_2CO_3$ (75 g/L) were added. The mixture was incubated for five min at 50˚C then cooled to temperature. For an effect sample, 0.5 mL of $H_2O$ was used. The absorbance was measured at 760 nm. Total phenolic content expressed as acid equivalent (GAE $g^{-1}$ of dried extract) was calculated using the subsequent equation supported the calibration curve:

$$y = 0.2269x + 0.4847 \quad (1)$$

$$R^2 = 0.992 \quad (2)$$

where y is that the absorbance and x is that the concentration (mg GAE $g^{-1}$ of dried extract). $R^2$=Correlation Coefficient.

### *2.2.4.Determination of Total Flavonoids Contents (TFC)*

The content of total flavonoid concentration of different antioxidant extracts was measured according to the method of Ordon et al. (2006) with some modification. 1.5 mL of $AlCl_3$ ethanolic solution (20 g $L^{-1}$) was added to 0.5 mL of every extract of samples (10 mg in 10 mL solvent) separately and incubated for one hour at room temperature. The absorbance was measured at 420 nm at room temperature and the yellow color indicates the presence of flavonoids. Total flavonoid content expressed as Quercetin equivalent (mg QE $g^{-1}$ of dried extract) was calculated using the following equation based on the calibration curve:

$$y = 0.3033x + 0.6511 \quad (3)$$

$$R^2 = 0.9987 \quad (4)$$

where x is the absorbance and y is the concentration (mg QE $g^{-1}$ of dried extract). R2=Correlation Coefficient.

### *2.2.5.DPPH˙ Free Radical Scavenging Assay*

The ability of different antioxidant extracts to decolorization the purple color of the DPPH˙ solution was measured according to the method of Gulcin et al. (2004). 0.1 ml of each extract (10 mg in 10 mL solvent) was added to 3 mL of 0.1 mM DPPH˙ dissolved in the same solvent to each extract, separately, and measured for two hours every 30 min at room temperature. The control of the assay was prepared according to

usage to negative control from DPPH˙ solution and only solvent without extracts, and the positive control by exchange the extracts by BHA synthetic antioxidants. The absorbance was determined against a negative control at 517 nm for every period, separately .

Percentage of antioxidant activity of DPPH˙ free radical was calculated using the following equation:

$$\text{Inhibition (\%)} = \left(\frac{A_c - A_t}{A_c}\right) \times 100 \qquad (5)$$

where $A_c$ is the absorbance of the negative control and $A_t$ is the absorbance of the sample and/or positive control. $IC_{50}$ is the antioxidant concentration that inhibits the DPPH reaction by 50% under experimental conditions.

### *2.2.6.β-Carotene/Linoleic Acid Bleaching (βCB) Assay*

The ability of various antioxidant extracts and artificial antioxidants (BHA) to stop the bleaching of β-carotene was assessed as described by Kayvan et al. (2007). In brief, 0.2 mg of β-carotene in 1 mL of chloroform, 20 mg linolic acid, and 200 mg of tween 20 were placed in a very flask. After removal of the chloroform, 50 mL of water was added, and also the resulting mixture was stirred vigorously. Aliquots (3 ml) of the emulsion were transferred to tubes containing extract or synthetic antioxidants. Immediately after mixing 0.5 mL of extract solution (10mg extract in10 mL solvent), an aliquot from each tube was transferred to a cuvette and therefore the absorbance at 470 nm was recorded ($A_0$). The remaining samples were placed within the water bath at 50 °C for 120 min, then the absorbance at 470 nm was recorded ($A_{120}$). An impression without added extract was also analyzed. Antioxidant activity was calculated as follow:

$$\text{A A (\%)} = \left(1 - \frac{(A_{S0} - A_{S120})}{A_{C0} - A_{C120}}\right) \times 100 \qquad (6)$$

where $A_{S0}$ is that the initial absorbance and $A_{S120}$ is that the absorbance at 120 min for samples. while $A_{C0}$ is that the initial absorbance and $A_{C120}$ is that the absorbance at 120 min for negative control.

### *2.2.7.Thiobarbituric Acid Reactive Substances (TBARs) Assay*

The capacity of various antioxidant extracts to inhibit lipid peroxidation was also evaluated by using the modified assay of thiobarbituric acid reactive substances (TBARs) (Gonzalez-Paramas et al., 2004). the tactic relies on the peroxidation of a liposome system (25 mL of fifty mg/ml phosphatidyl-choline in 1.5:1 (v:v) chloroform:ethanol) induced by 200 ml of 1 mM iron chloride containing 300 mM chloride within the presence of the extracts (50 ml). Peroxidation was started by adding ascorbate (125 ml at 0.16 mM) and incubating at 37 °C for twenty-four hr. The reaction was stopped by adding 0.75 ml of a combination 1.5:1 (v:v) of 9.4% TCA in 0.47 N acid (pH 1.5) with 1% TBA and 0.05 ml of BHT (760 mg/l in ethanol). the assembly of TBARS, fundamentally malonaldehyde, as a secondary product of peroxidation, was measured spectrophotometrically at 535 nm after incubation at 95 °C for 60 min.

A control without the extracts (with the various solvents employed in the extractions) was wont to evaluate the phosphatidylcholine peroxidation as inhibition ratio (IP, %):

$$\text{IP (\%)} = \left(1 - \frac{A_t}{A_t^0}\right) \times 100 \qquad (7)$$

where $A_t$ and $A_t^0$ are extracted and control absorbance after incubation for 60 min. The repetition variance of the procedure was always <10%.

### *2.2.8.Antimicrobial activity*

*2.2.8.1.Antibacterial activity*

The antibacterial activity was estimated according to Bayer et al. (1966) and Akl et al. (2020). Discs of filter paper were saturated with 30 µL of different antioxidant extract (800 µg/mL) and placed on Petri dishes containing agar media contaminated with pathogenic bacteria (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853), and incubated for 24 h at 37 °C. Then, measured the inhibition zone diameters

(mm). A disc saturated with distilled water was a negative control, and levofloxacin was a positive control.

*2.2.8.2.Minimum inhibitory concentration (MIC)*

The effect of the different antioxidant extracts on the visible turbidity of tubes contaminated with pathogenic bacteria before and after incubation was measured according to Andrews (2001). 30 µL of different antioxidant extract at different concentrations of (0, 200, 400, 800, and 1000 µg/mL) was incubated with broth media contaminated with pathogenic bacteria, then, observed the turbidity of tubes before and after incubation. The MIC was the lowest concentration exhibiting a clear zone on Muller–Hinton agar (MHA) plates according to Reda et al. (2020) and Sheiha et al.(2020) .

*2.2.8.3.Bacterial growth curve (turbidity test)*

The bacterial growth assays were estimated according to El-Saadony et al. (2020). 30 µL of the different antioxidant extract (800 µg/mL) was added to tubes containing 100 µL of tested pathogenic bacteria and 10 mL nutrient broth. Then, incubation at 37 °C for 6 h intervals of (0–24 h). The turbidity of tubes was measured at 600 nm and compared with distilled water as a negative control and levofloxacin as a positive control.

*2.2.8.4.Antifungal activity*

The inhibition of fungal growth of different antioxidant extracts was evaluated against four fungal species; *Aspergillus niger, Aspergillus ochraceus, Penicillium citrinum,* and *Fusarium oxysporum*, according to Elgorban et al. (2016) and El-Saadony et al. (2019). Five-millimeter discs of filter paper were saturated with 50 µL of different antioxidant extract (800 µg/mL) and applied on both sides of potato dextrose agar (PDA) plates. Carefully, the disc of mycelia was picked from the edge of fungal cultures and placed in each Petri dish center, then incubated at 28 °C for 3–5 days. The fungal mycelium's radial growth was measured by a ruler (cm/4 days). The PDA plates with 50 µL distilled water were a negative control, and Difenoconazole (800 µg/mL) was a positive control.

The minimum fungal concentration (MFC) was estimated according to Alizadeh et al. (2014). 50 µL of different antioxidant extract (0, 200, 400, and 800 µg/mL) was added to different PDA tubes containing fungi. Then, incubation at 28 °C for 48–72 h. The least concentration of the different antioxidant extract that removes fungal growth was considered as MFC.

***2.2.9.Statistical Analysis***

The tests were done in triplicate according to Steele and Torrie (1996), and the data were analyzed using the means, standard deviation by Microsoft Office Excel (2016), Paired sample t-test, and one-way ANOVA variance analysis by IBM SPSS version 25.0 software (SPSS Inc., Chicago, IL, USA) at the level of probability of ($P \leq 0.05$).

## 3.Results and discussions

Extraction of the antioxidant extracts depended on soak the fine ground powder of samples in extraction solvents where exposure huge area of a sample to extraction solvent leads to facilitation and increase efficiency extraction operation. While the magnetic stirrer's rotational speed expands the field of exposure area of the sample to extraction solvents and facilitates extracting the phytochemical compounds (the main source of the antioxidant act) to the solution of extraction. A difference in pressure in the sample flask increases cell wall permeability which gives more facilitates extracting in little time.

### 3.1.Total Phenols (TPC) and Total Flavonoids (TFC) Contents

Phenolic and flavonoid compounds are derived from compounds of the secondary metabolism of plants which have the ability to scavenger free radicals, protect food elements during the food processing chain, prolong the shelf-life of food products, and protect organs of the human body from oxidative stress (Granato et al., 2018). So, the effectiveness of the antioxidant activity of phenolic and flavonoid compounds was a very important incentive to determine the total contents of phenolic and flavonoid compounds. The concentration of phenolic and flavonoid

compounds of different extracts was expressed as mg Gallic acid (GAE) and Quercetin (QE); respectively per g of dried extracts (Table 1). The results showed a significant mean difference ($P \leq 0.05$) between the soak and extraction by difference in pressure methods, a positive effect to the difference in pressure methods on the concentration of phenolic and flavonoid compounds. The data also shows a significant mean difference ($P \leq 0.05$) between some samples and no significant mean difference ($P \leq 0.05$) between other samples of orange peels and potato peels of the different extracts for soak and extraction by difference in pressure methods, and only a significant mean difference ($P \leq 0.05$) between the samples of orange peels and potato peels of the different extracts for extraction method by the difference in pressure.

For the soak extraction (SE) method, orange peel extracts showed a higher concentration value of total phenolic contents than potato peel extracts for all different extracts. The absolute ethanolic extracts of orange peels ($OPE_{100}$) shows the highest concentration value of total phenolic compounds followed by 70% ethanolic extracts of orange peels ($OPE_{70}$) then 50% ethanolic extracts of orange peels ($OPE_{50}$) (231.43±1.43, 220.19±1.09, and 218.47±1.07 mg GAE $g^{-1}$ of dried extract; respectively), while the lowest concentration value of total phenolic compounds was 50% ethanolic extracts of potato peels ($PPE_{50}$) (180.72±1.02 mg GAE $g^{-1}$ of dried extract). Also, orange peel extracts showed a higher concentration value of total phenolic contents than potato peel extracts for all different extracts of the extraction method by the difference in pressure (DE). The absolute ethanolic extracts of orange peels ($OPE_{100}$) shows the highest concentration value of total phenolic compounds followed by 70% ethanolic extracts of orange peels ($OPE_{70}$) then 50% ethanolic extracts of orange peels ($OPE_{50}$) (262.19±1.19, 247.58±0.58, and 232.41±0.41 mg GAE $g^{-1}$ of dried extract; respectively), while the lowest concentration value of total phenolic compounds was 50% ethanolic extracts of potato peels ($PPE_{50}$) (191.19±1.19 mg GAE $g^{-1}$ of dried extract).

For the concentration of total flavonoid contents, 50% ethanolic extracts of orange peel ($OPE_{50}$) showed the highest concentration value of total flavonoid contents followed by 50% ethanolic extracts of potato peel ($PPE_{50}$) then 70% ethanolic extracts of potato peel (PPE70) (82.39±1.09, 81.48±1.08, and 61.42±1.42 mg QE $g^{-1}$ of dried extract; respectively), and absolute ethanolic of potato peel extracts ($PPE_{100}$) was the lowest concentration value of total flavonoid contents (29.73±1.03 mg QE $g^{-1}$ of dried extract), of soak extraction (SE) method. While the highest concentration value of total flavonoid contents of the extraction method by the difference in pressure (DE) was 50% ethanolic extracts of orange peel ($OPE_{50}$) followed by 50% ethanolic extracts of potato peel ($PPE_{50}$) then 70% ethanolic extracts of orange peel ($OPE_{70}$) (86.28±1.28, 83.43±1.03, and 78.16±1.16 mg QE $g^{-1}$ of dried extract; respectively), and absolute ethanolic of potato peel extracts ($PPE_{100}$) was the lowest concentration value of total flavonoid contents (36.87±0.87 mg QE $g^{-1}$ of dried extract).

The increase of the total flavonoid contents came with a decrease in the concentration of ethanolic solvent, an increase of total phenolic contents with an increase in the concentration of ethanolic solvent, and the highest efficiency was the extracts of the extraction method by the difference in pressure. The results agreed with the results reported by Brahmi et al. (2012), and Rosa et al. (2019), although the comparison is highly difficult because of the different extraction conditions used.

**Table 1.** The contents of total phenol (TPC), total flavonoids compounds (TFC), and yield of different antioxidants extracts

| | | TPC (concentration mg GAE $g^{-1}$ of dried extract) | | TFC (concentration mg QE $g^{-1}$ of dried extract) | | Yield of Extracts (g/100g of dried materials) | |
|---|---|---|---|---|---|---|---|
| | | SE | DE | SE | DE | SE | DE |
| OP | $E_{100}$ | 231.43±1.43$^{a}$ | 262.19±1.19$^{a}$ | 35.26±1.06$^{d}$ | 49.15±1.15$^{e}$ | 27.24±2.24$^{ab}$ | 29.15±1.15$^{ab}$ |
| | $E_{70}$ | 220.19±1.09$^{b}$ | 247.58±0.58$^{b}$ | 54.51±1.1$^{c}$ | 78.16±1.16$^{c}$ | 28.51±1.01$^{a}$ | 30.57±0.5$^{ab}$ |
| | $E_{50}$ | 218.47±1.07$^{b}$ | 232.41±0.41$^{c}$ | 82.39±1.09$^{a}$ | 86.28±1.28$^{a}$ | 30.19±1.09$^{a}$ | 32.49±1.4$^{a}$ |
| PP | $E_{100}$ | 186.73±1.03$^{c}$ | 207.43±1.03$^{d}$ | 29.73±1.03$^{e}$ | 36.87±0.87$^{f}$ | 21.38±1.08$^{c}$ | 22.42±1.02$^{c}$ |
| | $E_{70}$ | 181.27±1.07$^{d}$ | 198.45±0.45$^{e}$ | 61.42±1.42$^{b}$ | 70.42±0.42$^{d}$ | 25.6±1.2$^{b}$ | 27.04±1.04$^{b}$ |
| | $E_{50}$ | 180.72±1.02$^{d}$ | 191.19±1.19$^{f}$ | 81.48±1.08$^{a}$ | 83.43±1.03$^{a}$ | 29.4±1.2$^{a}$ | 31.07±1$^{a}$ |

Values mean ±SD; n = 3. Different letters in the same column indicate significant differences (P≤ 0.05).

**Table 2.** Assays of antioxidants activity of different antioxidants extracts

| | | $IC_{50}$ (µg $mL^{-1}$) | | % of inhibition lipid peroxidation | | | |
|---|---|---|---|---|---|---|---|
| | | | | β -Carotene | | TBARs | |
| | | SE | DE | SE | DE | SE | DE |
| OP | $E_{100}$ | 22.53±0.53$^{c}$ | 21.18±1.18$^{c}$ | 81.52±0.52$^{a}$ | 83.15±1.15$^{a}$ | 77.04±1.04$^{b}$ | 78.82±0.85$^{b}$ |
| | $E_{70}$ | 26.49±1.4$^{bc}$ | 24.19±1.1$^{b}$ | 76.35±1.35$^{b}$ | 78.51±0.51$^{b}$ | 70.21±1.2$^{c}$ | 72.89±0.89$^{d}$ |
| | $E_{50}$ | 29.45±0.45$^{a}$ | 27.94±0.94$^{a}$ | 69.23±1.2$^{d}$ | 71.54±0.54$^{c}$ | 69.31±1.31$^{c}$ | 71.62±0.62$^{d}$ |
| PP | $E_{100}$ | 24.61±0.61$^{c}$ | 22.97±0.97$^{c}$ | 78.85±0.85$^{ab}$ | 80.94±0.94$^{a}$ | 82.02±1$^{a}$ | 87.83±0.83$^{a}$ |
| | $E_{70}$ | 27.83±0.83$^{b}$ | 25.37±1.3$^{ab}$ | 73.26±1.2$^{c}$ | 77.82±0.82$^{b}$ | 77.25±1.2$^{b}$ | 79.42±1.02$^{b}$ |
| | $E_{50}$ | 29.03±1.03$^{a}$ | 27.49±1.4$^{a}$ | 69.36±1.3$^{d}$ | 73.04±1.04$^{c}$ | 70.15±1.15$^{c}$ | 76.12±1.1$^{c}$ |

Values mean ±SD; n = 3. Different letters in the same column indicate significant differences (P≤ 0.05).

**Table 3.** Anti-bacterial activity of different antioxidants extracts

| Anti-Bacterial Activity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ***Staphylococcus aureus*** | | ***Bacillus cereus*** | | ***Escherichia coli*** | | ***Pseudomonas aeruginosa*** | |
| | | **SE** | **DE** | **SE** | **DE** | **SE** | **DE** | **SE** | **DE** |
| **OP** | **$E_{100}$** | 30±0.5$^{b}$ | 32±0.2$^{b}$ | 26±0.4$^{b}$ | 29±0.5$^{a}$ | 24±0.4$^{b}$ | 26±0.1$^{b}$ | 28±0.9$^{b}$ | 28±0.4$^{b}$ |
| | **$E_{70}$** | 25±0.1$^{c}$ | 27±0.5$^{d}$ | 20±0.5$^{c}$ | 25±0.7$^{b}$ | 20±0.6$^{c}$ | 22±0.5$^{c}$ | 24±1$^{d}$ | 26±0.5$^{c}$ |
| | **$E_{50}$** | 20±1$^{d}$ | 24±0.5$^{e}$ | 17±1$^{d}$ | 20±0.3$^{c}$ | 15±0.5$^{e}$ | 18±0.6$^{e}$ | 19±0.8$^{e}$ | 21±1$^{d}$ |
| **PP** | **$E_{100}$** | 29±0.5$^{b}$ | 30±0.5$^{c}$ | 26±0.4$^{b}$ | 29±0.5$^{a}$ | 21±1$^{c}$ | 25±0.4$^{b}$ | 26±0.3$^{c}$ | 28±0.5$^{b}$ |
| | **$E_{70}$** | 25±1$^{c}$ | 26±0.3$^{d}$ | 21±0.4$^{c}$ | 25±0.5$^{b}$ | 18±0.7$^{d}$ | 20±0.5$^{d}$ | 23±1$^{d}$ | 26±0.5$^{c}$ |
| | **$E_{50}$** | 19±0.5$^{d}$ | 23±1$^{e}$ | 16±0.4$^{d}$ | 19±0.5$^{c}$ | 15±0.4$^{e}$ | 17±0.6$^{e}$ | 20±0.5$^{e}$ | 22±0.5$^{d}$ |
| **Cont.** | **PC** | 34±0.2$^{a}$ | | 30±0.1$^{a}$ | | 28±0.3$^{a}$ | | 31±0.7$^{a}$ | |
| | **NC** | ND | | ND | | ND | | ND | |

Values mean ±SD; n = 3. Different letters in the same column indicate significant differences (P≤ 0.05).

**Table 4.** Anti- fungal activity of different antioxidants extracts

| Anti-Fungal Activity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ***Aspergillus niger*** | | ***Aspergillus ochraceus*** | | ***Penicillium citrinum*** | | ***Fusarium oxysporum*** | |
| | | **SE** | **DE** | **SE** | **DE** | **SE** | **DE** | **SE** | **DE** |
| **OP** | **$E_{100}$** | 1.7±0.2$^{c}$ | 1.4±0.3$^{c}$ | 1.8±0.2$^{c}$ | 1.5±0.5$^{b}$ | 1.6±0.2$^{d}$ | 1.4±0.2$^{d}$ | 2±0.5$^{c}$ | 1.8±0.2$^{c}$ |
| | **$E_{70}$** | 1.9±0.1$^{b}$ | 1.8±0.2$^{ab}$ | 2.3±0.3$^{b}$ | 1.9±0.1$^{ab}$ | 2±0.5$^{c}$ | 1.8±0.2$^{c}$ | 2.6±0.2$^{b}$ | 2.2±0.2$^{b}$ |
| | **$E_{50}$** | 2.3±0.3$^{ab}$ | 2.1±0.1$^{b}$ | 2.8±0.2$^{a}$ | 2.5±0.5$^{a}$ | 2.6±0.2$^{ab}$ | 2.1±0.1$^{ab}$ | 3±1$^{a}$ | 2.9±0.1$^{a}$ |
| **PP** | **$E_{100}$** | 1.9±0.3$^{b}$ | 1.5±0.1$^{c}$ | 2±0.5$^{c}$ | 1.6±0.2$^{b}$ | 1.9±0.1$^{c}$ | 1.7±0.2$^{c}$ | 2.1±0.1$^{c}$ | 1.9±0.1$^{c}$ |
| | **$E_{70}$** | 2.3±0.3$^{ab}$ | 1.9±0.2$^{b}$ | 2.4±0.4$^{b}$ | 2.1±0.1$^{c}$ | 2.5±0.2$^{b}$ | 2±0.1$^{ab}$ | 2.8±0.2$^{ab}$ | 2.4±0.4$^{b}$ |
| | **$E_{50}$** | 2.9±0.1$^{a}$ | 2.6±0.2$^{a}$ | 2.9±0.1$^{a}$ | 2.7±0.2$^{a}$ | 2.9±0.1$^{a}$ | 2.8±0.2$^{a}$ | 2.3±0.3$^{a}$ | 2.9±0.1$^{a}$ |
| **Cont.** | **PC** | 1.2±0.2$^{d}$ | | 1.1±0.1$^{d}$ | | 1.2±0.2$^{d}$ | | 1.6±0.2$^{d}$ | |
| | **NC** | 9 | | 9 | | 9 | | 9 | |

Values mean ±SD; n = 3. Different letters in the same column indicate significant differences (P≤ 0.05).

### 3.2.The Antioxidants Activity of Different Antioxidants Extracts

The antioxidants activity of different antioxidant extracts was determined by some of the different methods; DPPH˙ scavenging radical activity, β-Carotene/Linoleic acid bleaching (βCB) assay, and thiobarbituric acid reactive substances (TBARs) assay and the results shown in table 2. The results showed a significant mean difference (P≤ 0.05) between both extraction methods (soak and extraction by the difference in pressure) for all the methods of antioxidants activity assays, a positive effect to the extraction method by the difference in pressure. The data also shows a significant mean difference (P≤ 0.05) between some samples and no significant mean difference (P≤ 0.05) between other samples of the different orange and potato peel extracts for both different methods of extraction.

DPPH˙ scavenging radical activity was used as a wide model to evaluate the scavenging radical activity of the natural antioxidant extracts. The antioxidant extracts are able to reduce the DPPH˙ free radical and change the color of the solution from purple to yellow, in the non-radical situation (Shen et al., 2016). Data in fig. 1 shows the increase in $IC_{50}$ with the decrease of ethanolic concentration solvent for both orange and potato peel extracts of both soak and extraction methods by the difference in pressure. Absolute ethanolic extracts give more stabilization of DPPH˙ scavenging radical activity more than 70% ethanolic extracts more than 50% ethanolic extracts for both orange and potato peel in both soak and extraction methods by the difference in pressure. The absolute ethanolic of orange peel extracts ($OPE_{100}$) give the most stabilization of DPPH˙ scavenging radical activity followed by absolute ethanolic of potato peel extracts ($PPE_{100}$) then 70% ethanolic of orange peel extracts ($OPE_{70}$) (22.53±0.53, 24.61±0.61, and 26.49±1.4 µg $ml^{-1}$; respectively), while 50% ethanolic of orange peel extracts ($OPE_{50}$) gives the lowest stabilization of DPPH˙ scavenging radical activity (29.45±0.45 µg $ml^{-1}$) of soak extraction method. Also, absolute ethanolic of orange peel extracts ($OPE_{100}$) give the most stabilization of DPPH˙ scavenging radical activity followed by absolute ethanolic of potato peel extracts ($PPE_{100}$) then 70% ethanolic of orange peel extracts ($OPE_{70}$) (21.18±1.18, 22.97±0.97, and 24.19±1.1 µg $ml^{-1}$; respectively) and 50% ethanolic of orange peel extracts ($OPE_{50}$) gives the lowest stabilization of DPPH˙ scavenging radical activity (27.49±0.49 µg $ml^{-1}$) for extraction method by the difference in pressure.

Determination of the antioxidant activity of the different extracts on inhibition lipid peroxidation by β-Carotene/Linoleic acid bleaching was dependent on the activities of lipid radicals as auto-oxidation products of linoleic acid which attack double bonds of β-carotene, and the ability of the antioxidative substance to protect β-carotene (yellowish-orange colour) (Zhang et al., 2015). In both orange and potato peel extracts, absolute ethanolic extracts give a high value of β-Carotene/Linoleic acid bleaching (βCB) assay more than 70% ethanolic extracts more than 50% extracts for both extraction methods (fig. 2). For the soak extraction method, both orange and potato peel extracts give approximate and nested of a significant and non-significant mean difference (P≤ 0.05) value of β-Carotene/Linoleic acid bleaching (βCB) assay in all of the ethanolic concentrations. Data shows no significant mean difference (P≤ 0.05) between absolute ethanolic of orange peel ($OPE_{100}$) and absolute ethanolic potato extract ($PPE_{100}$) (81.52±0.52, and 78.85±0.85% percentage; respectively), also no significant mean difference (P≤ 0.05) between the lowest value of β-Carotene/Linoleic acid bleaching (βCB) assay of both 50% ethanolic orange peel extract ($OPE_{50}$) and 50% ethanolic potato extract ($PPE_{50}$) (69.23±1.2, and 69.36±1.3% percentage; respectively), while 70% ethanolic concentration of both orange and potato peel give significant mean difference (P≤ 0.05) (76.35±1.35, and 73.26±1.2% percentage; respectively). Whilst, no significant mean difference (P≤ 0.05) between orange and potato peel in the same concentration of ethanolic solvent for extraction method by the difference

in pressure (83.15±1.15, and 80.94±0.94% percentage of absolute ethanolic orange peel extract ($OPE_{100}$) and absolute ethanolic potato peel extract ($PPE_{100}$); respectively, 78.51±0.51, and 77.82±0.82% percentage of 70% ethanolic orange peel extract ($OPE_{70}$) and 70% ethanolic potato peel extract ($PPE_{70}$); respectively, and 71.54±0.54, and 73.04±1.04% percentage of 50% ethanolic orange peel extract ($OPE_{50}$) and 50% ethanolic potato peel extract ($PPE_{50}$); respectively).

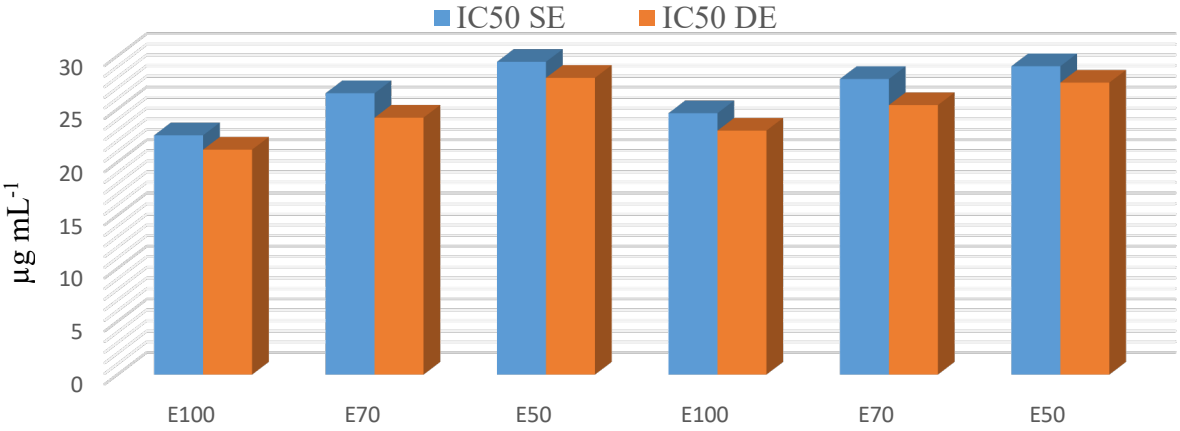


**Figure 1. DPPH˙ Free Radical Assay ($IC_{50}$)**

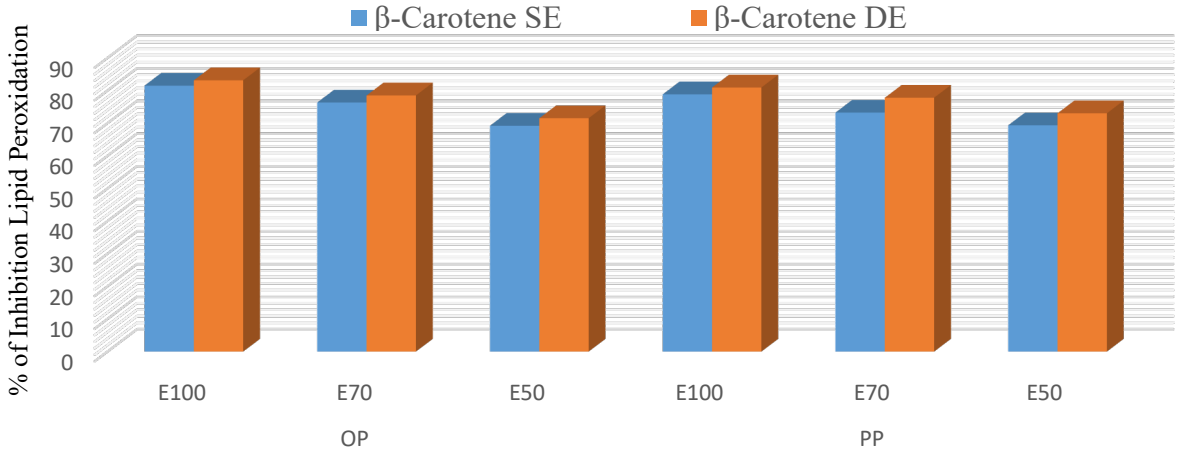


**Figure 2.** Inhibition Lipid Peroxidation; β-Carotene/Linoleic acid Bleaching (βCB) Assay

Determination of TBARs value was widely used to estimate the antioxidant activity and the ability of extracts to inhibit lipid peroxidation (Yim et al., 2013). For both soak and extraction method by the difference in pressure, absolute ethanolic extracts give the value of inhibiting lipid peroxidation more than 70% ethanolic extracts more than 50% ethanolic extracts for both orange and potato peel, and potato peel give the highest value per every type of ethanolic concentration separately (fig. 3). Absolute ethanolic potato peel extract ($PPE_{100}$) gives the highest value of thiobarbituric acid reactive substances (TBARS) assay followed by 70% ethanolic potato peel extract ($PPE_{70}$) with no significant mean difference ($P \leq 0.05$) between absolute ethanolic of orange peel extract ($OPE_{100}$) and 70% ethanolic potato peel extract ($PPE_{70}$), and no significant mean difference ($P \leq 0.05$) between 50% ethanolic potato peel extract ($PPE_{50}$) and both 70% and 50% ethanolic orange peel extracts ($OPE_{70}$ and $OPE_{50}$) (82.02±1, 77.25±1.2, 77.04±1.04, 70.21±1.2, 70.15±1.15, and 69.31±1.31% percentage; respectively) for soak extraction method. While, absolute ethanolic potato peel extract ($PPE_{100}$) gives the highest value followed by 70% ethanolic potato peel extract ($PPE_{70}$) with no significant mean

difference (P≤ 0.05) between 70% ethanolic potato peel extract ($PPE_{70}$) and absolute ethanolic orange peel extract ($OPE_{100}$), and no significant mean difference (P≤ 0.05) between 70% ethanolic orange peel extract ($OPE_{70}$) and 50% ethanolic orange peel extract ($OPE_{50}$) (87.83±0.83, 79.42±1.02, 78.82±0.85, 76.12±1.1, 72.89±0.89, and 71.62±0.62% percentage; respectively) for extraction method by the difference in pressure.

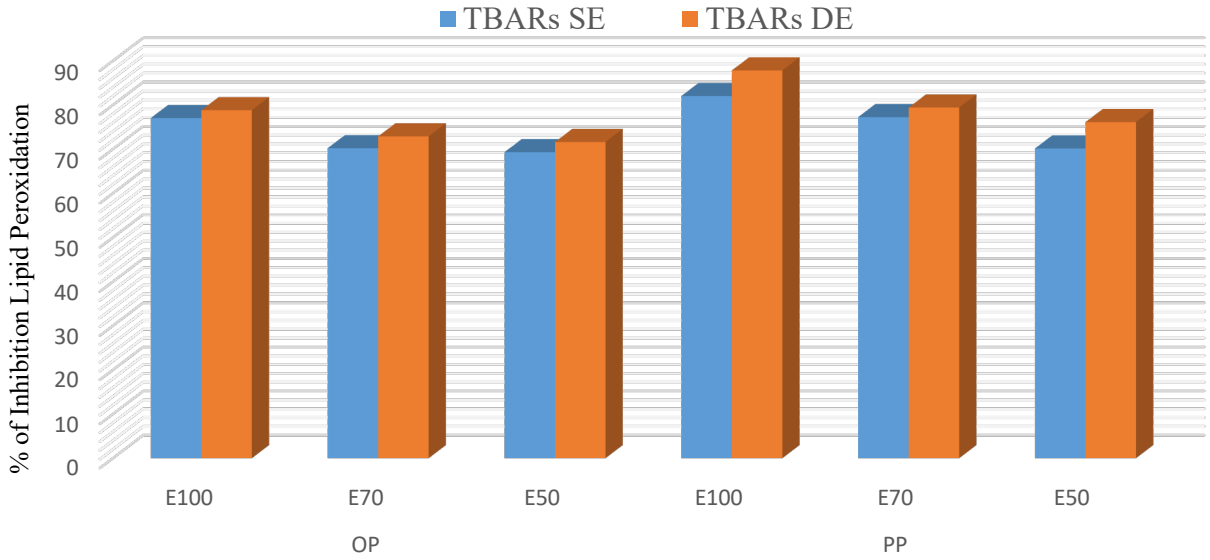


**Figure 3.** Inhibition Lipid Peroxidation; thiobarbituric acid reactive substances (TBARs) Assay

The increase of antioxidant activity by an increase in ethanolic solvents concentration came compatible with the increase of total phenolic and the highest efficiency of the extracts of the extraction method by the difference in pressure, and the results agreed with the results reported by Rosa et al. (2019), although the different extraction conditions used.

### 3.3.Antimicrobial activity

The effect of different orange and potato peel extracts on microbial activity was measured by different methods. The ability of different orange and potato peel extracts to inhibit bacterial zone and restrain fungal growth was estimated and the results are shown in tables 3 and 4, and fig. 4. The minimum bacterial and fungal inhibitory concentration was in (600 µg $mL^{-1}$) for all microbial growth in absolute and 70% ethanolic orange and potato peel extracts, and (800 µg $mL^{-1}$) for almost microbial growth in 50% ethanolic orange and potato peel extracts in both soak and extraction method by the difference in pressure.

The results showed significant mean differences (P≤ 0.05) between both extraction methods for all samples, the positive effect was for the extraction method by the difference in pressure, no significant mean differences (P≤ 0.05) between orange and potato peel extracts for the same ethanolic concentration of only gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, and *Bacillus cereus* ATCC 11778) in soak extraction method, no significant differences (P≤ 0.05) between positive control and both absolute orange and potato peel extracts, and no significant differences (P≤ 0.05) between orange and potato peel extracts for the same ethanolic concentration of only gram-negative bacteria (*Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) in the extraction method by the difference in pressure (Table 3). Positive control gives the highest value for inhibition of bacterial activities for all bacteria followed by absolute ethanolic extracts, then 70% ethanolic extracts, then 50% ethanolic extracts for both extraction methods.

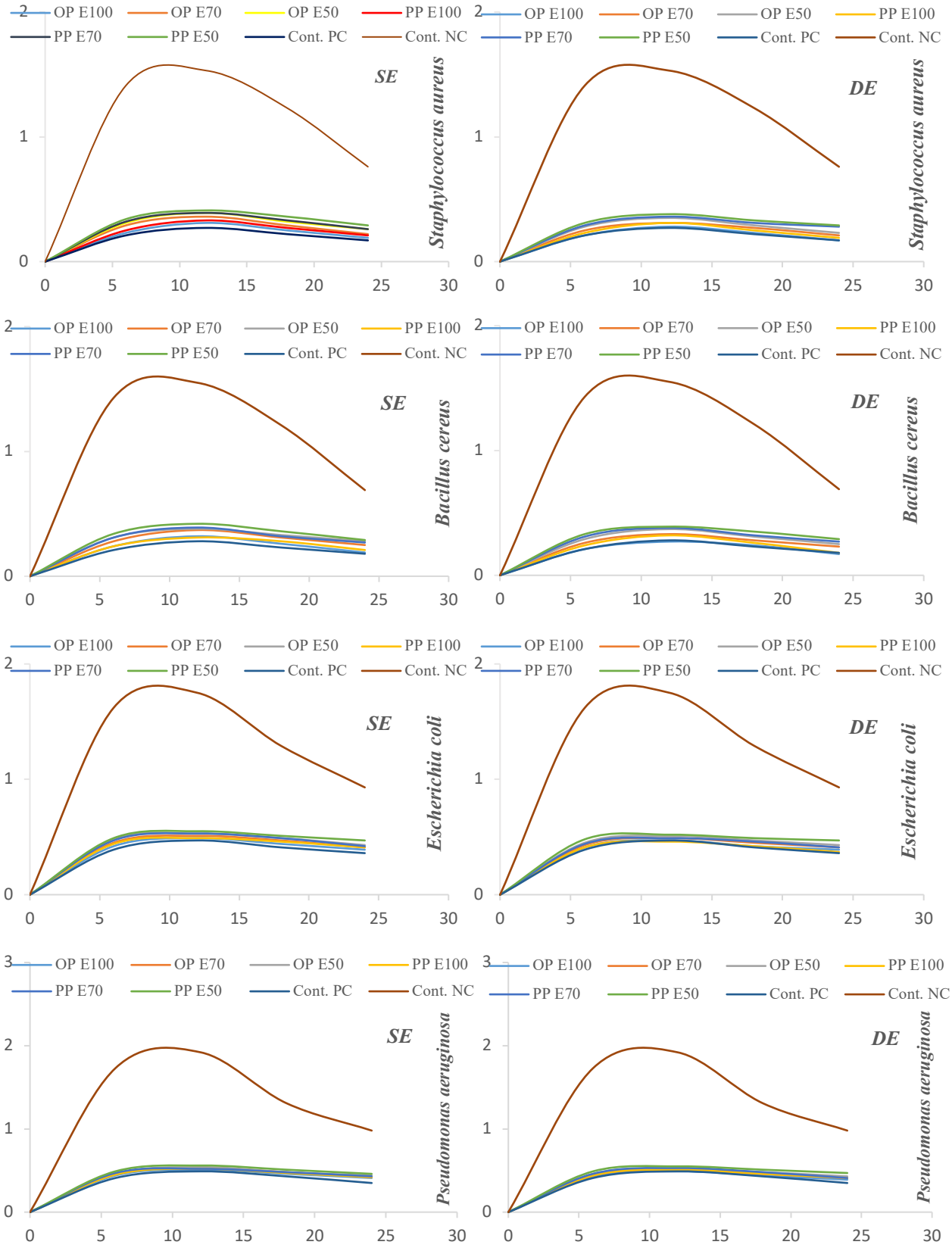


**Figure 4.** Growth curve of gram-positive and gram-negative bacteria in the presence of MIC of different orange and potato peel extracts (800 µg $mL^{-1}$).

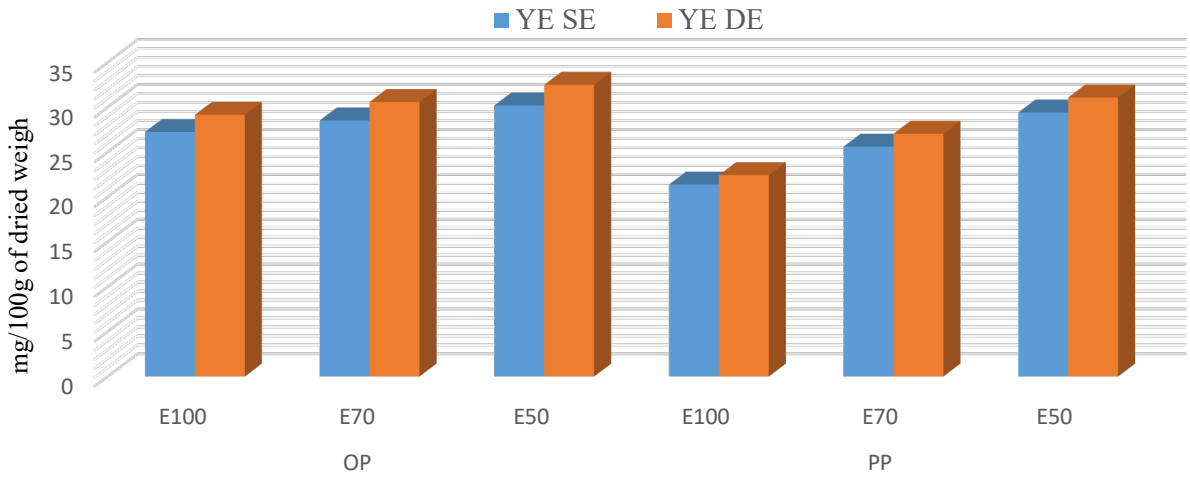


**Figure 5.** Yield of Extracts of Different Antioxidants Extracts

Table 4 showed fungal radial growth (cm) affected by different orange and potato peel extracts for the four fungal strains: *Aspergillus niger, Aspergillus ochraceus, Penicillium citrinum,* and *Fusarium oxysporum*. The extracts reduced the diameter colony of fungi from 9.0 cm for negative control to (1.4:2 cm) in the highest inhibition of fungal activities for different orange and potato peel extracts. The absolute ethanolic extracts give the highest value for inhibition of fungal activities for all fungal strains, followed by 70% ethanolic extracts, then 50% ethanolic extracts for both extraction methods, and positive control was more active against fungal activities than different orange and potato peel extracts .

The antimicrobial activities of different orange and potato peel extracts may originate from its high contents of phenolic compounds and flavonoids following Abdel-Shafi et al. (2019). The different orange and potato peel extracts were effective against the pathogenic bacteria (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853), and *fungal strains: Aspergillus niger, Aspergillus ochraceus, Penicillium citrinum,* and *Fusarium oxysporum*. The results indicated that gram-negative bacteria were more resistant than gram-positive ones, probably because of their more sophisticated membranes. The phenolics might alter the permeability and rigidity of the cell wall by inhibiting the cell wall enzymes.

### 3.4.The yield of Extract of Different Antioxidants Extracts

The yield of extracts resulted from different ethanolic concentrations of different methods of extraction was measured and the result shown in fig. 5. Data shows different amounts of extracts value between the different concentrations of ethanol, a significant mean difference ($P \leq 0.05$) between the soak and extraction method by the difference in pressure, a positive effect for the extraction method by the difference in pressure. In both orange and potato peel, 50% ethanolic extracts give the high amount value of yield extracts more than 70% ethanolic extracts more than absolute ethanolic extracts for both extraction methods. 50% ethanolic orange peel extract ($OPE_{50}$) shows the highest value of yield extracts followed by 50% ethanolic potato peel extract ($PPE_{50}$) then 70% ethanolic orange peel extract ($OPE_{70}$) ($OPE_{50}$) (30.19±1.09, 29.4±1.2, and 28.51±1.01 mg/100g of dried weight; respectively), while absolute ethanolic potato peel extract ($PPE_{100}$) gives the lowest value of yield extracts (21.38±1.08) for soak extraction method. Also, 50% ethanolic orange peel extracts ($OPE_{50}$) show the highest value of yield extracts followed by 50% ethanolic potato peel extracts ($PPE_{50}$) then 70% ethanolic orange peel extracts ($OPE_{70}$) (32.49±1.4, 31.7±1, and

30.57±0.5 mg/100g of dried weight; respectively), and absolute ethanolic of potato peel ($PPE_{100}$) gives the lowest value of yield extracts (22.42±1.02) for extraction method by the difference in pressure. These results are in agreement with the study of El-Naggar et al. (2017).

## 4.Conclusion

This study was executed to determine the impact of the difference in pressure on the antioxidants and antimicrobial activities for different concentrations of ethanolic of orang and potato peel extracts. The extraction method by the difference in pressure showed enhanced the antioxidants and antimicrobial activities with observed increases in the yield of extracts. The increase of the antioxidants and antimicrobial activities extraction by the differences in pressure method may be due to the increase of phenolic compounds concentration. We suggest that more studies on the effect of pressure on the antioxidants and antimicrobial activities for natural extracts and never use only soaking to the extraction of natural antioxidant extracts.

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# EFFICIENCY OF GREEN EXTRACTION BY AQUEOUS GLYCEROL ON ANTIOXIDANT AND ANTIRADICAL PERFORMANCE OF DANDELION (*TARAXACUM OFFICINALE*) AERIAL PART

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**ABSTRACT**

In this study, aerial parts of the dandelion were exposed to extraction by different solvents such as water, ethanol, methanol and glycerol and also their aqueous mixtures to compare the effect of extraction solvents on bioactive performance of the dandelion and also to show the effectiveness of hydroglycerolic extraction which is a green extraction process. Total phenolic content (TPC) and total flavonoid content (TFC) of the extracts were determined and also antiradical scavenging activities and antioxidant capacities of the samples were also evaluated. TPC and TFC of the samples ranged between 4.63-21.28 mg GAE/g and 1.16-14.38 mg CE/g, respectively. The highest TPC and TFC values were determined in aqueous extract of glycerol (75% w/w) compared to other solvents. Additionally, $ABTS^{\cdot+}$ and DPPH radical scavenging activity and ferric reducing capacity and antioxidant capacity values were determined for the extracts and the best solvent was also aqueous glycerol (75% w/w).

## 1.Introduction

*Taraxacum officinale* is one of the most popular medicinal plants belonging to the family of Asteraceae and known as dandelion which is a perennial plant and it was reported that the dandelion was rich in some flavonoids, triterpenes, coumarins, and phytosterols (You *et al*., 2010). It has been used in folk medicine for many years to threat fever, lactating and sore throat (Sun *et al*., 2014). In many pharmacological researches, it was showed that the dandelion extracts showed strong antioxidant, anti-inflammatory, anti-fertility and antitumor activities (Jeon *et al*., 2008; Park *et al*., 2010). In different studies, different parts of the dandelion have been investigated and their antioxidant properties (Hu and Kitts, 2005, Park *et al*., 2011), antimicrobial activities (Ionescu *et al*., 2013; Rodino *et al*, 2015; Oseni and Yussif, 2012) and antidiabetic properties (Hussain *et al*., 2004) were reported.

In many studies, solvent extraction was performed using some effective organic solvents such as ethanol, methanol, acetone, ethyl acetate, n-hexane etc. to evaluate the bioactivity of the medicinal plants (Ghaima *et al*., 2013; Oseni and Yussif, 2012). As is known, extraction of the bioactive substances from the plant structure is the main and important process for the medicinal and aromatic plants and the quality and quantity of the bioactive compounds depend on the selected extraction solvent and extraction process (Lucchesi *et al*. 2004). In recent years, the use of green solvents such as glycerol which is environment friendly matter increased because they were evaluated as good alternative to the synthetic and organic

chemicals, and they showed better yield and quality of the extracts (Azmir *et al*. 2013). Glycerol is a natural, non-toxic, biodegradable and recyclable viscous liquid which is produced from renewable sources (Wolfson *et al*., 2006). It shows no easy flammability due to very high boiling point (290 ºC) and it is really cheap solvent compare to other organic ones (Paleologou *et al*. 2016). Apostolakis *et al*. (2014) reported that the glycerol can favorably alter the polarity of the water and so, it can act as an effective co-solvent to increase the polyphenolic substance from the plant structure. In the literature, some studies regarding the efficiency of water/glycerol extracts were performed (Apostolakis *et al*. 2014; Karakashov *et al*. 2015a, Karakashov *et al*. 2015b; Eyiz et al., 2020). Taking into account all of this information, this study was planned to show the efficiency of hydroglycerolic extraction of dandelion aerial parts on some bioactive parameters. For this purpose, effect of hydroglycerolic extraction at different concentrations (25, 50 and 75% w/w) was compared to hydroethanolic and hydromethanolic extractions in terms of antiradical and antioxidant activities for dandelion.

## 2. Materials and methods

### 2.1. Materials

The dried aerial parts of dandelion (*Taraxacum officinale*) were procured from Karakaş Food Plant Co. (İstanbul). The moisture content of the dandelion was 9.93%. Glycerol was purchased from a local supplier in Turkey and ethanol and methanol were provided from Merck (Germany).

### *2.2 . Plant extraction process*

Two g of ground plant sample was weighed. Then 60 mL of solvent (glycerol, ethanol and methanol) at different concentrations (25, 50 and 75 and 100% w/w) as shown in Table 1 was incorporated into the sample and all samples were placed in shaking water bath to extract at room temperature (25±0.5 ºC) for 1 hour. After the process, the samples were centrifuged at 9000 g and 10 ºC for 5 min and the supernatant was filtrated using 0.45 µm and then the extract samples were stored for further analysis.

### *2.3. Analysis of total phenolic content (TPC)*

TPC of the samples was determined using the method suggested by Singleton and Rossi (1965). For this purpose, 200 µl of the extract was mixed with 1800 µl of distilled water. Then 1 mL of diluted (1/10) Folin Ciocelteue reagent and after 1 min later, 2 mL of sodium carbonate (2% w/v) was added into all tubes. The samples were incubated for 2 hours at room temperature and dark conditions. At the end of the incubation, the absorbance values of the samples were recorded at 765 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Total phenolic content of the samples was calculated as mg GAE/g sample using a calibration curve.

### *2.4. Analysis of total flavonoid content (TFC)*

TFC of the samples was measured according to method of Zhishen *et al.* (1999). For this aim, 0.5 ml of the sample was mixed with 2 mL of distilled water and then 150 µl of sodium nitrite (5% w/v) was added into the tubes and the samples were waited for 5 min. Then 150 µl of $AlCl_3$ (5% w/v) was incorporated into the samples and after 6 min waiting, 1 ml of NaOH (1 M) and 1.2 ml of distilled water was added and the final mixture was vortexed and the absorbance of these mixtures was recorded at 510 nm by a UV-Vis spectrophotometer (Shimadzu, Japan) and the total flavonoid content of the samples was calculated as mg catechin equivalent (CE)/g sample.

### *2.5. Determination of DPPH radical scavenging activity*

DPPH radical scavenging activity of the samples was determined as described by He *et al*. (2016). A 100 µL of the extract sample was mixed 3900 µL of DPPH radical solution in methanol (2 mM) and mixed well using vortex. After the incubation of the samples at room conditions in a dark place for 30 min, the absorbance values were recorded at 517 nm by a UV-Vis

spectrophotometer (Shimadzu, Japan). DPPH radical scavenging capacity was calculated as % inhibition using the following Equation 1):

% Inh. (Remaining) = 100-[($Abs_{control}$-$Abs_{sample}$) /$Abs_{control}$]x100 (1)

### *2.6. Determination of $ABTS^{\cdot+}$ radical scavenging activity*

Firstly, $ABTS^{\cdot+}$ radical was produced by preparation of $ABTS^{\cdot+}$ stock solution. For this purpose, 7 mmol/L $ABTS^{\cdot+}$ stock solution was prepared and mixed with 2.45 mmol/L potassium persulfate. It was kept at dark conditions at room temperature for 16 h to complete the radical occurrence. At the end of the time, the stock radical solution was diluted with the buffer solution (pH 7.4) until the absorbance value of 0.7±0.05 at 734 nm was obtained. After that, four different concentrations (15, 30, 45 and 60 µL) of diluted extracts (1:20) were placed into the spectrophotometer cuvettes and 2 mL of $ABTS^{\cdot+}$ solution was placed into the cuvettes having extracts and the samples were incubated for 6 min and the absorbance values of the samples were recorded at 734 nm using a spectrophotometer (Shimadzu, Japan). The radical scavenging activity of the samples as % inhibition was calculated using the following equation (Eq.2).

% Inh. = 100- [($Abs_{control}$-$Abs_{sample}$)/$Abs_{control}$]x100 (2)

where $Abs_{sample}$ is the absorbance of $ABTS^{\cdot+}$ with sample; $Abs_{control}$ is the absorbance of $ABTS^{\cdot+}$ without sample. The % inhibition values were converted into the Trolox values and all results were expressed as Trolox equivalent antiradical capacity (µg TEAC/g sample) (Gong *et al*., 2012).

### *2.7. Ferrous ion chelating activity*

Iron chelating activities of the samples were determined according to the method suggested by Rival *et al*. (2001). For this purpose, 1 mL of the sample extract diluted as 1/10 was taken and 3.7 mL of ethanol (95% v / v) was added. Then, 100 µL of $FeCl_2$ was added to the samples and immediately after vortexing the samples, 200 µl of ferrozine (5 mM) was incorporated into the tubes. The homogeneously mixed samples were allowed to incubate for 10 min at room temperature in the dark and the absorbance values of the samples were measured by UV-Vis spectrophotometer (Shimadzu, Japan) at 562 nm. Iron chelating activity values of the samples were calculated as % inhibition using the following equation.

% Chelating activity= 100-[($Abs_{control}$-$Abs_{sample}$)/$Abs_{control}$]x100 (3)

### *2.8. Ferric reducing antioxidant activity*

Reducing power, which gives an idea about the antioxidant capacity of the samples, was determined based on the method applied by Malomo *et al*. (2011). One ml of the sample extracts of various concentrations and standard (ascorbic acid) were mixed with 2.5 ml of 0.2 M phosphate buffer solution (pH = 6.6) and then 2.5 ml of 1% w/v potassiumferricyanide [$K_3Fe(CN)_6$] was added. The samples were incubated for 20 min at 50 ºC. After this step, 2.5 ml of trichloroacetic acid (10% w/v) was added to the reaction mixture and centrifuged at 1000 g for 10 minutes and 2.5 ml was taken from the top of the solution. 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ were added to the separated part of the solution (2.5 mL) and the samples were mixed by vortex. Then the absorbance values of the samples were measured by UV-Vis spectrophotometer (Shimadzu, Japan). The results were given in mg ascorbic acid equivalent (mg AAE / kg).

### *2.9. Antioxidant capacity by phosphomolybdenum reduction*

Antioxidant capacity of the samples was also evaluated by phosphomolybdenum reduction assays according to Prieto *et al*. (1999). In this regard, 400 µL of extract was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Then the tubes were mixed and placed in a water bath to incubate at 95 ºC for 90 min. At the end of the incubation, the tubes were cooled in an ice bath and the absorbance of the

samples was recorded at 695 nm using UV-Vis spectrophotometer (Shimadzu, Japan). The results were expressed as mg ascorbic acid equivalent (mg AAE/kg) using a calibration curve created by ascorbic acid standard.

### *2.10. Statistical analysis*

Statistical analysis of the data was evaluated using Windows based SAS 8.2 statistical analysis software (SAS Institute, Cary, North Carolina, USA). Duncan multiple comparison was performed with the significance level of 95%. All the analyses were carried out in duplicate with four repetitions.

Table 1. Solvent type and mixture ratios (g/g) used for the extraction

| Solvent type | W | GLY | EtOH | MeOH |
|---|---|---|---|---|
| GLY | - | 100 | - | - |
| GLY-75 | 25 | 75 | - | - |
| GLY-50 | 50 | 50 | - | - |
| GLY-25 | 75 | 25 | - | - |
| EtOH | - | - | 100 | - |
| EtOH-75 | 25 | - | 75 | - |
| EtOH-50 | 50 | - | 50 | - |
| EtOH-25 | 75 | - | 25 | - |
| MeOH | - | - | - | 100 |
| MeOH-75 | 25 | - | - | 75 |
| MeOH-50 | 50 | - | - | 50 |
| MeOH-25 | 75 | - | - | 25 |

GLY: Glycerol, EtOH: Ethanol, MeOH: Methanol

## 3.Results and discussions

### 3.1 Total phenolic and flavonoid content of dandelion extracts

TPC of the samples was illustrated in Fig.1 and it was seen that the TPC of the samples was in the range of 4.63-21.28 mg GAE/g sample. Solvent type affected the TPC of the samples significantly ($p<0.05$) and the highest TPC was recorded for the extract obtained by 75% glycerol (Glycerol: Water 75:25 w/w) while the lowest TPC was for sole glycerol. Ethanol and methanol and also their aqueous mixtures showed lower TPC compared to mixture of glycerol and water. Sole water or ethanol also showed similar TPC for the samples. As is seen from the Fig.1, there were no huge differences between 25, 50 and 75% for ethanol and methanol. This situation was also reported by Amyrgialaki et al. (2014) as both 40 and 60% levels of ethanol showed statistically similar TPC values for the samples. Rodino et al. (2015) reported the TPC of dandelion aerial parts was in the range of 15-19 mg GAE/g sample according to the extraction process type. Eyiz et al. (2020) investigated the bioactivity of red grape pomace extracted by aqueous glycerol and they reported that the TPC of the samples increased significantly with the increase of glycerol concentration ($p<0.05$). It was reported that the TPC of red grape pomace was measured as 16 mg GAE/kg for 10% glycerol level while it was 25.4 mg GAE/kg for the sample extracted by 50% glycerol. Increase of glycerol by fivefold provided an increase in TPC by 50% approximately. In another study, eggplant peel was extracted using an aqueous glycerol by ultrasound application and it was reported that the TPC of the samples increased by the increase of glycerol level in the solvent mixture (Philippi et al., 2016). Similar results for the effectiveness of the glycerol on the increase of TPC were also reported by Blidi et al. (2015).

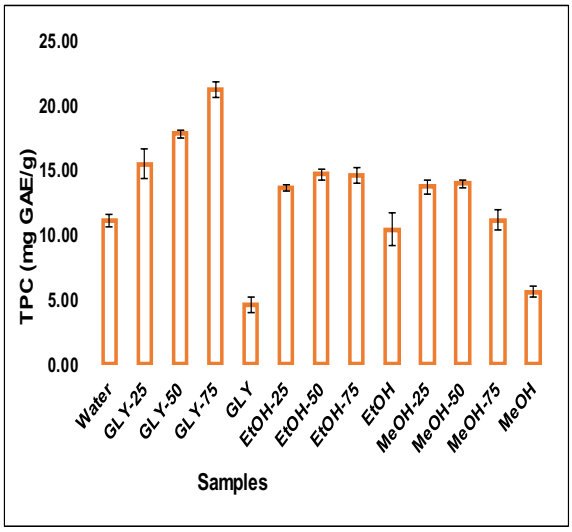


**Fig 1.** Change in total phenolic content (TPC) of the samples depending on solvent type and concentration

It was informed that the main reason behind the mechanism of the effect of higher glycerol levels on the increase of TPC was the lower dielectric constant of glycerol. Additionally, the solubility of the phenolics in the plant is affected by the hydrogen bonding and steric effect of the extraction solvent (Philippi *et al.,* 2016). Also, Apostolakis et al. (2014) reported that the glycerol in the extraction solvents could increase the recovery of the polyphenolic substances due to its ability to change the water polarity.

TFCs ranged between 1.16-14.38 mg CE/g sample (Fig. 2). The lowest TFC was determined in the extract sample obtained by sole glycerol while the highest TFC was for the sample extracted by 75% glycerol as similar to TPC of the samples. Increase of glycerol concentration also increased the TFC of the samples significantly ($p<0.05$). Rodino *et al.* (2015) informed that the TFC of aerial parts of the dandelion extracts was in the range of 5-6.5 RE/g sample as quite similar to the results in the current study. For ethanol, methanol or water, the highest TFC was for the sample extracted by 50% ethanol and it was seen that the 50% ethanol was same with 50% glycerol on the extraction ability of flavonoids.

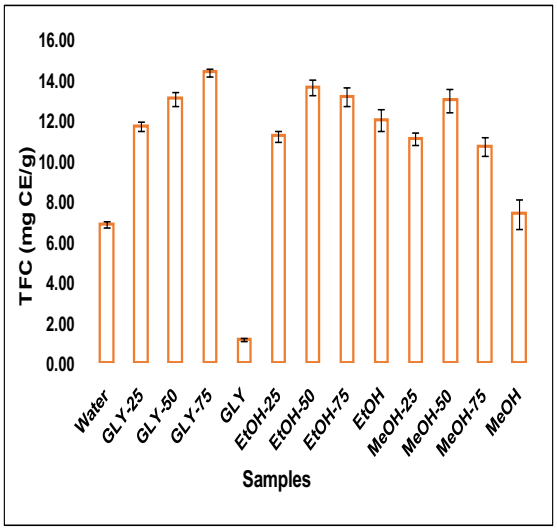


**Fig 2.** Change in total flavonoid content (TFC) of the samples depending on solvent type and concentration

It could be said that the TFC results according to the solvent type were correlated well with the results of TPC positively (r=0.813). Similar results were also reported as the increase in glycerol concentration increased the TFC of the samples due to the reduced polarity, increased hydrogen bonding capacity and steric effects (Eyiz *et al*., 2020, Philippi *et al*., 2016).

### 3.2 Antiradical activity of dandelion extracts

Radical scavenging activity of the dandelion extracts obtained by different solvent was evaluated by DPPH and $ABTS^{\cdot+}$ radical scavenging tests. It was observed that the strong antiradical activity was observed for the samples. Fig.3 shows the change in the remaining DPPH after the scavenging activity of the extract samples obtained by different solvents and as is seen clearly, a significant difference was determined in terms of antiradical performance of the samples ($p<0.05$). The highest remaining DPPH ratio was calculated for the solvent of sole methanol, glycerol and water which also having the lowest total flavonoid and total phenolic content compared to other solvents.

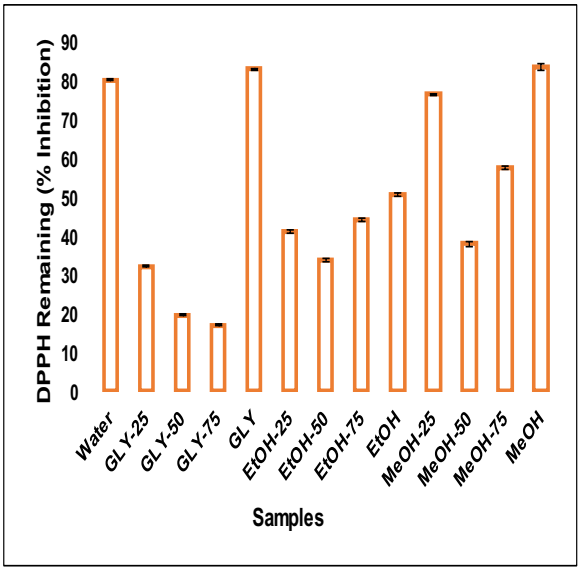


**Fig 3.** Change in DPPH radical scavenging activity of the samples depending on solvent type and concentration

It was concluded that the sole solvent usage had no significant effect on the extraction of the strong bioactive compounds because the lowest remaining DPPH ratio which means the strong antiradical activity was determined for the aqueous glycerol (75%). As compared to aqueous ethanol and methanol, a significantly higher DPPH radical scavenging activity was observed for the aqueous glycerol. It was calculated that there was a negative and significant correlation with the remaining DPPH and total phenolic content ($r=-0.86$, $p<0.05$) and total flavonoid content ($r=-0.81$, $p<0.05$). Similar to the DPPH radical scavenging activity, the samples also showed $ABTS^{\cdot+}$ radical scavenging performance. Fig. 4 illustrates the change in $ABTS^{\cdot+}$ radical scavenging activities of the samples and it is clear from the results that the change in antiradical activities of the samples showed significant differences ($p<0.05$).

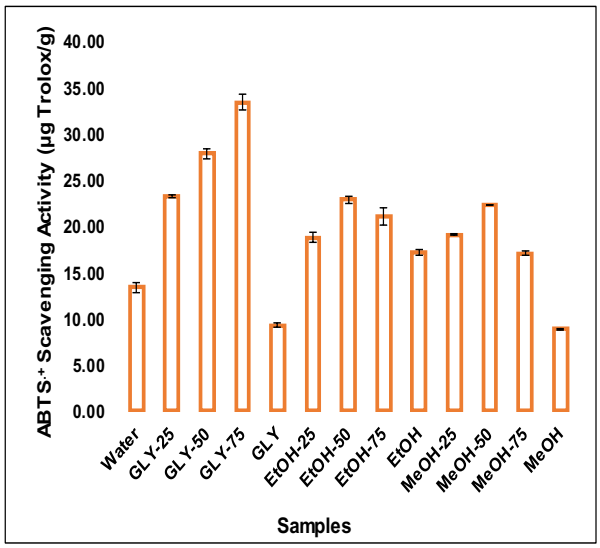


**Fig 4.** Change in $ABTS^{\cdot+}$ radical scavenging activity of the samples depending on solvent type and concentration

The lowest $ABTS^{\cdot+}$ radical scavenging activity (9.29 µg Trolox/g) was calculated for the solvent of sole glycerol and also methanol while the highest $ABTS^{\cdot+}$ radical scavenging activity (33.45 µg Trolox/g) was determined for the aqueous glycerol (75%) as similar to the DPPH radical activity. So, the strong antiradical performance of the aqueous glycerol (75%) was validated by two common antiradical scavenging test. A significant correlation was also observed between TPC and $ABTS^{\cdot+}$ radical scavenging activity ($r=0.97$, $p<0.05$). Also the correlation between two studied radical scavenging test (DPPH and $ABTS^{\cdot+}$) was also high ($r=0.91$, $p<0.05$). Eyiz *et al*. (2020) reported that the glycerol increase showed a higher antiradical activity for the grape pomace extracted due to the increased glycerol provided a higher total phenolic yield because a significant and quite high correlation ($r=0.869$, $p<0.05$) was observed between TPC and antiradical activity of red grape pomace (Eyiz *et al*. 2020). Shehata *et al*. (2015) also reported that the aqueous mixtures of glycerol up to 90% (w/v) showed quite high satisfactory yields for total phenolics from two Artemisia species, at the same liquid-to-solid and the glycerol levels were correlated with the antiradical activities of the samples.

### 3.3 Antioxidant capacity of dandelion extracts

Antioxidant performance of the samples was evaluated by ferric reducing power, ferrous ions chelating activity and phosmolybdenum antioxidant activity test procedures because the antioxidant system is quite complex and it is affected by many factors and so, one method is not enough to describe the antioxidant activity of a sample (El Jemli *et al*., 2016). Fig. 5 shows the ferrous ions chelating ability of the samples. As is seen, the samples showed different chelating performance and the differences among the activities were significant ($p<0.05$). The highest chelating activity was for the samples of aqueous methanol, ethanol and glycerol, respectively. Sole methanol and glycerol showed the weakest chelating performance. Ferrous ions chelating activity shows the antioxidant power of the samples because it was reported that the ferrous state of iron stimulate lipid peroxidation and iron was known to be the most powerful pro-oxidant

among the various species of metal ions (Salar et al., 2015).

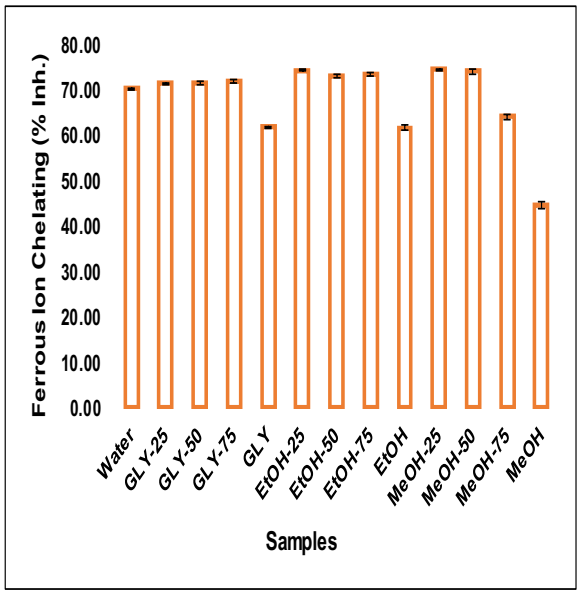


**Fig 5.** Change in ferrous ions chelating activity of the samples depending on solvent type and concentration

It was observed that there was a positive correlation between the chelating performance and TPC of the samples ($r=0.73$, $p<0.05$) and the correlation was also verified by Budhiyanti *et al*. (2011) who reported that the ferrous ions chelating ability of the extracts increased by the increase of TPC. Khokhar and Apenten (2003) reported that the performance of the phenolic substances was related to the ortho-dihydroxy polyphenols. Similarly, it was also informed that the metal chelating activities of the phenolic compounds change with the phenolic structure and the hydroxyl group location and number (Santoso *et al*., 2004; Andjelkovic *et al*., 2006).

Ferric reducing antioxidant power of the samples was showed in Fig. 6. Ferric reducing activity shows the antioxidant performance of the sample extracts. As is seen, aqueous glycerol solution exhibited the strongest ferric reducing activity (31.01 mg AAE/(kg) compared to other solvents to extract the samples. In addition to that, the other aqueous solvents namely ethanol and methanol also showed a weaker antioxidant capacity at all water:alcohol ratios compared to glycerol. As similar to ferrous ion chelating activity, the lowest ferric reducing power was measured for the sole solvents namely glycerol (6.15 mg AAE/kg), methanol (7.78 mg AAE/kg) and water (8.44 mg AAE/kg). It was calculated that there was quite high and significant correlation between ferric reducing antioxidant activity and TPC ($r=0.941$), TFC ($r=0.821$), DPPH radical scavenging activity ($r=0.94$) and $ABTS^{\cdot+}$ radical scavenging activity ($r=0.983$). Similar results were also reported by El Jemli *et al*. (2016) and they found that ferric reducing activity was directly depended on TPC and they reported that there was high correlation between ferric reducing activity and TPC ($r=0.911$), TFC ($r=0.986$), DPPH radical scavenging activity ($r=0.957$) and $ABTS^{\cdot+}$ radical scavenging activity ($r=0.848$). Amin *et al.* (2013) also reported that these tests were important to show the antioxidant power of the extracts because the chelating capacity provided a reduction in the concentration of the catalyzing transition metal.

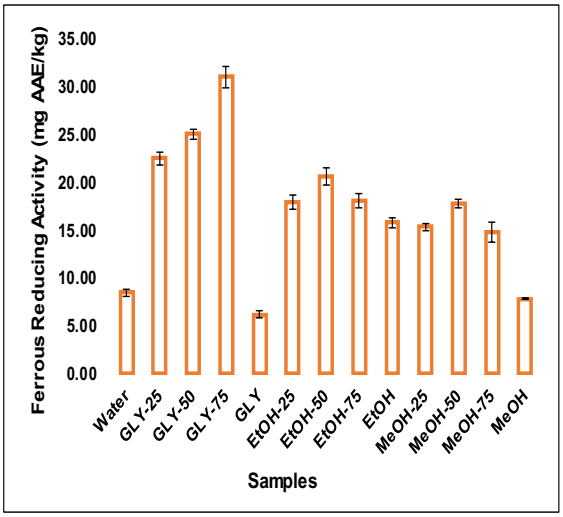


**Fig 6.** Change in ferrous reducing antioxidant activity of the samples depending on solvent type and concentration

Hossain *et al.* (2014) reported that the ferrous ions chelating or reducing activities of the plant extracts show their bioactive performance because iron plays a significant role in the generation of free radical substances and the extracts showing ferrous reducing or chelating performance delays the oxyradical

generation and the consequent oxidative damage.

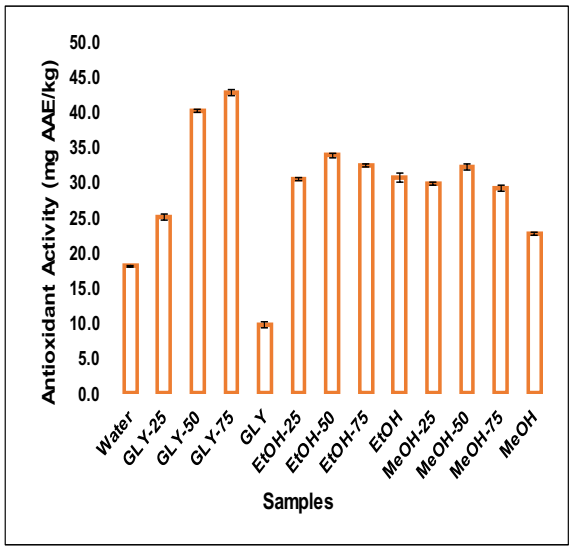


**Fig 7.** Change in antioxidant capacity of the samples depending on solvent type and concentration

Antioxidant activity of the extract samples obtained by different solvents was also evaluated by phosphomlybdenum approach. Sole glycerol extracts showed the weakest antioxidant activity while the strongest antioxidant performance was also for aqueous glycerol (75%) (Fig.7).

Also the mixture of glycerol:water at 50:50 showed quite similar activity in terms of antioxidant performance of 75% glycerol solvent. Antioxidant activity of the samples was well correlated with TPC ($r=0.85$, $p<0.05$), TFC (0.93, $p<0.05$), ferric reducing activity ($r=0.87$, $p<0.05$) and antiradical activity ($r=0.87$, $p<0.05$).

## 4. Conclusions

The present study showed that the extraction of dandelion by using aqueous glycerol is quite efficient process to increase the bioactivity of the produced extracts compared to other solvents such as water, ethanol, methanol or their aqueous mixtures. Among the studied concentrations namely 25, 50, 75 and 100% for the solvents, the best effect in terms of total phenolic content, total flavonoid content, $ABTS^{\cdot+}$ and DPPH radical scavenging activities, and also antioxidant capacities was determined for the solvent of 75% glycerol. It was observed that the hydroglycerolic extraction is the best way to produce green extract from the medicinal plants like dandelion and the effect of aqueous glycerol should be investigated for other important plants used in phytomedicine.

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# HEAT FLUX DENSITY AS THE MAIN VECTOR IN THERMAL CONDUCTIVITY PROBLEMS

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**ABSTRACT**

The advantages of boundary conditions of the second kind in the problems of thermal conductivity and the method of presenting the results of analytical or research work in the form of heat flux density not as a product of the driving force of the process and resistance, but their ratio are shown. For the first time an analogue of the vector Terms for temperature fields – the vector of heat flux density was found. A brief overview of the development of thermometry in Ukraine and the transit calorimetry on its basis is presented. For closed-type calorimeters, recommendations are given for their design and fabrication using the Gauss-Ostrogradsky theorem, which relates the integral flow of a continuously deferenced vector field through a closed surface and the integral of the divergence of this field over the volume bounded by this surface. The Gauss's theorem for a isothermic-shell calorimeter (ISC) states that the total heat flux through its surface and the heat release or absorption capacity in the substance of the sample in the shell are the same, even if these fluxes are non-uniform over the surface and in the volume. The development of heat meters as small-sized, low-inertia sensors of heat flux density allowed the creation of thermal calorimeters-shells which common feature is the combination of the functions of the shell and calorimeter system. Operation of various types of ISC has confirmed their advantages over other calorimeters, namely: ISC shell has a small thermal resistance and inertia compared to the resistance and inertia of the sample, which allows to correctly study nonstationary processes; the calibration process is greatly simplified; the temperature differences are not measured at all; there is no need for differential measurements with a comparison sample etc.

## 1. Introduction

The modern development of food technology equipment is at a fairly high level. An important factor in improving existing and designing new food equipment is the study and study of the properties of food, in particular and thermophysical.

Thermophysical properties determine the nature and speed of the process of heating or cooling the product. These include specific heat, coefficients of thermal conductivity and thermal conductivity. Knowledge of thermophysical characteristics is required to calculate the amount of thermal energy

required for cooling or freezing food during transportation, storage and processing.

The research objective is to show the advantages of boundary conditions of BC-2 of the second kind in thermal conductivity problems and to find the thermal analogue of vectors for energy flows of different types.

## 2. Materials and methods

Of the most common boundary conditions, let us mention the conditions of the first kind (BC-1), when the surface temperature of the solid body $t_b$ is given, and of the second kind BC-2 – the given heat flux density through the surface $q$, the third kind BC-3 – respectively the ambient temperature $t_a$ and the intensity of interaction between the body and the medium, and the fourth kind, when there is perfect thermal contact between the neighboring bodies. The most widespread are BC-3. In the research (Pekhovich and Zhidkikh, 1976) conditions of the fifth and sixth kind are proposed if there is a thin layer of solid or liquid substance with high heat capacity $c\rho$ with or thermal conductivity λ, and $q$ or $t$ are set already on the inner surface of this layer.

In solving problems of thermal conductivity with any boundary conditions, they are often reduced to BC-3

$$q = \alpha \cdot (t_b - t_a). \quad (1)$$

This equation is still called the Newton's law, although BC-3 was introduced by Fourier. In addition α very often depends on $\Delta t = t_b - t_a$ (for example, under free convection conditions) or on $q$ (during condensation on a solid surface). In addition, $t_a$ in (1) is the temperature of the liquid (gas) outside the wall layer, and its thickness also depends on $q$ and $\Delta t$. Therefore, Newton's relation (1) is an identical definition of $\alpha \equiv q/\Delta t$. The only convenience is that we can write $\alpha = 1/R_\alpha$, where $R_\alpha$ is thermal resistance of heat transfer and add $R_\alpha$ to other $R$ in the heat transfer equation of Peclet.

E. F. Adiutori proposed to abandon the concept of "heat transfer coefficient" (Adiutori, 1977) and to investigate all heat transfer processes using the equation

$$q = f_1 \cdot f_2. \quad (2)$$

where $f_1$ – system parameters; $f_2$ – thermal driving force.

For thermal conductivity, the thermal driving force is the temperature gradient, for heat transfer by radiation $T_{source}^4 - T_{receiver}^4$ ($T$ is absolute temperatures of the source and receiver), and for convective heat transfer $t_b - t_a$.

The author (Adiutori, 1977) proposes to abandon also the numbers and similarity equations, the only exception is for free convection by introducing the Jenner number $Je$

$$Je = Nu\ Re = \frac{q\beta g l^4}{\nu\lambda\alpha}, \quad (3)$$

that is, the dimensionless quantity $q$ (the remaining quantities in (3) are the parameters of the system), which allows the experimental data to be processed in the form of equation (2).

For thermal conductivity, the functions $f_1$ and $f_2$ are unambiguous and equal to one

$$q = -\lambda \frac{dt}{dx}, \quad (4)$$

is an equation of Fourier law (or the first Fourier law). This law also has limitations under conditions of unsteady intensive modes of heat transfer, its notation is complicated (Lykov, 1972):

$$q = -\lambda \frac{dt}{dx} - \tau_\rho \frac{dq}{d\tau}, \quad (5)$$

where $\tau_p$ is the relaxation time – an analogous to the relaxation time of stresses that occur in the body under the action of deforming forces. It takes into account the rate of transfer of internal energy, which is not infinitely high, as is customary in the derivation of Fourier's law. For gases $\tau_p \sim 10^{-9}$ c for solids and liquids

is even smaller (for metals $\tau_p \sim 10^{-11}$ c), so when considering the processes of heat power engineering and heat technology, the addend in (5) can always be neglected.

The gradient of any parameter in both space and time can play a greater role in a process than the parameter itself, not only in inanimate nature, but also in living organisms. It is written in the journal "Discoveries and Hypotheses" No. 9 of 2015 on page 22: "Living things in general tend to respond to the gradient rather than the modulus".

Despite the clear advantages of considering separate heat flux density and motive force instead of their ratio (heat transfer coefficient by radiation does not make physical sense at all), the new approach has not yet found a proper place in theory and practice, in monographs and textbooks.

In his monograph (Lykov, 1972) L. V. Lykov considers BC-2 only "when there is heating of bodies in high-temperature furnaces according to the Stefan-Boltzmann law", and for joint heat and mass transfer also under the same conditions of bringing the problem to BC-2. In the monograph (Carslow and Eger, 1964) all thermal conductivity problems of solids are solved from BC-3. In a very useful for researchers and designers work (Pekhovich and Zhidkikh, 1976) only 9 out of 78 problems reduced to computational graphs are devoted to BC-2. We hope that the development of thermometry will provide a proper impetus.

## 3. Results and discussions

Thermometry as a branch of thermophysics and metrology, designed to measure heat flux density $q$, W/m², originated in Ukraine 65 years ago. The first heat meters – the small-sized inertial heat flux density sensors were made in 1955 by spraying paired thermoelectrode materials on the pipe of a laboratory installation for the study of steam drying of Ukrainian earthy brown coal (Fedorov and Herashchenko, 1959). The signal of these so-called single heat meters was small, and they are still made for devices with high $q$. A sharp increase in sensitivity has been achieved in battery sensors of various types. The first author's certificate was obtained for a wafer-type heat meter (Fedorov and Herashchenko, 1963), where the principle of "parallel connection by heat flow and series connection by electrical signal" was implemented (Fig. 1). In the following years, these thermoelectric heat meters of the "auxiliary wall" type began to be mass-produced in Ukraine and other European countries, in the USA and Japan.

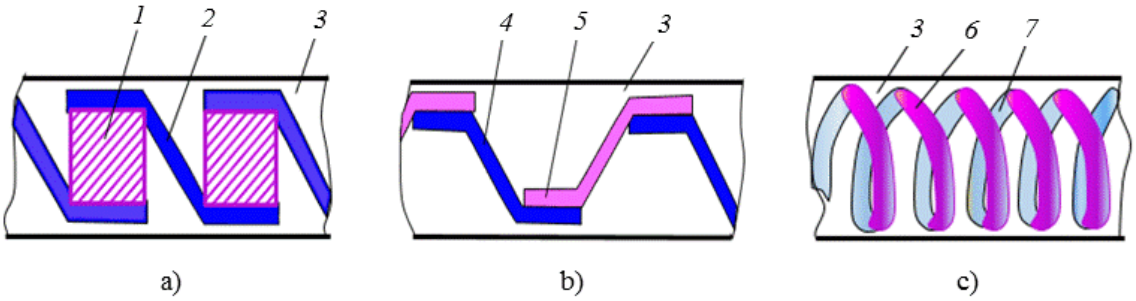


**Figure 1.** Sensitive elements of different calorimeters-shells
a) – wafer-type; b) – stepped; c) – spiral shells:
*1 – constantan, 2 – copper, 3 – insulation, 4 – chromel, 5 – alumel, 6 – constantan covered with copper, 7 – pure constantan wire*

On the basis of heat meters, several dozen types of derived devices have been created and implemented for measuring heat loss, determining thermal conductivity and heat capacity, radiation pyrometry, medical and biological research etc. Their use allowed

reducing heat loss, insulation costs, determining the effective thermophysical characteristics (TPC) of new substances, correctly assessing the heat balance in thermal installations, effectively control and automating new technological processes (Herashchenko, 1971). The results of the study of processes in the food and refrigeration industry are summarized in (Fedorov, 1974), and in agriculture – in (Draganov *et al.*, 1993).

New information is not limited to heat transfer phenomena. Thus, the correlation between $\lambda$ and the strength of fiberglass (Herashchenko, 1971), the hysteresis between $\lambda$ and $c\rho$ of milk fat in (Fedorov *et al.*, 2020), is found. The results (Fedorov *et al.*, 2020) and many others were obtained using the flow thermometric calorimeters, the theory of thermal regimes, which is presented in (Fedoriv *et al.*, 1997).

A separate group among these calorimeters consists of closed flow isothermic-shell calorimeter (ISC). A fundamentally new feature of these ISCs is the combination of the functions of the shell and the calorimetric system. One of the first closed ISCs with a thermometric shell is shown in Fig. 2.

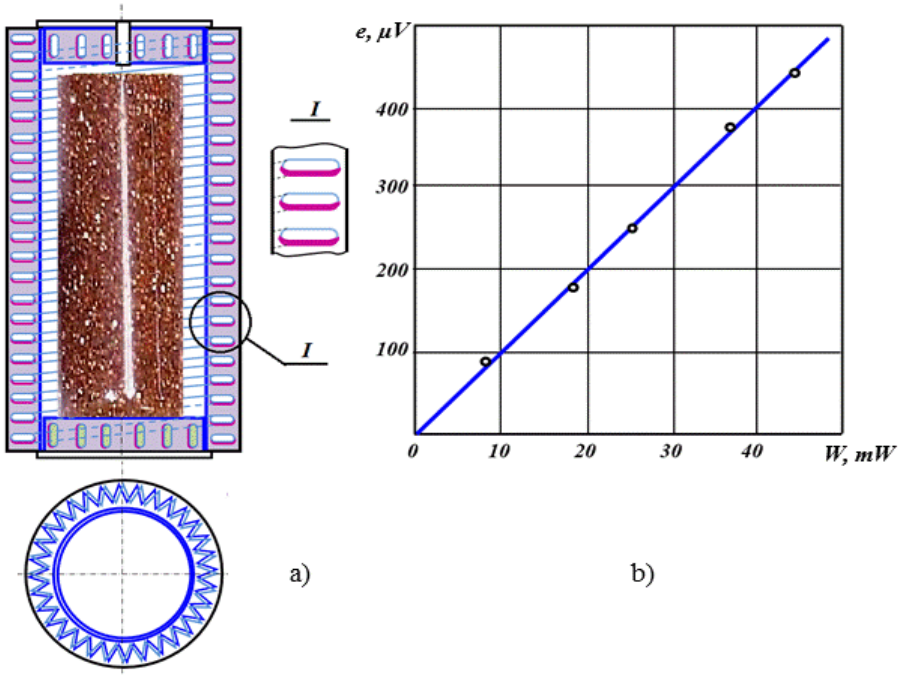


**Figure 2.** Scheme of microcalorimeter with thermometric shell (a) and its calibration characteristics (b)

The whole shell, including the cover, is made of series-connected heat meters, the electrical signal of such a battery is proportional to the average value of $q$ passing through the shell to the sample in it, or vice versa. The issues of thermal interaction of the sample and the shell, the introduction of the necessary corrections to the output signal, as well as reference samples etc. led to the theoretical considerations. They were based on the Gauss-Ostrogradsky theorem. The Gauss-Ostrogradsky theorem connects the integral flux of a continuously differentiated vector field $F$ through a closed surface $S$ and the integral of the divergence of this field over a volume $V$ bounded by this surface:

$$\iiint_V divF = \iint_S (F, n), \qquad (6)$$

where *n* are the coordinates.

Vector Condition is a vector of energy flux density of a physical field, which is transferred per unit time through a unit plane, which is perpendicular to the direction of energy flow at a given point (Ohorodnyk and Fedorov, 2020). Thus, the Vector Condition is a general concept of the quantitative characteristics of the transfer of different types of energy in any physical process. In particular, the vector of the flux density of the electromagnetic field is called the Poynting vector.

In thermophysics in general and in calorimetry separately, when considering thermal conductivity processes, such a vector should be the heat flux density $q$, W/m² – the amount of thermal (internal) energy transferred per unit time through a unit plane perpendicular to the direction of energy flow.

The use of the Gauss-Ostrogradsky theorem in this case is that it can be argued that the sums of heat fluxes through the surface of the shell calorimeter and inside, which is a sample in the shell, are the same, even if these fluxes are uneven on the surface and in volume.

The value of the surface integral in (6) should be determined by the signal of the shell – a calorimetric system consisting of a large number of elementary heat flux sensors connected in series.

Since the vector field $F = q$ is non-uniform, the components $F$ must take $q_x$, $q_y$ i $q_z$. By definition, the divergence of a field $q$ is a scalar, which is a three-dimensional derivative of this field:

$$divq = \lim_{V \to 0} \frac{\int qdS}{V}. \quad (7)$$

This value has a clear physical meaning – volumetric heat flux density, it has units of W/m³ and is denoted by $q_V$. We have the equation in Cartesian axials (8) for a closed shell-calorimetric system ISC:

$$\iiint_V \left(\frac{\partial}{\partial x} + \frac{\partial q_y}{\partial y} + \frac{\partial q_z}{\partial z}\right) dxdydz = \iint_S \left(q_x dydz + q_y dxdz + q_z dxdy\right). \quad (8)$$

Both formally and physically, the result of summing both parts (8) is the integral heat flux $Q$, W, which passes through the shell and is equal to the thermal power, i.e. it is the amount of heat released or absorbed by the sample volume $V$ and surface $S$ per unit time.

The equality of the left and right parts (8) must be maintained during the design and operation of ISC, but this requirement is difficult to implement. The left part (8) requires equality of the sample volume and the internal capacity of the shell, which is possible only if the sample is a liquid, pasty or granular substance. A solid sample can be only if the energy enters it not by thermal conductivity, but in another way, for example, ionizing radiation. In this case, the signal of the thermometric shell must be corrected in the form of the ratio of the volumes of the sample and the shell. In other cases, the property of the air or other gas that fills the shell must be taken into account. The most accurate way to make this correction is calibration.

The right part of equation (8) can be a source of inequality of both parts due to the finite thickness of elementary heat meters and hence the entire heat meter shell. This part is a surface integral of the 2nd kind, i.e. the surface has two sides. Its orientation depends on the chosen direction of the vector $q$, but the thickness of this surface should ideally be zero. In all shell calorimeters, this thickness is an order of magnitude less than the characteristic size of the sample, but if the vector $q$ is directed inside the shell, we must take the inner $S$ and vice versa, which is outside then the outer $S$ shell.

Summation and averaging of the shell signal is performed using modern electronic equipment.

If the design conditions do not allow making completely closed calorimeters, for example, when energy is supplied to the sample radially along a large length of pipe, the role of the end surfaces is played by the "protection zones" of the shell.

The prototype of the ISC is not a Mueller calorimeter, which measures the heat flux from the combustion chamber through a massive heat-conducting wall. Closer to the ISC are the Tian-Calvet calorimeters, in which the measurement of the temperature difference between the sample cell and the thermostated shell provides information about the heat flux.

The operation of different types of ISC has confirmed their advantages over other calorimeters:

a) the ISC shell has low thermal resistance and inertia compared to the resistance and inertia of the sample, which allows to conduct correct experiments during non-stationary processes;

b) difficulties in calibration are significantly reduced or disappear;

c) temperature differences are not measured at all;

d) there is no need for differential measurements with a comparison sample etc. ISCs are competitive in the global calorimeter market (Knauss H. *et al.*, 2006; Knauss H. *et al.*, 2009).

## 4. Conclusions

It is shown that during the theoretical or experimental solution of problems of thermal conductivity in a solid body it is necessary to pay more attention to boundary conditions of the second kind. For the first time on the basis of the Gauss-Ostrogradsky theorem for the processes of thermal conductivity an analogue of the Vector Condition is found – a surface heat flux density. The thickness of the thermometric calorimeter-shell can be reduced to millimeter, for example in modern oblique heat meters.

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# QUALITY CHANGES OF 'CEMPEDAK' (*Artocarpus integer*) FRUIT POWDER PACKAGED IN ALUMINUM-LAMINATED POLYETHYLENE POUCHES

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**ABSTRACT**

'Cempedak' powder was produced by spray-drying of juice produced from Celluclast® 1.5 L-treated 'cempedak' fruit puree, to which 15% (w/w) maltodextrin DE 10 and 0.66% (w/w) calcium phosphate have been added. Spray-drying took place at an inlet air temperature of 160 °C. The powder was packed in aluminum-laminated polyethylene pouches and subjected to accelerated storage at a temperature of 38±1 °C and 90% relative humidity (RH) for 49 days. Spray-dried 'cempedak' fruit powder was found to have a more pronounced hygroscopicity and caking tendency with the increase of storage time-apart from becoming darker, more reddish but less yellowish. The kinetics of most quality parameters monitored was of zero-order, indicating that the 'cempedak' fruit powder degradation was constant: while hygroscopicity and water solubility index was of the first order. Under accelerated storage conditions, the shelf-life was extrapolated to be 60.43 days, based on the Guggenheim-Anderson-de Boer (GAB) model for water activity-moisture content.

## 1. Introduction

Protection of powder in packaging with a good barrier is essential, as, during storage and distribution, the product may be exposed to high temperature, light, oxygen and humid environment that leads to food degradation (Henríquez *et al.*, 2013). The selection of storage conditions is also important as it influences the food quality and shelf life (Yu *et al.*, 2015). A film such as laminated metalized films that are made from polyethylene and aluminum foils are commonly applied in the packaging of snacks and high-value food, as it has good protection to dried foods (Zorić *et al.*, 2016). Besides, aluminum-laminated polyethylene has low permeability to water vapor, thus prolonging the shelf-life of the product stored in the packaging pouch (Dak *et al.*, 2014).

Aluminum-laminated polyethylene has been applied in the packaging of aloe vera powder, coconut milk powder and also mango

soy fortified yogurt powder (Ramachandra and Rao, 2013; Kumar and Mishra, 2004). In these studies, aluminum-laminated polyethylene was reported to be more superior compared to high-density polypropylene (HDPP), biaxially oriented polypropylene (BOPP) and polypropylene (PP), in retaining powder qualities and having longer product shelf-life (Ramachandra and Rao, 2013; Kumar and Mishra, 2004). Recent study from Loo and Pui (2020) concluded that aluminum-laminated polyethylene (ALP) is better compared to polyethylene terephthalate (PET) in retaining the properties of spray-dried Kuini mango powder, where the powder in ALP has lower water activity, moisture content, hygroscopocity and caking.

The shelf-life of a food product is the maximum time it can be stored without any deterioration in acceptability and quality, where the prediction of shelf life of a package-product was performed in the area of water transmission rate and water uptake that (Jena and Das, 2012). On the other hand, there are other definitions of shelf-life where it is considered as the time where the food reaches critical moisture content, thus causing caking to start (Labuza, 1982; Robertson, 2010). Shelf-life testing is performed where samples were subjected to conditions that mimic conditions prone to be encountered before consumption (Brown and Williams, 2003). The application of accelerated storage methods should be used to develop moisture ingress and storage time relationship quickly (Pua *et al.*, 2008). It involves the application of high humidity 90% relative humidity (RH) and temperatures at 38 °C.

Accelerated storage study has been incorporated in the work of on storage stability of apple peel powder, coconut milk powder and pomegranate arils, respectively (Henríquez *et al.*, 2013; Dak *et al*., 2014; Jena and Das, 2012). Apple peel powder packaged in metalized films of the high barrier under conventional and accelerated condition was reported to have had shelf-life of 298 and 120 days, respectively, indicating a reduction of 2.5-fold of shelf-life with the application of accelerated storage study (Henríquez *et al.*, 2013). Based on accelerated storage tests, previous studies predicted aloe vera powder and mango soy fortified yogurt powder packaged in aluminum-laminated polyethylene to be 51.05 and 54 days, respectively (Dak *et al.*, 2014; Ramachandra and Rao, 2013).

Kinetic modeling is used to predict the changes against time (Ramachandra and Rao, 2013; Van Bockel, 1996). The deterioration of kinetics is measured under either environmental or accelerated conditions (Hough *et al.*, 2006). It can be determined according to equations that involve the rate of reaction against time. Common changes such as in food color either follow zero or first-order degradation reaction kinetics (Singh, 2000; Kumar and Mishra, 2004). The zero-order rate normally described the effect of reaction that is caused by enzymatic degradation or non-enzymatic browning and lipid oxidation (Singh, 2000). Food deterioration reactions involving vitamin, protein loss and microbial growth showed the first-order loss.

'Cempedak', also known as *Artocarpus integer*, is a smaller fruit that is similar to jackfruit (*Artocarpus heterophyllus*) (Subhadrabandhu, 2001). It has green, yellow or brown skin that is either round or spiky, while its pulp is soft and golden yellow to orange in color (Chong *et al.*, 2008). 'Cempedak' pulp can either be consumed fresh, processed into a refreshing juice or creamed to make jams and cakes (Janick and Paull, 2008; Subhadrabandhu, 2001). To increase product availability, 'cempedak' juice can be spray-dried into powder, as powder form can serve as an ingredient to the various food product while reducing transport cost, as compared to fresh fruits (Chew *et al*., 2019). As 'cempedak' fruit is high in sugar, the encapsulation of the fruit puree with maltodextrin is essential to produce a powder that is non sticky and free-flowing. In our previous work, optimization of spray-drying of 'cempedak' powder were carried out, with the recommended condition of inlet air temperature and maltodextrin concentration of

160 °C and 15% (w/w), respectively (Pui *et al*., 2020a). Among different anti-caking agents (calcium silicate, silicon dioxide and calcium phosfate), Pui *et al.* (2020b) reported that Calcium phosfate (0.66 % w/w) yielded powder with the best properties: lowest moisture content, water activity, hygroscopicity and change in cake height ratio.

This research aimed to study the storage stability of spray-dried 'cempedak' fruit powder that has been packed in aluminum-laminated polyethylene pouches. The 'cempedak' fruit powder was kept under accelerated temperature conditions, namely 90% relative humidity (RH) and 38 °C, and the moisture content, water activity, hygroscopicity, color, degree of caking, water solubility index and total carotenoid content of the powder were monitored over time of storage.

## 2. Materials and methods

### 2.1. Materials

'Cempedak' variety CH28 was procured in 3 different batches (n = 3), with ten fruits per batch from the Department of Agriculture, Serdang, Selangor, Malaysia. Ripe 'cempedak' fruit was cut into half, pulp separated from the seeds and then vacuum-packed in transparent polyethylene bags (200 g per bag) and stored at -20 °C in the dark. Frozen 'cempedak' pulp was thawed at room temperature before the experiment and homogenized to puree form using a commercial blender at low speed (Pui *et al*., 2018).

The 'cempedak' puree was then diluted with water at 1:2 ratio and treated with Celluclast® 1.5 L (1.2% v/w) at 45 °C for 1 hour. After filtration through a piece of muslin cloth, the filtrate ('cempedak' juice) was spray-dried using a Büchi B-290 mini spray-dryer (Büchi Labortechnik AG, Flawil, Switzerland) with the addition of 15% (w/w) maltodextrin and 0.66% (w/w) calcium phosphate (Pui *et al*., 2020b). Spray-drying was conducted at an inlet air temperature of 160 °C, with flow rate, dryer aspirator rate and pump rate of 900 $m^3$/min air, 100% and 10%, respectively. Outlet air temperatures used ranged from 85-95 °C, with a feed flow rate of 5 mL/min (Pui *et al*., 2020a). The resultant powder was then used to study the effect of accelerated storage.

### 2.2. *Packaging of spray-dried 'cempedak' fruit powder*

'Cempedak' fruit powder (25±0.5 g per package) was sealed in aluminum-laminated polyethylene pouches (155×135 mm, Infra Plastic Sdn. Bhd. Selangor) by heat sealing using a vacuum packager (DZQ400/500, YuSheng, China), avoiding any air pockets. The pouches were then placed in a desiccator (maintained at 90±1% relative humidity by using saturated potassium nitrate solution), and the desiccator was placed in a convection oven (UFB 500, Memmert GmbH & Co. KG., Schwabach, Germany) at 38±2 °C temperature for 49 days. The pouches were properly arranged to avoid overlapping of the pouches (Kumar and Mishra, 2004). Physicochemical analyses were carried out on the stored powder after spray-drying and at 7 days intervals, in a total of 49 days (Ramachandra and Rao, 2013). The analyses carried out were water activity, moisture content, hygroscopicity, color, caking, water solubility index and total carotenoid content.

### 2.3. *Assessment of spray-dried 'cempedak' fruit powder quality during storage*

#### *2.3.1 Water activity and moisture content*

The measurement of water activity was carried out using a water activity meter (PRE 00207, AquaLab Pre, Decagon Devices, Inc., Pullman, USA). AOAC (2000) method was applied to determine the moisture content of stored 'cempedak' fruit powders (Chang *et al.*, 2020). Calibration was conducted before sample measurement, in which potassium sulfate ($K_2SO_4$) and potassium chloride (KCl) solution were used for calibration.

#### *2.3.2. Hygroscopicity*

Hygroscopicity of the 'cempedak' fruit powder was determined by placing 'cempedak' fruit powder (2 g) (placed in a pre-weighed petri dish) in an airtight desiccator containing 500

mL of a saturated solution of $Na_2SO_4$, for one week at room temperature (Cai and Corke, 2000). Hygroscopicity of the powder was then calculated by weight difference, expressed as grams of adsorbed moisture per 100 g dry solids.

***2.3.3. Color***

A Hunter Lab ColorFlez Ultra-Scan spectrophotometer (Hunter Associate Laboratory Inc., Reston, USA) was used to determine the color values of stored 'cempedak' fruit powder (Wong *et al.*, 2015). The instrument was calibrated against a white tile and black tile before sample measurement. Color value readings were expressed in L* (lightness-darkness), a* (greenness-redness) and b* value (blueness-yellowness).

***2.3.4. Caking properties***

The caking test was performed using a powder rheometer, TA.HD Plus Powder Flow Analyzer (Stable Micro Systems, Godalming, UK) based on the method described by Janjatović *et al.* (2012). 'Cempedak' fruit powder (40 g) was added to the apparatus power column, and the rheometer's blade first leveled the top of the powder column to measure column height, and then move down at a speed of 20 $mms^{-1}$ to compact the powder to 200 g force. After that, the blade was moved downwards at 10 $mms^{-1}$ to slice through the powder. The compaction was performed in five cycles. From the software, the cake strength, mean cake strength and change in cake height ratio were obtained.

***2.3.5. Water Solubility Index (WSI)***

The method described by Grabowski *et al.* (2006) was employed to determine the water solubility index (WSI) of spray-dried 'cempedak' fruit powder. 'Cempedak' powder (1 g) was mixed vigorously (30 seconds) with 10 mL water in a 15 mL centrifuge tube, and the powder suspension was incubated in a 37 ºC water bath for 30 minutes. It is then centrifuged at 2000 *x g* for 10 minutes at room temperature (Beckman J2-21M/E, Beckman Coulter, Inc., California, USA). The supernatant was placed in an aluminum tray (1 cm height x 3.5 cm diameter) and was dried overnight in an oven (UFB 500, Memmert GmbH & Co. KG., Schwabach, Germany) at 105 °C. WSI was expressed as the percentage of the total dry solids over the original weight of 'cempedak' fruit powder used in the analysis.

The calculation of WSI was shown in Eq. (1):

$$WSI = \frac{\text{Weight of residue}}{\text{Weight of 'cempedak' fruit powder}} \times 100 \quad (1)$$

***2.3.6. Carotenoid content***

'Cempedak' fruit powder (1 g) mixed with distilled water (10 mL) was subjected to incubation at room temperature for 30 minutes, following which 20 mL of cold acetone was added and mixed. The mixture was left to stand for 15 minutes before filtration with suction through a Whatman No. 1 filter paper, and filtrate collected. The residue was then placed in a mortar, and 15 mL cold acetone was added. A pestle was used to grind the residue to form a suspension, which was then filtered. All the filtrate was pooled, and 1/3 of the total volume was added with 20 mL petroleum ether into a 500 mL separation funnel, followed by 300 mL of distilled water. After mixing and separation of phase, the bottom colorless aqueous layer was discarded. Another 1/3 of the filtrate was added to the separation funnel, and the process of extraction was again repeated as described above. After the third extraction, the yellow-colored organic phase (upper layer carotenoid extract) was collected. The organic phase was evaporated to dryness at 35 °C, and the 10 mL acetone was added to dissolve the carotenoids. The absorbance of the solution was then read at 450 nm (Rodriguez-Amaya and Kimuram, 2004). A standard curve was constructed using different concentrations of the standard solution (0 to 6 mg/mL).

***2.3.7. Kinetics of property changes during accelerated storage***

The degradation constant (K) of 'cempedak' fruit powder was determined based on the moisture content, hygroscopicity, color change,

caking (change in cake height ratio), water solubility index and carotenoid content while considering zero-order or first-order kinetics for these aspects according to Ramachandra and Rao (2013). The zero and first-order kinetics were determined using the Eq. 2 and 3 below:

$$[A]t = -kt + [A]0 \quad (2)$$

$$\text{In } [A]t = -kt + \text{In}[A]0 \quad (3)$$

Where [A]t = Concentration of the chemical of interest at a particular time (t), and [A]0 = Initial concentration, with the k = Order rate constant.

***2.3.8. Measurement of permeability of packaging material***

The water vapor permeability rate, k ($kg.m^{-2}$ $day^{-1}$ $Pa^{-1}$), was calculated using Eq. 4 (Labuza, 1984).

$$k = \frac{dw/d\theta p}{Ap\ P^*} \quad (4)$$

In which $dw/d\theta p$ = the slope of the straight-line plot between the time θp (day) and weight (kg) of the silica gel, Ap = Surface area of the packaging material ($m^2$), and P* = Saturation vapor pressure of water.

***2.3.9. Assessment of shelf-life of spray-dried 'cempedak' fruit powder***

The shelf-life of the powder was calculated according to the equation below (Eq. 5) (Crank, 1999).

$$\int d\theta = \frac{W_s}{P^* k A_p}\left(\int_{X_i}^{X_o} \frac{dX}{RH - a_w}\right) \quad (5)$$

where θ = Shelf-life (days), Ws = Weight of the dry solids (g), P* = Saturated vapor pressure of water at ambient temperature (Pa), k = Permeability of packaging material ($kg.m^{-2}day^{-1}Pa^{-1}$), Ap = Surface area of the packaging material ($m^2$), RH = Relative humidity of the environment in which the package is placed (%), aw = Water activity of the product, Xi = Initial moisture content (%, d.b.) and Xc = Critical moisture content (% d.b.).

**2.4. *Statistical Analysis***

The statistical program used was Minitab 17 software (Minitab Inc., Pennsylvania, USA) was used to analyze the data obtained from this study using one-way ANOVA and significant differences ($p \leq 0.05$) using Tukey's test. All measurements were conducted in triplicates. The results were expressed as mean ± standard deviation.

## 3. Results and discussions

Table 1 presents the effects of accelerated storage (38 °C, 90% RH) on several properties of 'cempedak' fruit powder packed in aluminum-laminated polyethylene (ALP) and stored at accelerated condition (90% RH and 38±2 °C). The properties assessed included water activity, hygroscopicity, color, degree of caking and water solubility index

### 3.1. Water activity and moisture content of stored 'cempedak' powder

Water activity is an important index for spray-dried powder, as it can greatly affect its shelf-life (Thankitsunthorn *et al.*, 2009). It can be concluded that the increase of water activity (to 0.25) was negligible as the range of powder was in the range of 0.2-0.3, where the powder is considered as stable (Yu *et al*., 2015).

Figure 1 shows that the moisture content of 'cempedak' fruit powder packaged in aluminum-laminated polyethylene pouches showed a 50% increase at the end of the storage period (Week 7), as compared with the moisture content of initial 'cempedak' fruit powder (0 day storage time). This value (7.0%) was lower than the maximum moisture content of 10% for food powders to remain stable (Tze *et al.*, 2012).

Also, moisture content increase in 'cempedak' fruit powder followed zero-order kinetics (Table 2), indicating a constant rate in the uptakes of water by the powder during the

storage period. A similar pattern was also observed by Kumar and Mishra (2004) in the mango/soy yogurt powder, where it is dependent on the packaging material as well (Kumar and Mishra, 2004). Hence, to keep the quality of powder during storage, utilizing the packaging material with a good moisture barrier property is essential (Rao *et al.*, 2011). Temperature and RH also significantly affected the moisture gain was also reported in jackfruit powder (Pua *et al.*, 2008).

### 3.2. Hygroscopicity of stored 'cempedak' powder

Hygroscopicity measures the material's ability to absorb moisture from its environment (Vidović *et al.*, 2014). Table 1 shows the effects of accelerated storage on the hygroscopicity of the packaged 'cempedak' fruit powder. It can be concluded that increasing the storage time led to the hygroscopicity of 'cempedak' fruit powder to increase 1.3-fold at Week 7 as compared to Week 0. The moisture uptake depends on the water vapor permeability of the packaging material (Dak *et al.*, 2014). Besides, the rate of hygroscopicity increase followed first-order reaction kinetics (Table 2), suggesting the degradation of powder compounds (Singh, 2000). However, it is hard to predict the time when the powder deteriorates enough to be unacceptable to the consumers, as there were no guidelines on the maximum hygroscopic range.

The increment of moisture content decreases the water retention capacity, thus deteriorating its physical, chemical and technological properties. The hygroscopicity of mango powder was also reported to increase with storage time (Jaya and Das, 2005). From Figure 1 and Table 1, it can be observed that moisture content increase also leads to an increase of hygroscopicity in 'cempedak' powder.

### 3.3. Color of stored 'cempedak' powder

Color is an important quality indicator of food, and it is used to determine its acceptance, while the quality of deterioration was indicated by retention of color (Shin and Bowmik, 1995).

From Table 1, it is observed with increased storage time, the L*and b* values of 'cempedak' fruit powder decreases (reduction of 21% and 13%), while a* values increases (14%). This indicates that as the storage time increased, the stored 'cempedak' fruit powder becomes darker, more reddish and also yellowish. The decreasing of L* values and increasing of a* value indicates browning (Pua *et al.*, 2008). Sornsomboonsuk *et al.* (2019) suggested that the elevated storage temperature increases the rate of oxidation, which in turn causes the rise a* and lowering L* of the bael powder stored.

Figure 2 shows the total color change across the storage period, where an increase of 16.0 was noted. However, there was no limit in which range a product should be rejected, although the changes should be as the minimum possible. Total color changes (∆E) increased with the increase of storage time, while storage temperature did not have much effect (Yu *et al.*, 2015; Idham *et al.*, 2012). The increase in yellowness indicates the powder's tendency to become brown. At a water activity level of 0.3-0.7, Maillard, or non-enzymatic, the browning reaction takes place (Yu *et al.*, 2015). From Table 1, it can be observed that the increase of water activity also leads to the changes in color (increase in darkness and redness, while the decrease in yellowness), probably due to browning.

**Table 1.** Effects of accelerated storage (38 °C, 90% RH) on properties of 'cempedak' fruit powder packaged in aluminum-laminated polyethylene pouches

| Powder property | Storage time (week) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Water activity | 0.19± 0.01$^{a}$ | 0.19± 0.01$^{ab}$ | 0.20± 0.01$^{ab}$ | 0.21± 0.01$^{ab}$ | 0.22± 0.00$^{ab}$ | 0.23± 0.00$^{ab}$ | 0.24± 0.00$^{b}$ | 0.25± 0.02$^{b}$ |
| Hygroscopicity (g/100 g) | 20.7± 0.6$^{a}$ | 22.3± 1.2$^{ab}$ | 22.0± 1.0$^{ab}$ | 22.7± 1.3$^{b}$ | 23.0± 1.0$^{bc}$ | 24.7± 1.2$^{bc}$ | 25.0± 1.0$^{c}$ | 23± 0.6$^{d}$ |
| Color | | | | | | | | |
| *L** | 73.42± 0.99$^{a}$ | 69.55± 1.02$^{b}$ | 66.56± 1.20$^{c}$ | 64.72± 1.34$^{c}$ | 64.27 ± 1.49$^{cd}$ | 61.67± 1.35$^{d}$ | 59.91± 0.63$^{de}$ | 58.00± 1.90$^{e}$ |
| *a** | 8.61± 0.36$^{a}$ | 5.56± 0.34$^{ab}$ | 8.77± 0.34$^{ab}$ | 8.84± 0.10$^{ab}$ | 9.01± 0.49$^{ab}$ | 9.10± 0.55$^{ab}$ | 9.20± 0.56$^{ab}$ | 9.82± 0.69$^{b}$ |
| *b** | 34.49± 1.02$^{a}$ | 32.01± 1.71$^{ab}$ | 31.85± 1.27$^{a}$ | 31.55± 0.17$^{ab}$ | 31.45± 1.83$^{ab}$ | 31.49± 1.25$^{ab}$ | 30.42± 1.59$^{b}$ | 29.97 ± 1.07$^{b}$ |
| Cake strength | 840± 129$^{a}$ | 2175± 272$^{b}$ | 2547± 263$^{b}$ | 2444± 292$^{ab}$ | 1164± 137$^{b}$ | 3702± 364$^{c}$ | 5895± 842$^{d}$ | 12990± 1293$^{e}$ |
| Mean cake strength | 159.04± 38.50$^{a}$ | 198.42± 30.57$^{ab}$ | 195.97± 59.40$^{ab}$ | 151.78± 27.81$^{ab}$ | 132.22± 149$^{a}$ | 159.47± 22.61$^{ab}$ | 212.24± 8.27$^{c}$ | 232.40± 18.27$^{d}$ |
| Water Solubility Index (WSI) | 89.74± 0.35$^{a}$ | 86.05± 0.5$^{b}$ | 85.85± 1.03$^{ab}$ | 85.72± 1.06$^{ab}$ | 83.88± 0.49$^{ab}$ | 83.53± 0.75$^{bc}$ | 81.27± 1.30$^{bc}$ | 78.64± 2.16$^{c}$ |

Each value represents the mean of triplicate samples ± standard deviation. Values within the same row with different superscript (a-e) are significantly different at $p \leq 0.05$, as measured by Tukey's HSD test.

**Table 2.** Non-linear regression analysis of physicochemical property kinetics in 'cempedak' fruit powder

| Powder property | Zero-order | | | First-order | | |
|---|---|---|---|---|---|---|
| | $K_0$ | $[A]_0$ | $R$ | $K_1$ | $[A]_0$ | $R$ |
| Moisture content | 0.340 | 4.659 | 0.991 | 0.057 | 4.786 | 0.977 |
| Hygroscopicity | 0.809 | 20.635 | 0.950 | 0.341 | 20.747 | 0.957 |
| Total color change | 2.096 | 1.444 | 0.992 | 0.185 | 4.707 | 0.956 |
| Change in cake height ratio | 0.028 | -0.005 | 0.999 | 0.147 | 0.149 | 0.822 |
| Water solubility index | -1.164 | 88.85 | 0.912 | -0.014 | 88.29 | 0.953 |
| Carotenoid content | -0.114 | 1.068 | 0.956 | -0.235 | 1.296 | 0.863 |

Abbreviations: K0, Zero order rate constant; K1, the first-order rate constant; [A]0, initial concentration; R, the Regression coefficient of the reaction.

Table 2 shows the color change kinetics during accelerated storage. It can be observed that the degradation of 'cempedak' fruit powder color is followed by zero-order kinetics. Change in the total color difference that followed zero-order kinetics, indicating enzymatic degradation or non-enzymic browning, was also reported for mango soy fortified yogurt powder (Kumar and Mishra, 2004). A study on the kinetics of color change in dried aloe vera gel powder during storage for 49 days at 38±1 °C and 90±1% RH showed the color change of powder stored in ALP is 19.62 (Ramachandra and Rao, 2013). The kinetic parameter of color change was obtained from first-order kinetics. The value of the rate constant for AF pouches (k1) was −0.0444, in which the negative sign indicates it reduces with the increase in storage time. Aluminum foil above 17 microns in thickness is considered as a good barrier against moisture, gases and light (Emblem, 2000).

### 3.4. Caking of stored 'cempedak' powder

The caking properties in terms of cake strength and mean cake strength are also presented in Table 1, while the change in cake height ratio is presented in Figure 3. Results obtained that the cake strength, mean cake strength and change in cake height ratio increased about 15.5, 1.3 and 2.2-fold, respectively, after 7 weeks of storage. The increase in change in the cake height ratio shows that it is more prone to caking (Shah *et al.*, 2008). However, there was no range reported on the maximum limit of the cake height ratio in defining its caking properties.

Pua *et al.* (2008) reported that lumpiness in jackfruit powder increases with the increase of storage time due to an increase in moisture content. Mango and soy fortified yogurt powder showed caking at a moisture content between 6.8% and 2% (Kumar and Mishra, 2004). The caking is attributed to the migration of moisture from the environment, increasing the water activity that caused the plasticizing of compounds and collapse in structure (Lee *et al.*, 2013). Also, Durakova *et al.* (2019) attributed the change in granulometric composition in Lucuma powder to packaging permeability.

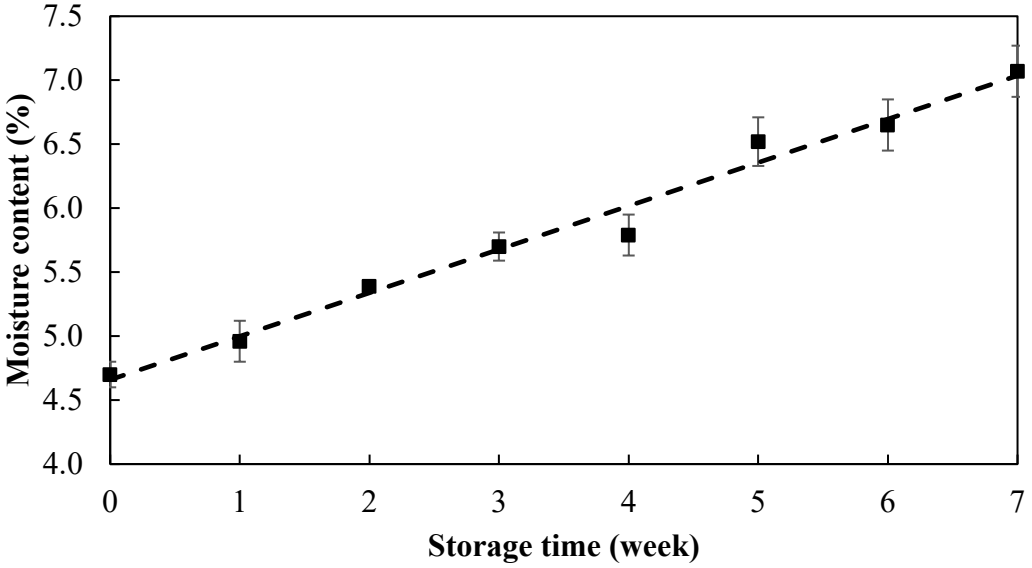


**Figure 1.** Effects of accelerated storage (38°C, 90% RH) on moisture content (%) of 'cempedak' fruit powder packaged in aluminum-laminated polyethylene pouches

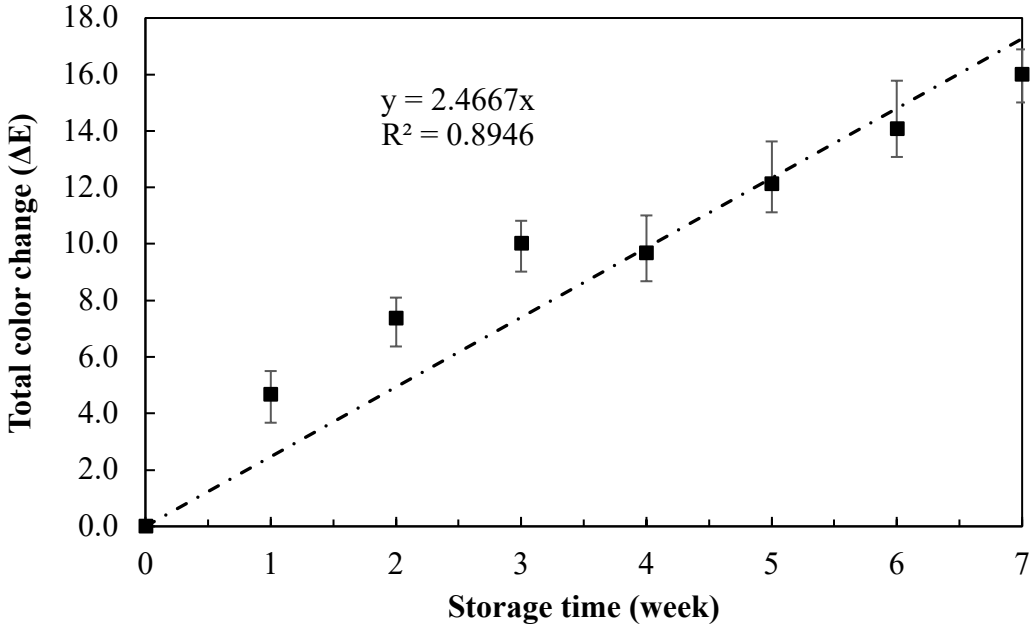


**Figure 2.** Effects of accelerated storage (38 °C, 90% RH) on total color change (ΔE) of 'cempedak' fruit powder packaged in aluminum-laminated polyethylene pouches

The kinetics of caking in terms of change in cake height ratio changes in stored 'cempedak' fruit powder is presented in Table 2. It can be concluded that the caking of stored powder followed by zero-order kinetics. This may be due to the continuous increase in moisture uptake during storage (Pua *et al.*, 2008).

### 3.5. Water solubility of stored 'cempedak' powder

The water solubility index of 'cempedak' fruit powder packaged and subjected to accelerated storage is shown in Table 1. It can be seen that the water solubility index of stored 'cempedak' fruit powder decreased following the increase of storage time. However, the reduction of 12% in the water solubility index after Week 7, indicates that the stored 'cempedak' fruit powder still retains its water-soluble properties. Gavarić *et al.* (2019) also observed a decrease in solubility of spray-dried basil extract with storage time, in which after 50 days of storage, dehydration time increased from 6-9.1s to 11.2 to 18.3 s.

The slight reduction of the water solubility index of stored 'cempedak' fruit powder may occur due to the caking or lump formation of powder (Laokuldilok and Kanha, 2015). The caking of powder from absorbed moisture as the time of storage increases was affected by its water vapor permeability of ALP pouch (Pua *et al.*, 2008). Besides, the first-order reaction kinetics shown in Table 2 indicates microbe or loss of powder component such as protein or vitamins (Singh, 2000).

### 3.6. Carotenoid content of stored 'cempedak' powder

Figure 4 provides information on the stability of the carotenoid content of packaged 'cempedak' fruit powder at accelerated storage. It can be observed that there is a decrease in carotenoid content with an increase in storage time, incurring a reduction of 63% and 88% after 6 and 7 weeks of storage, respectively. The loss in carotenoid content compromised the quality of the fruit powder. The storage of mango powder was packed in metalized polyester/ polyester poly packaging, where after 6 months of storage, the carotene amount was still in the acceptable and good range (50%

of pigment retention) (Hymavathi and Khader, 2005).

Table 2 shows the kinetics of changes in carotenoid content of the 'cempedak' fruit powder during accelerated storage. As can be observed, the degradation of carotenoids in the 'cempedak' fruit powder followed zero-order kinetics, indicating the degradation of carotenoid pigments (Singh, 2000). Although there is scarce information on the carotenoid degradation kinetics with storage of powder, anthocyanin retention and degradation in accelerated storage has been examined by a few researchers (de Oliveira *et al.*, 2009; Idham *et al.*, 2012; Tonon *et al.*, 2010). The kinetic degradation of anthocyanin in dried pomegranate arils was found to be following zero-order kinetics (Dak *et al.*, 2014).

On the other hand, Tonon *et al.* (2010), in their work on spray-dried acai powder, observed two first-order kinetics for the anthocyanin degradation, the highest rate at 45–60 days and lower degradation rate until 120 days. The first order of anthocyanin degradation was exhibited by the pomegranate aril (Dak *et al.*, 2014). Degradation of total carotenoid in jackfruit powder also followed a first-order reaction (Saxena *et al.*, 2012).

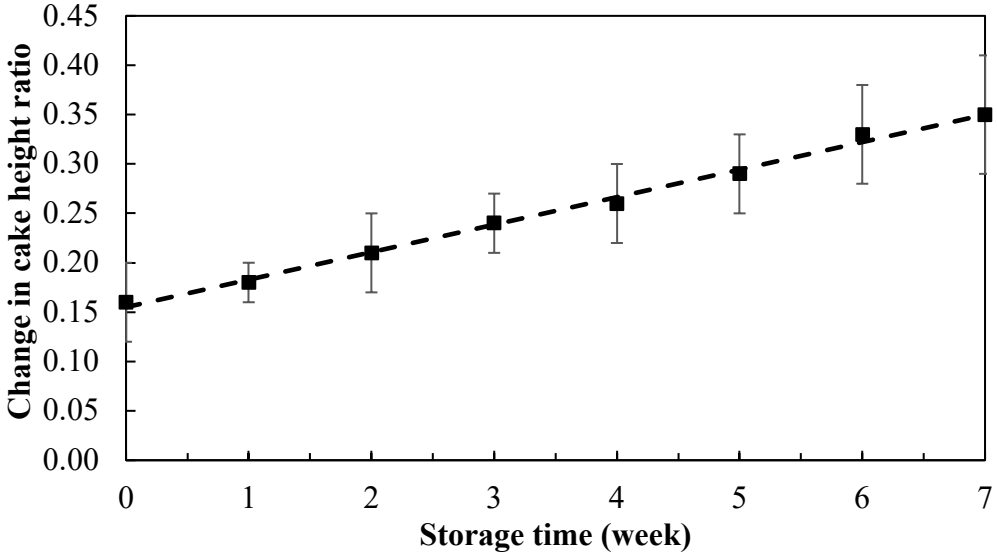


**Figure 3.** Effects of accelerated storage (38°C, 90% RH) on change in cake height ratio of 'cempedak' fruit powder packaged in aluminum-laminated polyethylene pouches.

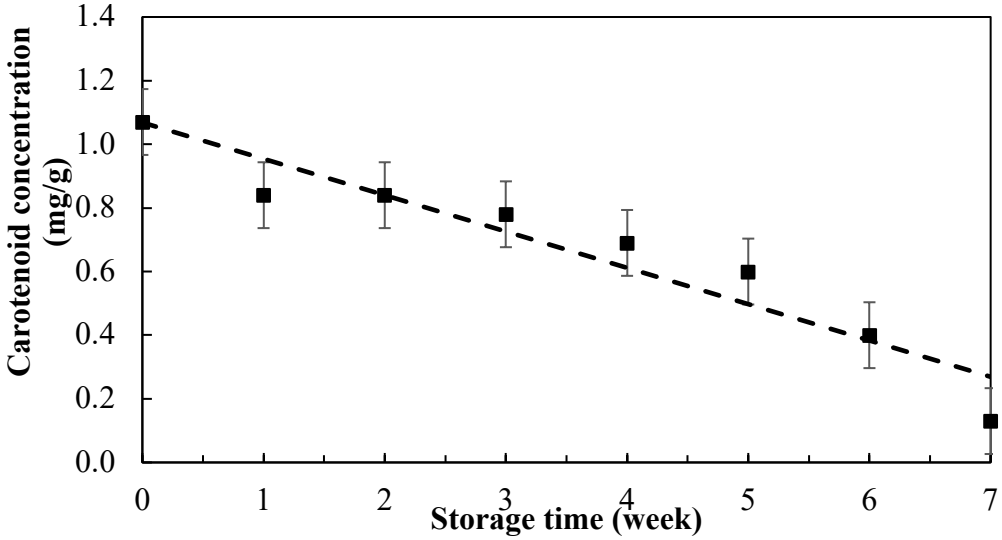


**Figure 4.** Effects of accelerated storage (38 °C, 90% RH) on carotenoid content (mg/g) of 'cempedak' fruit powder packed in aluminum-laminated polyethylene pouches

A higher degradation rate is caused by the presence of non-encapsulated material that has contact with oxygen or the presence of oxygen in the pores (Ferrari *et al.*, 2013; de Oliveira *et al.*, 2009).

### 3.7. Shelf-life prediction of stored 'cempedak' powder

Figure 5 shows the weight gain or uptake by silica gel that is packed (aluminum-laminated polyethylene) that is stored in an environment maintained at 90±1% relative humidity and 38±1 °C temperature. It should be noted that aluminum-laminated polyethylene used in this study has a water vapor transmission rate (WVTR) of $8.64\times10^{-6}$ kg.m$^{-2}$ day$^{-1}$ Pa$^{-1}$. The WVTR obtained for the aluminum-laminated polyethylene packaging for dried pomegranate arils and mango soy fortified yogurt powder has been reported to be $6.16 \times 10^{-8}$ kg m$^{-2}$ day$^{-1}$ Pa$^{-1}$ (Dak *et al.*, 2014; Kumar and Mishra, 2004), while those for jackfruit powder and bovine colostrum powder were 1.21 × 10-6 and $1.58 \times 10^{-8}$ kg m$^{-2}$ day$^{-1}$ Pa$^{-1}$, respectively. The WVTR obtained in this study is still considered as a higher WVTR (Yu *et al.*, 2015; Pua *et al.*, 2008). This may be caused by thinner packaging material (40 μm), as compared to 90 μm utilized in the work of Jaya and Das (2005).

In this study, the values of Guggenheim-Anderson-de Boer (GAB) parameters, Mo (monolayer value of powder), Cg (GAB model constant) and Kg (GAB model constant) for aluminum laminated polyethylene were 0.0862, 0.226 and 21.848 (Jena and Das, 2012). The surface area (Ap) of the aluminum-laminated polyethylene pouches used in this study was $13.5\times10^{-3}$ m$^2$, while the amount of the dry solids (Ws) was 0.020726 kg. From the calculation in Eq. 4, shelf-life (θs) of the spray-dried 'cempedak' fruit powder was determined to be 60.4 days base on the free-flowing properties as subjected to accelerated storage under 90% relative humidity (RH) at 38 °C.

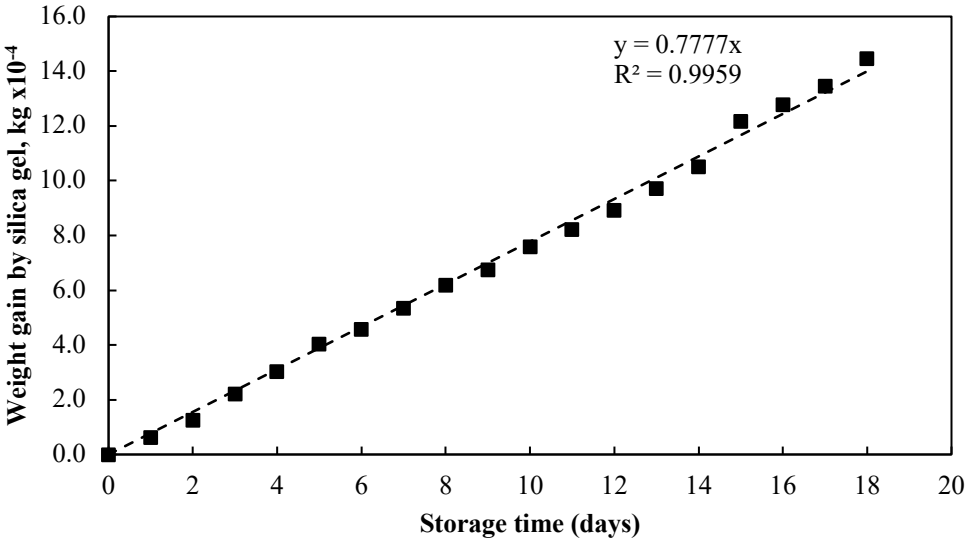


**Figure 5.** Permeability of aluminum-laminated polyethylene pouches

This result is in agreement with the shelf-life (based on free-flowness) of mango soy fortified yogurt and aloe vera gel powder, which has shelf lives of 54 and 51.05 days, respectively (Ramachandra and Rao, 2013; Kumar and Mishra, 2004). However, the shelf-life obtained in this study was shorter as compared to the 187 days shelf-life of pomegranate aril packed in aluminum-laminated polyethylene under accelerated storage (Dak *et al.*, 2014). This may be due to the different properties used in the determination of shelf-life, where the previous

study adapted color change as the factor for shelf-life determination (Dak *et al.*, 2014).

## 4. Conclusions

This study demonstrated the accelerated storage of 'cempedak' fruit powder packaged in aluminum-laminated polyethylene pouches and subjected to 90% RH at 38 °C for 49 days. As the storage time increase, 'cempedak' fruit powder increased in its moisture content, water activity, hygroscopicity, total color change and caking (change in cake height ratio), while decreased in its water solubility index and carotenoid content. In general, it can be concluded that the accelerated storage resulted in spray-dried 'cempedak' fruit powder that is more hygroscopic and susceptible to caking. The powder is darker, more reddish, but less yellowish after storage. Moisture content, total color change, caking and degradation of carotenoid of 'cempedak' fruit powder during storage followed zero-order reaction kinetics. In contrast, its hygroscopicity and water solubility index followed first-order reaction kinetics. Permeability in terms of water vapor transmission (WVTR) of aluminum-laminated polyethylene was found to be 8.64 x $10^{-6}$ kg.$m^{-2}$ $day^{-1}$ $Pa^{-1}$. Besides, during storage under accelerated conditions (38±1 °C, 90% RH), the predicted shelf-life of spray-dried 'cempedak' fruit powder was found to be 60.43 days in aluminum-laminated polyethylene pouches based on free-flowness with the Guggenheim-Anderson-de Boer (GAB) model. 'Cempedak' fruit powder produced can be used as an ingredient for other food products, where sensory evaluation can be proposed to determine the acceptability of spray-dryer feed (enzyme-treated 'cempedak' juice), 'cempedak' fruit powder and its final products (representative food systems).

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# EFFECT OF Ph AND HEATING TECHNIQUES ON EXTRACTION OF PECTIN FROM DIFFERENT SOURCES AVAILABLE IN PAKISTAN

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**ABSTRACT**

Pectin is complex heteropolysaccharide primarily present in the cell wall of terrestrial plants cell wall. Although it is obtained from citrus peels and apple pomace, but new sources have been investigating to fulfill the increased demand of pectin. The current study aimed to evaluate the effect of three parameters (pH level and heating methods) on the yield of pectin from peels of five different sources. Pectin was extracted from peels of sapodilla, banana, muskmelon, orange and apple at different pH levels (pH 1-pH 7) and with two heating methods include heating on Bunsen burner and microwave heating and after keeping the extracting mixture for 24hrs at room temperature before final precipitation of pectin. Although the results of the current study showed highest pectin yield from orange peels but among the three new sources (sapodilla, banana and muskmelon), banana peels pectin was found to show highest yield at pH 3. While the lowest yield was resulted from muskmelon peels among the five fruits peels. Pectin yield was found to be significantly influenced by pH level and heating method after 24hrs curing. Microwave heating showed significantly increased yield of pectin from all the investigated fruits peels. Thus concluded that these new sources of pectin can play promising role in order to fulfill the global requirement of pectin production.

## 1. Introduction

Pectin is complex heteropolysaccharide that is primarily present in the cell wall of dicotyledonous plant (Hamidon 2017). The higher concentration of pectin is present in middle lamella of cell wall of plants. It has wide range of applications in food, pharmaceutical (Sandarani 2017), cosmetic products (Marić et al., 2018), personal care and nutraceutical products (Ciriminna, 2016). In addition it has versatile gelling property because of source dependent verity of molecular size, degrees of methylation and acetylation and amount of galacturonic acid and neutral sugar moieties (Gawkowska, 2018). Demand of pectin is increasing due to wide range of applications. On commercial scale apple pomace and citrus peels are used for production of pectin (Hamidon, 2017).

Different pectin polysaccharides are present in plant cell wall which are covalently linked domains that may be distinguished as homogalacturonan(HG), hamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), xylogalacturonan (XGA), apiogalacturonan (AGA), arabinan, galactan, arabinogalactan I (AGI) and arabinogalactan II (AGII) whereas 65% of HG (homogalacturonan) is present

which is most abundant, 20-35% of RG-I (rhamnogalacturonan-I) and less than 10% covers RG-II (rhamnogalacturonan-II) and XGA (xylogalacturonan) is present (Harholt, 2010). Pectin can be divided into two types according to degree of methylation (Vanitha, 2019).The pectin with more than 50% of degree of methylation referred to as high methoxy pectin. It can form gels in acidic medium in pH from 2 to 3.5, in presence of sucrose with more than 55% by weight. Pectin referred to as low methoxy pectin when it has degree of methylation less than 50%. It also forms gels in higher range of pH from 2 to 6 in presence of divalent cations such as calcium. Pectin is also treated with ammonia which results amidated pectin. Pectin contains 65% of galacturonic acid units in food industries (Ciriminna, 2016). Commercially, pectin produced from citrus sources contributes 85% of pectin production where as 14% of pectin is obtained from apple pomace and little amount is obtained from sugar beet in addition commercially pectin extraction involved acid extraction, filtration and precipitation with ethanol (Gawkowska, 2018). Different types of pectin extraction techniques are well described by Sandarani (2017). Acid extraction of Pectin, Microwave assisted extraction of Pectin, Enzymatic extraction of Pectin. In Acid extraction of pectin the method involved utilization of chemical agents which include water, buffer, acid, base and calcium ion chelating agents. Among these, acid is found to be most effective extracting agent for pectin while commonly used acids are hydrochloric acid, acetic acid, citric acid and tartaric acid. By increase in strength of acid it causes increase in galacturonic acid content. The yield, physicochemical and functional properties of pectin also depend on the type and concentration of acid used for extraction of pectin. Nitric acid is also use in common practice for acidifying hot water for pectin extraction.

In microwave assisted extraction of pectin dielectric heating of plant molecules through exposure of microwave is carried out. Microwave energy absorption is taken place causing dipolar rotation of water which leads to heat generation inside the plant tissue. Studies showed improved pectin yield by microwave assisted pectin extraction, the inactivation of pectin esterase enzyme due to microwave radiation causes better pectin extraction. Further due top disintegration of parenchyma cells causing increase in specific surface area which improve the water absorption capacity of plant cell which leads to decease extraction time and energy. Yield of pectin is directly related to the power of microwave. The increase in microwave irradiation improves penetration of solvent into plant matrix and when molecular irradiation interacts with electromagnetic field results rapid transfer of energy to the solvent and matrix that facilitates dissolution of components for extraction. As water is a polar solvent, it effectively absorbs microwave energy and thus promotes effective heating the cells rupture increases due to sudden increase of temperature and pressure rise inside the plant cells which further potentiates the exudation of pectin within plant cells into the surrounding solvents. Enzymatic extraction of pectin is not only safe environmentally but also important for good pectin yield. In this type of extraction, cell wall degrading enzymes which have minimum pectinolytic activity are used to hydrolyse non pectic components present in plant cell wall where different enzymes are used in this pectin extraction such as polygalacturonase, hemicellulose, protease and microbial mixed enzymes, alpha amylase, cellulose, alpha amylase and neutrase, xylase, b-glucosidase, celluclast, alcalase, pectinesterase and endopolygalacturonase. These enzymes are responsible for degradation of pectin and modification of its physicochemical properties.

Objectives of Study Considering the eco-friendly environmental and economical value of pectin sources and it has wide range of applications

## 2. Materials and Methods

### 2.1. Sources of Samples

Sapodilla, Orange, Banana, Muskmelon and Apple.

### 2.2. Extraction of Pectin

Pectin extraction is carried out by following the method, with slight modification, adopted by Siddiqui (2018). The fruits were taken from local market which were identified by experts and their herbarium numbers were deposited in the department of pharmacognosy. The following steps were taken place to extract pectin from sapodilla, orange, banana, muskmelon and apple. Fruits were washed and peeled off with sharp knife. Peels were sliced of a few mm in thickness. 40 g of pieces of each fruit peels was taken in beaker and 200 ml of IMS was added to each. Now allow these to boil for 5 minutes in a water bath. IMS was carefully decanted and 120 ml DI water was added and these were blended for 30 seconds in a mechanical blender to form slurry. The slurry of each sample was then boiled for 10 minutes using Bunsen burner and microwave heating. The contents were then allowed to cool and pH from 1 to 7 was for each sample by either 0.1 NH4OH or 0.1N solution of HCl. The contents were kept overnight for 24hrs at room temperature. The solids of each mixture were removed by filtration with the help of muslin cloth. The pectin for each sample was then precipitated by adding ethanol in a ratio of 1:4.The precipitated pectin for each sample was separated by using Buchner funnel and weighed. Wet pectin was freeze dried by and then weighed. Percentage yield of pectin from each source was calculated by using following formula,%Yield = Weight of dried pectin/ weight of peels × 100.The dried pectin from all the three sources were then sieved with mesh number 60 and then desiccated.

### 2.3. Identification Tests for Pectin

A few tests like stiff gel test, test with ethanol, iodine and potassium hydroxide were performed to identify the extracted pectin according to the method followed by (Qadir, 2019)

### 2.4. Statistical Analysis

Statistical analysis was performed using SPSS 13 and Minitab 13.1. The mean comparison was done by using Tukey HSD (Steel , 1997) at a 5% level of significance.

## 3. Results and discussion

The non-edible parts of plants are the most focusing area for the researchers for the isolation and evaluation biological activities of bioactive molecules/compounds. Pectin is one of them which is also obtained from the waste of various fruits. It is reported that global market has increased upto 60,000 tonnes per year. The reported growth rate is 6% with price esteemed at $12.90/kg for LM pectin whereas for HM pectin, it is esteemed at $11.00/kg (Ciriminna et al., 2016).

Different extraction factors affecting the yield of pectin are playing effective roles for the optimization of extraction of pectin and can improve the yield of pectin from such economical source of pectin. Although apple pomace and citrus fruits peels are the commercial sources of pectin but researchers are now searching for new sources of pectin.

Current study designed to put great attention toward the economical source of pectin and optimization of pectin extraction to improve the yield of pectin. Considering the economical and eco-friendly environmental value of pectin source, the study involved the extraction of pectin from peels of five fruits. For factors affecting the yield of pectin, pH level heating techniques and curing time were used to optimize the extraction process for evaluating the positive effect on yield of pectin. The current study involved extraction of pectin from peels of five different fruits (sapodilla, banana, muskmelon, apple and orange) as shown in table 2. After extraction of pectin, few identification tests were performed for qualitative assessment which were stiff gel test, test with ethanol, iodine test and test with potassium hydroxide.

These tests for qualitative analysis exhibited positive results for pectin from each source (table 1). The gel forming property of pectin was confirmed for each pectin by stiff gel test. The

formation of translucent and gelatinous precipitate with 95% ethanol was resulted for each pectin which also confirmed the presence of pectin. Test of each pectin showed no blue coloration which differentiated pectin from starch. Each pectin was also differentiated from most gum by their test with potassium hydroxide due to their positive response. Yield of pectin was primarily supposed to be affected by its source ( Siddiqui, 2018). Aina (2012) also found influence of source on yield among three citrus fruits peels pectin (orange, lemon and grape fruit).Five sources are used in this study, although the results of the current study showed highest pectin yield from orange peels but among the three new sources (sapodilla, banana and muskmelon), banana peel pectin exhibited the highest yield while the lowest yield was resulted from muskmelon peels (table 2).

**Table1.** Results of tests of pectin identification

| Test | Sapodilla | Banana | Muskmelon | Apple | Orange |
|---|---|---|---|---|---|
| Stiff gel test | + | + | + | + | + |
| Test with ethanol | + | + | + | + | + |
| Iodine test | + | + | + | + | + |
| Test with potassium hydroxide | + | + | + | + | + |
| | | | | | |

**Table 2.** Yield of pectin from different fruits

| pH | Sapodilla | | Banana | | Muskmelon | | Apple | | Orange | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B | M | B | M | B | M | B | M | B | M |
| 1 | 1.45 | 2 | 5.5 | 5.8 | 0.75 | 0.8 | 1.55 | 4.5 | 3.45 | 4.25 |
| 3 | 3.5 | 2.5 | 8.5 | 10 | 0.75 | 0.4 | 4.25 | 4.45 | 8.45 | 9.35 |
| 5 | 2.5 | 3 | 4.5 | 7.5 | 0.4 | 0.9 | 2.05 | 3.55 | 6.35 | 7.5 |
| 6 | 1.5 | 2 | 4 | 3.5 | 0.9 | 2.1 | 1.35 | 2.2 | 5.7 | 7.25 |
| 7 | 0.5 | 0.5 | 6 | 7 | 0.35 | 0.7 | 4.65 | 4.9 | 5.05 | 6.05 |

The current results also favor the results of previous investigation on pectin yield from same fruits peels which also showed the highest pectin yield from banana peels and lowest from muskmelon peels (Siddiqui , 2018). As the experiment involved two heating methods for each pectin source, it was noted that the resulted high yield of banana obtained when the heating mode was microwave. Similarly the lowest yield of muskmelon was resulted when heating was carried out by the use of Bunsen burner. The experimental results favor the use of microwave heating for improved quantity of pectin as compared to the heating technique of utilizing Bunsen burner.

Previously sapodilla was assisted for total dietary fibers (Mahattanatawee et al., 2006) but recently pectin extraction from sapodilla was also investigated from its peels ( Siddiqui , 2018). The yield of pectin from sapodilla peels by using heating technique of Bunsen burner was in the range of 0.5% to 3.5 % while the yield of pectin from the same source by the use of microwave heating was found to be in the range of 0. 5% to 3% keeping all the parameters same for both heating techniques (Fig.1). These ranges are almost comparable to each other.

In case of banana pectin yield after the use of Bunsen burner was found to be from 4% to 8.5% but yield range was widen by the use of microwave heating as 3.5% to 10% (Fig.2) with same experimental conditions. Similarly microwave heating resulted broader range of pectin yield from muskmelon peels with same

experimental conditions, ranged from 0.4% to 2.1%, as compared to the pectin yield obtained by the use of heating on Bunsen burner which was ranged from 0.35% to 0.9% (Fig.3). Although muskmelon was previously investigated for total dietary fibers (Mahattanatawee, 2006) but recently it was also investigated for pectin extraction from its peels (Siddiqui , 2018). Earlier recorded yield of pectin from muskmelon peels (2.1 to 3.8) was relatively higher extracted at pH, temperature and duration ranges from 1 to 1.5, 70 to 90°C 30 to 60 minutes. So variation could be due the differences in the experimental conditions (Muthukumaran, 2017). The lowest yield could also be due to the variation in the cultivar and maturity state which affect the polysaccharide composition in the cell wall (Simandjuntak, 1996), so ultimate effect produces on the pectin yield (Bhardwaj et al., 2012). The experimental results favor the use of microwave heating for improved quantity of pectin as compared to the heating technique of utilizing Bunsen burner.

The two other sources, apple and orange, which are commonly used for commercial pectin (Ciriminna,2016; Srivastava 2011) were used investigated for evaluation of pectin yield from their peels with same experimental conditions. Apple peels contain 16.95% of pectin (Virk , 2004). In the present study pectin yield from apple peels was found to be from 2.2% to 4.9% which was higher with the use of microwave heating technique than the pectin yield obtained with Bunsen burner heating technique (1.35% to 4.65%) (Fig.4). Pectin from orange peels also exhibited the higher yield with microwave heating technique (4.25% to 9.35%) as compared to the pectin yield resulted by the use of Bunsen burner heating technique (3.45% to 8.45%) (Fig.5).

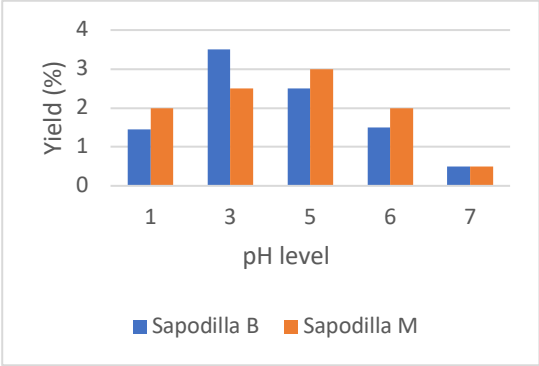


**Figure 1**.Pectin yield extracted from Sapodilla.

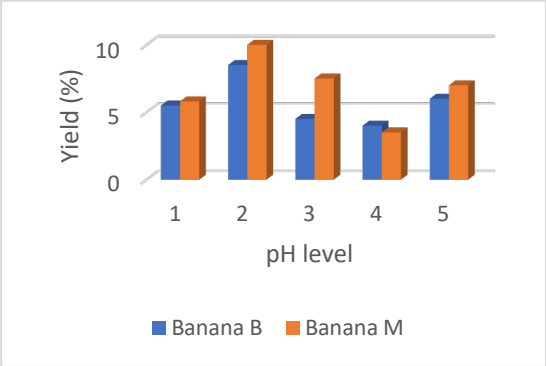


**Figure 2**. Pectin yield extracted from banana

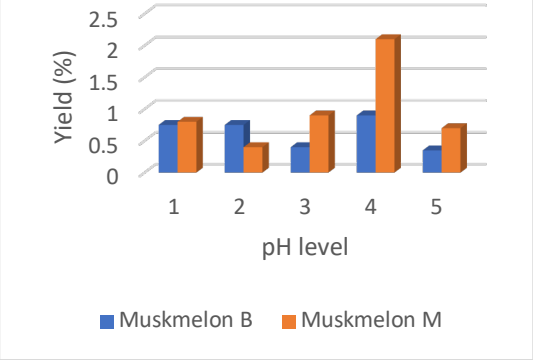


**Figure 3**.Pectin yield extracted from Muskmelon.

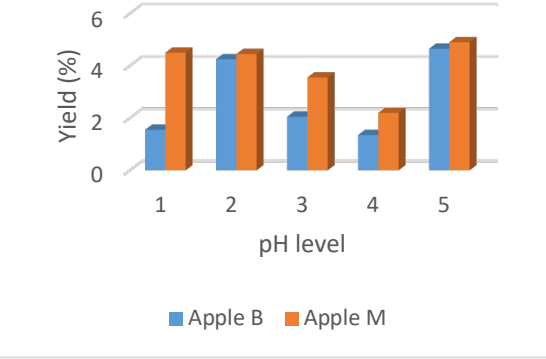


**Figure 4**. Pectin yield extracted from Apple

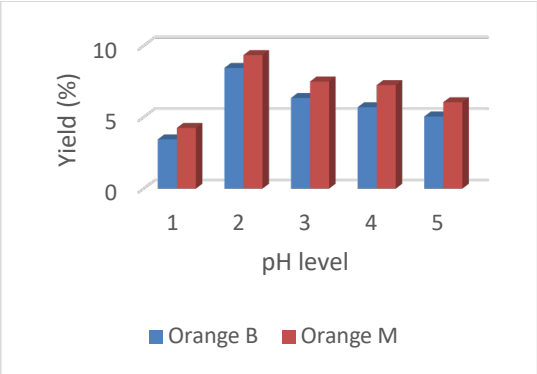


**Figure 5.** Pectin yield extracted from Orange

**Table 3.**Analysis of variance (mean squares) of yield for different fruits

| Source of Variation | Degrees of Freedom | Mean Squares | | | | |
|---|---|---|---|---|---|---|
| | | **Sapodilla** | **Banana** | **Muskmelon** | **Apple** | **Orange** |
| pH level | 4 | 5.9105** | 23.599** | 0.95887** | 8.8789** | 20.607** |
| Heating Method | 1 | 0.0919** | 8.427** | 0.91875** | 9.9188** | 8.748** |
| pH level x Heating method | 2 | 0.6530** | 2.615** | 0.49687** | 1.9369** | $0.129^{NS}$ |
| Error | 20 | 0.0059 | 0.054 | 0.00111 | 0.0154 | 0.046 |
| Total | 29 | | | | | |

S = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)

**Table 4.**Heating method × pH interaction mean±SE for Sapodilla.

| **Heating Method** | **pH Level** | | | | | **Mean** |
|---|---|---|---|---|---|---|
| | **1** | **3** | **5** | **6** | **7** | |
| **B** | 1.45±0.02e | 3.50±0.05a | 2.50±0.05c | 1.50±02e | 0.50±0.01f | 1.89±27B |
| **M** | 2.00±0.05d | 2.50±0.05c | 3.00±0.08b | 2.00±0.05d | 0.50±0.02f | 2.00±22A |
| **Mean** | 1.73±0.13C | 3.00±0.23A | 2.75±0.12B | 1.75±0.11C | 0.50±0.01D | |

B: Heating in Bunsen burner, M: Heating in microwave
Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

The effect of pH was also evaluated with heating methods by extracting pectin from five sources with pH range from 1 to 7. For sapodilla highest yield was 3.5% obtained at pH 3 with heating on Bunsen burner and with microwave heating the highest yield was 3% at pH 5 which was closed to the yield obtained at pH 3 by heating on Bunsen burner. Sapodilla showed lowest pectin yield at pH 7 (Fig.1). The highest yield of pectin obtained from banana at pH 3 were 10% and 8.5% with microwave heating and heating on Bunsen burner while the lowest yield was found to be 0.5% at pH 7 by heating on Bunsen burner and 4% at pH 6 by microwave heating (Fig.2). Muskmelon peels exhibited highest pectin yield 0.9% and 2.1% at pH 6 but the lowest recorded yield was 0.35% at pH 7 and 0.4% at pH 3 by heating on Bunsen burner and

microwave heating respectively (Fig. 3). Highest pectin yield from apple peels were 4.65% and 4.9% at pH 7 while the lowest pectin yield was 1.35% and 2.2% at pH 6 by heating on Bunsen burner and microwave heating respectively (Fig. 4). The highest pectin yield from orange peels was 8.45% and 9.35% at pH 3 and the lowest yield obtained at pH 1 which were found to be 3.45% and 4.25% by heating on Bunsen burner and heating on microwave heating respectively (Fig.5). These results of current study showed that heating methods and pH are the factors influencing the yield of pectin from different sources of pectin investigated.

Statistical analysis was applied for better evaluation of effects of heating method and pH on yield of pectin. The analyzed results indicated that heating method and pH level have significant impacts on the pectin yield obtained from five fruits at 1 % level of significance. Analysis of variance (ANOVA) was applied which showed positive effects of interaction of heating method and pH level on the yield of pectin extracted from each fruit peels (Table 3).

The interaction effect of heating method and pH level for sapodilla peels pectin using applying Tukey's test is represented in table 4. The overall means of factors investigating in the current study showed that pH 3 and microwave heating have significant impact ($P<0.05$) on pectin yield from sapodilla peels. The alphabetical order was used to indicate the effectiveness on pectin yield. The letter A was used for the highest effect on pectin yield and subsequent letters were used to show lesser effect. The lowest yield was resulted with pH 7 and heating with Bunsen burner according to the overall means. Previous study on the pectin from same source also showed highest yield at pH 3 and lowest at yield at pH 7 (Siddiqui, 2018). So the results showed the inverse relationship of pectin yield from acidic to basic medium which is in accordance with the previous studies (Tiwari, 2017; Zaid , 2016). The current investigation also showed significant effect on pectin yield from sapodilla peels by microwave heating method which also authenticate the previous studies on pectin of same source (Siddiqui., 2018) and from other source (Mosayebi, 2015; Wang., 2007).

For banana peels pectin, the effect of interaction of heating method and pH level using Tukey's test is shown in table 5. Like sapodilla alphabetical order is used for effectiveness on pectin yield. Letter A is used for highest effect on pectin yield while the subsequent letters refer the subsequent lesser effect on pectin yield. Results showed that pH 3 and microwave heating have significant effect ($P<0.05$) on pectin yield while the lowest yield extracted from banana peels was obtained by heating on Bunsen burner at pH 6. The previous study also showed highest pectin yield from banana peels at pH 3 (Swamy,2017). The current and the previous studies favored acidic medium for higher pectin yield from banana peels. The significant effect on pectin yield from banana peels was also observed previously by microwave heating technique (Siddiqui , 2018) while the power of microwave was found to have direct relationship with the pectin yield from banana peels (Swamy, 2017).

**Table 5.**Heating method × pH interaction mean±SE for Banana.

| Heating Method | pH Level | | | | | Mean |
|---|---|---|---|---|---|---|
| | 1 | 3 | 5 | 6 | 7 | |
| **B** | 5.50±0.5d | 8.50±0.19b | 4.50±09e | 4.00±0.03ef | 6.00±0.09d | 5.70±0.42B |
| **M** | 5.80±0.1d | 10.00±26a | 7.50±0.13c | 3.50±0.09f | 7.00±0.13c | 6.76±0.57A |
| **Mean** | 5.65±0.08C | 9.25±36A | 6.00±.67CD | 3.75±0.12 | 6.50±0.24B | |

B: Heating in Bunsen burner, M: Heating in microwave

Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

**Table 6.**Heating method × pH interaction mean±SE for Muskmelon.

| Heating Method | pH Level | | | | | Mean |
|---|---|---|---|---|---|---|
| | 1 | 3 | 5 | 6 | 7 | |
| B | 0.75±0.01cd | 0.75±0.01cd | 0.40±0.01e | 0.90±0.03b | 0.35±0.01e | 0.63±0.06B |
| M | 0.80±0.01c | 0.40±0.01e | 0.90±0.02b | 2.10±0.05a | 0.70±0.01d | 0.98±0.16A |
| Mean | 0.78±0.01B | 0.58±0.08D | 0.65±0.11C | 1.50±0.27A | 0.53±0.08D | |

B: Heating in Bunsen burner, M: Heating in microwave
Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

The investigated effect of variables on muskmelon peels pectin showed significant effect (P<0.05) on pectin yield by microwave heating at pH 6 and lowest impact was observed by heating on Bunsen at pH 7 (0.53±0.08) and pH 3 (0.58±0.08). The same alphabetical order representing the order of effectiveness on pectin yield from muskmelon peels. Where highest letter A indicated the highest yield while the lowest yield was indicated by the lowest used letter D (table 6). In contrast, significant effect at pH 5 and while the lowest effect at the same pH 7 was found in the previous study buts significant effect with microwave heating was observed (Siddiqui., 2018) as observed in the current study.

In the present study the interaction effect of heating method and pH on apple peels pectin is represented in table 7. Where the alphabetical order represented the impact of heating method and pH level on pectin yield. In case of apple peels pectin, the highest impact (P<0.05) on pectin yield was also observed by microwave heating method as indicated by letter A. Unlike pectin from sapodilla, banana and muskmelon, the apple pectin showed significant effect (P<0.05) on yield was exhibited at pH 7. The second highest yield obtained at pH 3 indicated by letter A which is closed to the pectin yield at pH 7. As discussed in the previous study that both alkaline and acidic media with accelerated temperature can promote release and hydrolysis of protopectin (Siddiqui., 2018) but the previous study favor acidic medium for higher pectin yield (Ziari,, 2010). The lowest yield was found to be at pH 6 as observed in case of banana peels pectin. Previously reported positive effect on pectin yield from apple pomace (Sandarani, 2017) and apple peels (Siddiqui , 2018) by the use of microwave assisted extraction as compared to conventional extraction method is also confirmed by the present study.

**Table 7.**Heating method × pH interaction mean±SE for Orange.

| Heating Method | pH Level | | | | | Mean |
|---|---|---|---|---|---|---|
| | 1 | 3 | 5 | 6 | 7 | |
| B | 3.45±0.06h | 8.45±0.13b | 6.35±0.15d | 5.70±0.10e | 5.05±0.14f | 5.80±0.44b |
| M | 4.25±0.06g | 9.35±0.10a | 7.50±0.08c | 7.25±0.20c | 6.05±0.13de | 6.88±0.45A |
| Mean | 3.85±0.18E | 8.90±0.21A | 6.93±0.27B | 6.48±0.36C | 5.55±0.24D | |

B: Heating in Bunsen burner, M: Heating in microwave
Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

**Table 8.**Heating method × pH interaction mean±SE for Apple.

| Heating Method | pH Level | | | | | Mean |
|---|---|---|---|---|---|---|
| | 1 | 3 | 5 | 6 | 7 | |
| **B** | 1.55±0.02f | 4.25±0.02c | 2.05±0.03e | 1.35±0.01f | 4.65±0.16ab | 2.77±0.37B |
| **M** | 4.50±0.03bc | 4.45±0.10bc | 3.55±0.08d | 2.20±0.05e | 4.90±0.06a | 3.92±0.26A |
| **Mean** | 3.85±0.66C | 4.35±0.06B | ±0.34D | 1.78±0.19E | 4.78±0.09A | |

B: Heating in Bunsen burner, M: Heating in microwave
Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

The investigated overall interaction effects of boiling method and pH level on pectin yield from orange peels pectin showed significant effect (P<0.05) on pectin yield by microwave heating as observed in previous described fruits peels pectin (sapodilla, banana, muskmelon and apple) at pH 3 as observed in case of banana and sapodilla peels pectin. The lowest yield was shown by heating on Bunsen burner techniques as observed by previous discussed fruits pectin (sapodilla, banana, muskmelon and apple) at pH 1 (table 8). Recorded highest pectin yield from orange peels was also resulted in acidic pH (2 to 2.5) with temperature and duration of 70°C and 30 minutes respectively ( Khan,2015). Previous investigation on pectin from orange peels also found better yield by microwave heating as compared to the conventional method (Alwan, 2016).

The overall interaction effect of pH level and heating techniques showed significant impact on pectin yield of each extracted pectin. Microwave assisted extraction which is preferable heating method because of large handling capacity, less duration of processing with good purity (Sandarani, 2017) showed significant effect on pectin yield from five sources in the current investigation as observed in the earlier studies of pectin (Mosayebi, 2015; Siddiqui., 2018; Wang ., 2007). The mechanism demonstrated previously in case of orange pectin extraction, is that when orange peels were subjected to microwave irradiation, there is inactivation of enzyme pectin esterase responsible for interaction with pectin substance in the orange peels and reduction of their solubility and destruction of orange skin cells which ultimately improve the pectin extraction Further more disintegration of parenchyma cells increases surface area to improve the water absorption capacity of the plant cells which contributes to minimize extraction time and energy (Sandarani, 2017).

The present study showed highest pectin yield in acidic medium from all the sources except apple pectin where highest yield was found at pH 7 but followed by pH 3 and yield at these pH levels were found to be closed to each other. So it may be due to any experimental effect. But overall effect of pH on pectin yield was found to show highest yield in acidic medium as observed in the earlier works ( Siddiqui , 2018; Tiwari., 2017; Zaid , 2016). The scientist also justified this relation of low acidic pH with high yield by stating that at low pH high concentration of hydrogen ions causing conversion of hydrated carboxylate groups to less hydrated carboxylate groups thus the loss of carboxylic groups associated with reduction of repulsion of polysaccharide molecules which accelerating the gelatinous ability of pectin that promotes precipitation of pectin. Different acids are used in pectin extraction such as nitric, oxalic, phosphoric, acetic, citric, lactic, malic, tartaric (organic), hydrochloric, phosphoric and sulfuric acids. But in the current study hydrochloric acid was preferred because it was found to show highest yield as compared to citric and nitric acids in case of guava, papaya, citrus fruits, banana, and cocoa pods in acidic medium and temperature ranged from 1 to 3 and 60°C-85°C (Sandarani, 2017). Another reason of using low strength (0.1N) HCl in the present

study was to maintain environment friendly ( Siddiqui , 2018)

## 4. Conclusions

It is concluded from the current investigation that pectin market can be improved by utilizing the new sources for pectin production on commercial scale.

## 5. References


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