CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

Vol. 16(2)
2024

Technical University of Cluj Napoca
U.T.Press Publishing House

Carpathian Journal of Food Science and Technology

Print : ISSN 2066-6845
Online : ISSN 2344-5459
ISSN-L 2066-6845

Vol. 16, Nr.(2) 2024

UNIVERSITATEA TEHNICĂ DIN CLUJ-NAPOCA

Technical University of Cluj Napoca
U.T.Press Publishing House

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY
ISSN-L 2066 -6845

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

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IMPACT OF TRADITIONAL COOKING METHODS ON THE ANTIOXIDANT ACTIVITY OF ALGERIAN CARROTS CULTIVARS (*Daucus carota* L.)

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https://doi.org/10.34302/crpjfst/2024.16.2.1

Article history:
Received: July 15th, 2023
Accepted: April 15th, 2024

Keywords:
Carrot;
Cooking;
Cooking water;
Antioxidant;
Antioxidant activity;

ABSTRACT

Fruits and vegetables are rich in antioxidants, and carrots, in particular, are a source of phenolics, vitamin A, and carotenoids. Carrots are regularly consumed raw, cooked, or in juice form for their particular taste, sweet flavor, and their high carotenoid content. The objective of the present study was to assess the effect of two common domestic cooking methods (steaming and boiling) on the content of the antioxidant compounds and the antioxidant power of two varieties of orange carrots cultivated in Algeria. The results showed that both steaming and boiling led to an increase in the total carotenoid content and reducing power. Additionally, the amount of phenolics, flavonoids, and antiradical activity increased in the steamed samples. However, a slight decrease in phenolic content was noted in the studied cultivar after boiling. Analysis of cooking water revealed that thermal treatment promoted the release of some antioxidant compounds into the water cooking, thus contributing to their antioxidant activity.

1.Introduction

Vegetables are an important part of a healthy diet and the principal source of natural antioxidants such as vitamin C, α-tocopherol, and phenolic compounds (Ames *et al.,*1993). These antioxidants derived from fruits and vegetables have been linked to the protection against many diseases (Harasym & Oledzki, 2014; del Río-Celestino & Font, 2020; Van der Merwe, 2021).

Carrot (*Daucus carota* L.) is a vegetable belonging to the *Apiaceae* family. Apart from the common orange-colored varieties, various other pigmented varieties of carrot such as white, yellow, red and black (purple) are available. Although, the orange and red carrots are popularly consumed everywhere and also possess various dietary benefits (Elham & Shahriar, 2013). Carrots are rich in a diversity of phytochemicals including carotenoids with provitamin A activity, phenolic compounds, ascorbic acid, α-tocopherol, vitamins D, K, B_1, and B_6, and polyacetylenes, many of which have antioxidant and other health promoting effects (Southon & Faulks, 2003). Crop improvement programs to breed antioxidant rich cultivars such as mixed color carrots have improved the diversity of available carrots, which could potentially increase the intake of health

promoting compounds through diet and help in the prevention of chronic human diseases (Char, 2018). In order to improve the sensory characteristics, most vegetables need to be cooked before consumption. Consequently, there is a growing interest in the effect of cooking on the nutritional and sensory quality of vegetables.

During the processing of vegetables, the most commonly used technique is cooking. While boiling is the most popular cooking method, various alternatives such as steaming, baking, roasting and microwaving are also used, depending on the vegetable and consumer preferences. It is well known that the cooking process induces changes in the chemical composition of vegetables, influencing the concentration and bioavailability of bioactive compounds such as total phenolics and other antioxidants. Changes in bioactive compound content during cooking may be the effect of two contrary phenomena: thermal treatment causes the denaturation of enzymes that are involved in the degradation of nutrients and bioactive compounds as well as cooking resulting in a softening effect, which increases the extractability of bioactive compounds, resulting in a higher level in cooked products as compared to the raw material (Palermo *et al.,* 2014). It is therefore reasonable to gain more knowledge about the final concentration of bioactive compounds after food processing to assess the availability of them within a diet.

The effects of cooking on several vegetables have been studied by various researchers, using different cooking techniques (Xu & Chang, 2009; Patras *et al.,* 2011; Leong & Oey, 2012; Arkoub-Djermoune *et al.,* 2016; Koç *et al.,* 2017; Arkoub-Djermoune *et al.,* 2019; Buratti e*t al.,* 2020; Wang *et al.,* 2021; Sharma *et al.,* 2022; Kosewski *et al.,* 2023). However, it is difficult to come to unique conclusions about the advantages/disadvantages of a particular cooking method when the nutritional quality of vegetables is concerned. Therefore, the purpose of this study was to evaluate the impact of two common domestic cooking methods (boiling and steaming) on the antioxidant content and the antioxidant activity of two Algerian orange carrot (*Supermuscade* and *Touchon*) cultivars.

2. Materials and methods

2.1. Chemicals

The aluminium chloride, potassium ferricyanide and Folin-Ciocalteu reagent was from Biochem, Chemopharma (Montreal, Quebec); gallic acid and β-carotene were from Prolabo (Montreuil, France); sodium carbonate was from Sigma-Aldrich (Switzerland) and 1,1-diphenyl-2- picrylhydrazyl (DPPH) was from Sigma-Aldrich (Germany).

2.2. Carrot samples

Two fresh orange carrot cultivars (*Supermuscade* and *Touchon*) were purchased from the local market of Bejaia city-Algeria.

2.3. Samples treatment

The peels were separated from carrots with a manual peeler and washed by distilled water. After that, the peeled carrots were cut into small slices following with cooking in two ways which were boiling and steaming at 100°C for 30 min. The tested parameters were determined before and after thermal processing.

The remaining cooking waters of carrot were collected after heat treatment and concentrated using a BÜCHI rotavapor (R-200, Germany) at 35°C up to a volume of 10 mL, then stored at -10°C for analysis.

2.4. Preparation of extracts

The fresh and cooked carrots (10 g) were grinded and extracted with 50 mL distilled water. The homogenate was then centrifuged at 4500 g for 15 min at 5°C (Sigma 2-16 K; Germany) after 30 min agitation in order to recover the supernatant. The residue was additionally extracted with 50 mL distilled water. Afterward, the collected supernatants were mixed and concentrated under vacuum using a BÜCHI rotavapor (R-200, Germany) at 35°C until reaching a volume of 10 mL, and then stored at -10°C until analysis.

2.5. Analysis of samples

In order to determine the effect of cooking, the fresh carrots, cooked carrots and water

cooking were analyzed regarding to their antioxidant contents and antioxidant capacities as follows:

2.5.1. Total phenolic content determination (TPC)

The TPC of raw, cooked carrots extract was evaluated by the method described by Naithani *et al.* (2006). Briefly, 100 µL of the diluted extract (1:1, v:v) was added to 2.2 mL of sodium carbonate (2%) then mixed. After that, 100 µL of Folin-Ciocalteu reagent (50%) was added after 3 min. Finally, the absorbance of the mixture was determined spectrophotometrically at 750 nm by using a spectrophotometer (UV-mini 1240 Shimadzu, China). The results were expressed as milligram Gallic Acid Equivalent per one hundred grams of the fresh weight (mg GAE/100g FW).

2.5.2. Total flavonoid content determination (TFC)

The TFC of both raw and cooked carrot extract was estimated according to the method of Djeridane *et al.* (2006). Specifically, 1.5 mL was mixed with 1.5 mL of 2 % (w/v) aluminium chloride. The absorbance was then measured at 410 nm after 10 min. The TFC was reported as milligram Quercetin Equivalent per one hundred grams of the fresh weight (mg QE/100g FW).

2.5.3. Total carotenoid content determination (TCC)

Carotenoids were collected from the raw and cooked samples according to the method described by Sass-Kiss *et al.* (2005). A mixture of hexane-acetone-ethanol (2:1:1, v: v: v) (20 mL) were homogenized with fresh and cooked carrots samples (0.5 g). The supernatant was collected after 30 min agitation and the residue was additionally extracted with hexane (10 mL). The carotenoid contents were estimated by measuring the absorbance of the combined hexane layers at 450 nm. The TCC in carrot samples were expressed as milligram β-Carotene Equivalent per one hundred grams of the fresh weight (mg βCE/100g FW).

2.5.4. Antioxidant capacities

2.5.4.1. Free radical scavenging activity against DPPH (DPPH-FRSA)

The antioxidant activity of carrot extracts against DPPH (1,1- Diphenyl- 2 Picryl-Hydrazyl) free radical was estimated by the method of Peschel *et al.* (2006). An aliquot of the extract (500 µL) was mixed with methanolic solution of DPPH radical (2 mL). The absorbance of the mixture was measured at 517 nm after 90 min. The inhibition percentage of DPPH was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(A_c - A_e)/ A_c]. 100 \quad (1)$$

Where A_c was the absorbance of the control and A_e was the absorbance in the presence of the sample extracts.

2.5.4.2. Ferric reducing antioxidant power (FRAP)

The reducing power of carrot extracts was evaluated according to the method of Bhandari & Kawabata (2004). In a test tube, raw and cooked carrot extracts (1 mL) was mixed with phosphate buffer (0.2 M, pH 6.6) (0.5 mL), potassium ferricyanide solution (1% w/v) (2.5 mL) and the mixture was incubated at 50°C for 20 min. After cooling, 0.5 mL of trichloracetic acid (10%) was added then the mixture was centrifugated at 3000g during 10 min (Sigma 2-16 K; Germany). Briefly, 1 mL of supernatant was mixed with distilled water (1 mL) and ferric chloride (0.1% w/v) (100 µL), allowing the reaction to proceed 10 min. Finally, the absorbance was measured at 700 nm. The FRAP of carrots extracts were expressed as milligram Trolox Equivalent per one hundred grams of the fresh weight (mg TE/100 g FW).

2.6. Statistical analysis

The antioxidant content and the antioxidant activity tests were performed in triplicate and the results were expressed as means ± standard deviation. ANOVA using the least significant difference (LSD) test at $p<0.05$ was performed

to statistically analyze the data obtained using STATISTICA 5.5. The correlation matrix was performed at three different significant levels (*0.05, 0.01* and *0.001*) using STATISTICA 5.5 software.

3. Results and discussions

3.1. Total Phenol Content (TPC)

The content of polyphenolic compounds in plant raw materials is affected by a number of factors, such as climatic conditions and agro-technical practices, the stage of maturity, the time of harvest, storage conditions, genetic factors, varietal diversity, and the extent of damage to the vegetable tissue (Ninfali & Bacchiocca, 2003). The low-acid conditions in carrot (pH 6.0-6.5) and carrot products allow the rapid increase of microbial infection and the pH conditions are advantageous (Patterson et al., 2012). Therefore, unprocessed carrot products have a short shelf life and should normally be consumed within 1-2 days, limiting its market potential and perhaps also leading to microbiological safety problems (Alklint *et al.,* 2004). Thermal processing is one of the main technologies used to destroy food-borne pathogens and ensure the safety of vegetable and fruit-based products. The content of phenolic compounds in vegetables is influenced by storage time, temperature and the type of culinary and technological processing (Kapusta-Duch *et al.*, 2017).

The TPC recorded in raw *Supermuscade* and *Touchon* carrots varieties were 12.74 ± 0.65 and 31.81 ± 0.44 mg GAE/100 g fresh weights, respectively (Fig. 1). The results obtained regarding the effect of thermal processes on TPC concentrations indicated that steaming increases significantly ($p<0.05$) the TPC of carrot varieties from 5.88 % to 53.57% (Fig.1). Arscott & Tanumihardjo (2010) have reported that the main phenolic compounds found in carrots are chlorogenic acids, which are hydroxycinnamic acid derivatives formed by the esterification of cinnamic acids, such as caffeic, ferulic, and *p*-coumaric acids, with (-)-quinic acid. The predominant phenolic acids in carrots are 5′-caffeoylquinic acid, 3′-caffeoylquinic acid, 4′-p-coumaroylquinic acid, 3′,4′-dicaffeoylquinic acid, 3′,5′-dicaffeoyl-quinic acid and others.

Chlorogenic acid, a major hydroxycinnamic acid, present in every color of carrot cultivars, it represents 42% to 62% of the total phenolic compounds detected in different carrots tissues.

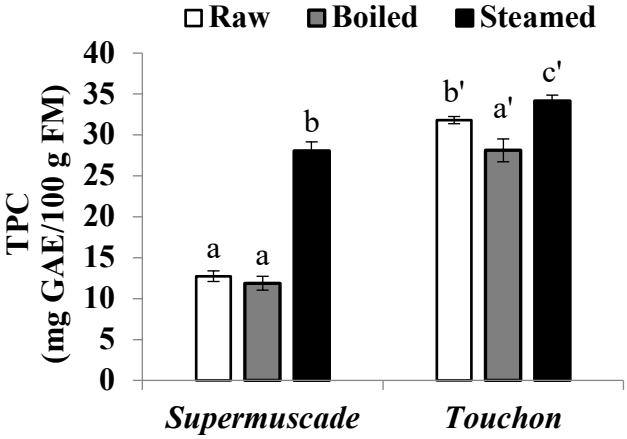


Figure 1. Total phenolic content of raw and cooked carrot.

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; b>a and c'>b'>a'.

The order of presence is as follows: peel > phloem > xylem (Zhang & Hamauzu, 2004).

The results obtained from the current study were similar to data published by Mazzeo *et al*. (2011) who reported that the steaming process significantly increased the level of these compounds. Several other studies have shown that cooking increases the TPC in vegetables, Sultana *et al.* (2008) in carrots, Arkoub-Djermoune *et al.* (2016) in eggplant, Arkoub-Djermoune *et al.* (2019) in tomato, Buratti *et al.* (2020) in cauliflowers, carrots and orange-fleshed sweet potatoes, Kosewski *et al.* (2023) in some selected vegetables. An increase in total polyphenol content might be due to the release of phenolics from intracellular proteins, changes in plant cell structure, matrix modifications, or the inactivation of polyphenol oxidase (Kao *et al.,* 2014). An increase in polyphenol content is also associated with the reaction of plants to

mechanical tissue damage, infection or other stress factors (Sikora et al., 2008). As indicated by Provesi *et al.* (2011), this increase was the result of the simultaneous action of several mechanisms. This include the facility which polyphenols are extracted in cooked samples, after the strong weakening of cell walls by heat. There is an increase in the availability of phenols physically and chemically linked to the microstructure of the processed vegetables in comparison to the raw. This may be attributed to the decomposition of phenolic compounds linked to the fiber (cellulose and pectin) (Martínez-Hernández *et al.,* 2013). The breaking of phenol sugar glycosidic links, giving raise to aglycons, also contributes to the increase in phenol concentration. This last mechanism perhaps the main one concerned in the increase of phytonutrient concentrations, which has been suggested to explain the variations during the culinary preparation (del Pilar Ramírez-Anaya *et al.,* 2015). Moreover, Arkoub-Djermoune *et al.* (2019) reported that the increases of TPC in cooked samples can be explained by the enhanced extractability and, therefore, increased the bioavailability of phenolic compounds. This would be the consequence of softening and breaking of cell walls, leading to a higher concentration of these compounds.

On the other hand, the result obtained in the present study showed either that boiling decreased the TPC in *Touchon* variety with a rate of 12.5 % but without a significant ($p<0.05$) effect on *Supermuscade* variety. These results are in agreement with those found by Mazzeo *et al.* (2011) and Oghbaei & Prakash (2021) who have shown that traditional cooking decreased the content of phenolic compounds in tomato, carrot and brown chickpea, respectively. A decrease in these compounds is usually caused by the leaching component, complexation with other compounds or oxidation (Grajek, 2007). This divergence recorded in cooking effect on the phenolic content may be explained by the differences in the cooking methods and conditions such as time and temperature.

3.2. Total Flavonoid Contents (TFC)

Flavonoids are the most phenolic compounds studied in foods with a large number of different molecules and several biological activities. The predominant flavonoids identified in orange carrots are quercetin, luteolin, kaempferol, and myricetin (Bahorun *et al.*, 2004).

The mean values of TFC of fresh and cooked samples are presented in Figure 2. The results showed that boiling has no significant effect ($p<0.05$) on TFC but steaming increase them with respective rates of 57.10 % and 6.15 % in *Supermuscade* and *Touchon* varieties. These results are in line with those reported in our pervious study on the effect of cooking (frying, baking and grilling) on the flavonoid content of tomato (Arkoub-Djermoune *et al.,* 2019). This increase in the TFC was related to the loss of tissue integrity, the cells and organelles membranes after heat treatment which facilitates their release or leaching in the cooking water (Olivera *et al.,* 2008; Arkoub-Djermoune *et al.,* 2019).

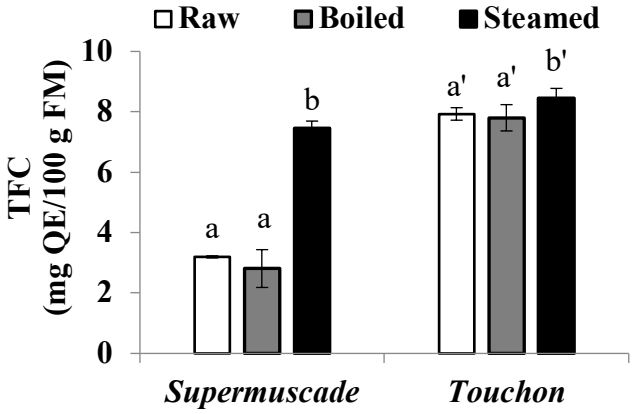


Figure 2. Total flavonoid content of raw and cooked carrots.

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; b>a and b'>a'.

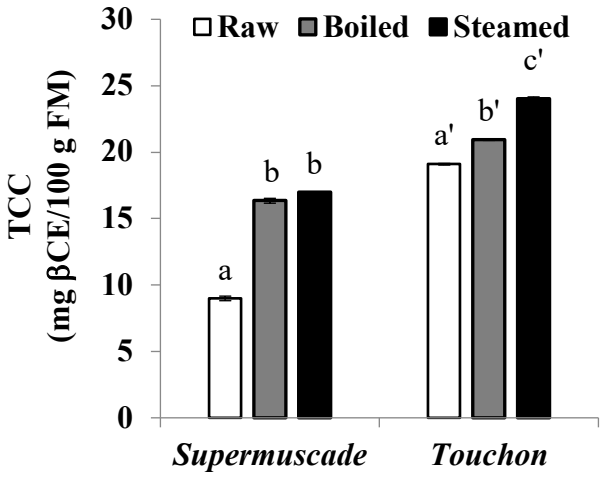
Nevertheless, studies conducted by Arkoub-Djermoune *et al.* (2016), Singh *et al.* (2018), and Oghbaei & Prakash (2021) have reported a

decrease in the flavonoid contents in some food matrices after thermal treatment of eggplant, black carrot and Chickpea, respectively. According to Yuan et *al.* (2009), this loss was due to their leaching to the cooking water and/or their thermal degradation. This divergence recorded in cooking effect on the flavonoid content may be explained by the differences in the cooking method, time and temperature.

3.3. Total Carotenoid Content (TCC)

Carotenoids are the most important micronutrients in fruit and vegetables. Several epidemiological studies have consistently shown that the consumption of diets rich in carotenoids is associated with a lower incidence of cancer, cardiovascular diseases, and cataract formation. In general, carotenoids are found particularly in orange, red, and yellow colored fruits and vegetables. The carrot root is one of the richer sources of these pigments, with the orange-rooted variety being the most familiar nowadays; this contains predominantly β-carotene (Gonzalvez *et al.,* 2014). Carotenoids are susceptible to degradation by chemical and physical factors, including exposure to light, oxygen, elevated temperature and others. Therefore, depending on the conditions of thermal processes, such as time and temperature, these compounds can be more or less affected, resulting in a decrease or increase of their amounts (Murador *et al.,* 2014).

The TCC of raw and cooked carrots were significantly different ($p<0.05$) (Fig. 3). The rates obtained in fresh carrots were 8.99 ± 0.17 mg ßCE/100 g FW and 19.09 ± 0.0.06 mg ßCE/100 g FW in *Supermuscade* and *Touchon*, respectively. Following boiling, the levels of TCC increased, respectively by 44.98% in *Supermuscade* and 8.83% in *Touchon*. In the same trends, the TCC in steamed samples raised with a rate of 47.02% (*Supermuscade*) and 20.62% (*Touchon*). Similarly, it has been reported that the β-carotene content in spinach and pumpkin were also increased by boiling (Azizah *et al.,* 2009, Bunea *et al.*, 2008). Furthermore, the contents of lutein and zeaxanthin in orange-fleshed sweet potato also



increased after thermal treatment (Donado-Pestana *et al.,* 2012).

Several other authors have registered a raise in carotenoids after thermal processing in some food matrix (Knockaert *et al.,* 2012; Zaccari *et al.*, 2015; Zhang *et al.,* 2020 Nartea *et al.,* 2021)

Figure 3. Total carotenoid content of raw and cooked carrots.

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; b>a andc'>b'>a'.

which could be attributed to the improved solubility of carotenoids due to heat treatment (Mayer-Miebach & Spiess, 2003). It has been suggested that the boiling process generally increases the carotenoid content in most vegetables. In addition, it has been reported that heat treatment might break down the cell walls, which further enhanced the release of carotenoids from the food matrix (Hwang *et al.,* 2012). In carrots, β-carotene is located in the chromoplasts where it is often associated with proteins and/or residual membranes. Chromoplasts have a double bilayer membrane and are located inside the plant cells (surrounded by a cell membrane and a cell wall) (Hornero-Méndez & Mínguez-Mosquera, 2007). As a result, several physical barriers have to be broken before β-carotene can be released from the carrot matrix and made accessible for absorption. Cooking partially dissolves

cellulose-thickened cell walls, freeing up nutrients by breaking down the cell membranes. As processing can have an effect on the food matrix and on these barriers, it can affect the β-carotene bioaccessibility and bioavailability (Char, 2018).

However, several authors have reported that thermal processing can decrease the carotenoid content in some vegetables (Arkoub-Djermoune *et al.,* 2016; Kapusta-Duch *et al.,* 2017; Arkoub-Djermoune *et al.,* 2019; Zhang *et al.,* 2020, Mehmood *et al.,* 2023). This was explained by Rodriguez-Amaya & Kimura (2004), that heat treatment induces *Cis*/*Trans* isomerization of carotenoids, altering their biological activities which causes the reduction of total carotenoid content in cooked sample. Furthermore, Zhang *et al.* (2020) have reported that the oxidation may be the main factor influencing carotenoid losses, a process that is stimulated by both light and heat. In addition, carotenoid oxidation also depended on available oxygen and the type of carotenoid. After the milling process, the exposed carotenoids, especially β-carotene, were more sensitive and vulnerable to heat treatment.

3.4. Antioxidant activity

Carrots are a unique vegetable crop rich in most of the natural antioxidants including carotenoids, phenolics, vitamin C and tocopherol. This antioxidant power protects against the free radicals generated endogenously through normal diet and metabolic activity as well as from environmental sources (Char, 2018). Both phenolic compounds and carotenoids are strong in vitro antioxidants. Phenolic fractions are potent free radical scavengers and β-carotene is considered a strong quencher of singlet oxygen (Schafer *et al.,* 2002).

The antioxidant capacity of raw and cooked carrots extract was evaluated by two methods: the radical scavenging activity against DPPH free radical (FRSA-DPPH) and the ferric reducing antioxidant power (FRAP).

3.4.1. DPPH free radical scavenging activity (DPPH-FRSA)

The Figure 4 shows the inhibition percentage of the DPPH free radical by the extract. The results obtained show a variation in the inhibitory activity of different cooked samples. Both cooking methods tested (boiling and steaming) have no significant effect ($p<0.05$) on the antiradical activity of *Touchon* variety. However, the antiradical activity raised in *Supermuscade* variety after steaming with a rate of 44.32%; but a slight decrease was noted after boiling with a percentage of 8.46%. These results are consistent with those reported by McDougall *et al.* (2010), Kapusta-Duch *et al.* (2017), Singh *et al.* (2018), Arkoub-Djermoune *et al.* (2019) concerning the effect of cooking on some vegetables. Moreover, Buratti *et al.* (2020), have detected a high good antioxidant activity in the steamed and microwaved carrots, whereas the minimum values were associated with long-boiled products. Nevertheless, Lin & Chang (2005) noted that cooking has no significant effect on the antioxidant properties of broccoli.

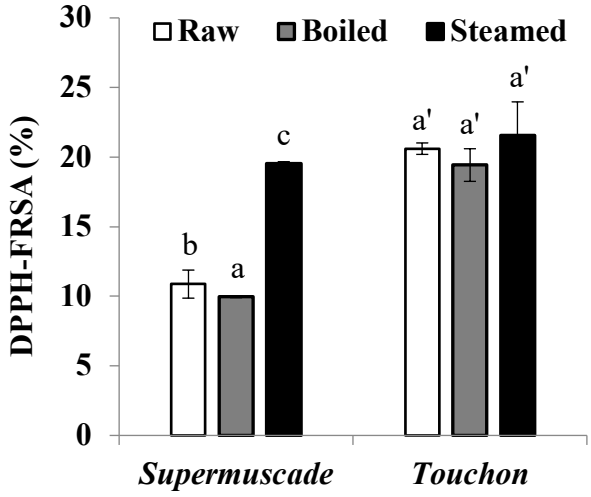


Figure 4. Antioxidant activity of raw and cooked carrots.

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; c>b>a.

This explains that during food processing, phytochemicals, which have additive or

synergistic effects on antioxidant activity, could be released from food matrix (Dewanto *et al.,* 2002). Due to thermal process, cell walls may break down, weakening the bonds between phytochemicals and tissue matrix (Dewanto *et al.,* 2002; Chang *et al.,* 2006). Thus, the bioavailability of these phytochemicals and, correspondingly, antioxidant activity could increase. Additionally, enzymatic degradation resulting from the heating process can increase carotenoid content due to the weakening of protein-carotenoid aggregates (Sahlin *et al.,* 2004). In addition, Faller & Fialho (2009) reported that different cooking methods (boiling, microwaving and steaming) reduce the antiradical activity of some vegetables (potato, carrot, onion, broccoli, white cabbage). Also, similar results were observed by Amin *et al*. (2006) in spinach, Arkoub-Djermoune et al. (2016) in eggplant and Ozer (2021) in homemade tomato sauces. The divergence registered concerning the effect of cooking on the antioxidant activity can be related to the differences on phenolic compounds content in the raw samples and/or the differences on the method, temperature and time of cooking.

3.4.2. Ferric reducing antioxidant power (FRAP)

The FRAP assay has been reported to be suitable for the monitoring of total antioxidant activity in the plant extracts (Benzie & Strain, 1996). The results of reducing power expressed as milligram Trolox Equivalent per 100 grams Fresh Weight are shown in the Figure 5. As it can be noted, both cooking methods tested (boiling and steaming) have significant effect ($p<0.05$) on reducing power of the studied varieties. The reducing power raised with rates ranging from 10.64 to 24.46 % after boiling and 38.35 to 44.31 % after steaming. Similar results were found by Arkoub-Djermoune *et al.* (2016), Teixeira-Guedes *et al.* (2019), Tuersuntuoheti *et al.* (2020) and Deyalage *et al.* (2021) in various cooked foods.

This increase can be due to the high content of antioxidant compounds in cooked samples after cell walls softening comparatively to the raw one. According to Arkoub-Djermoune *et al.* (2016), the observed increase in reducing power may be due to various factors. These include the liberation of high amounts of antioxidant components resulting from the thermal destruction of cell walls and sub cellular compartments, the production of stronger

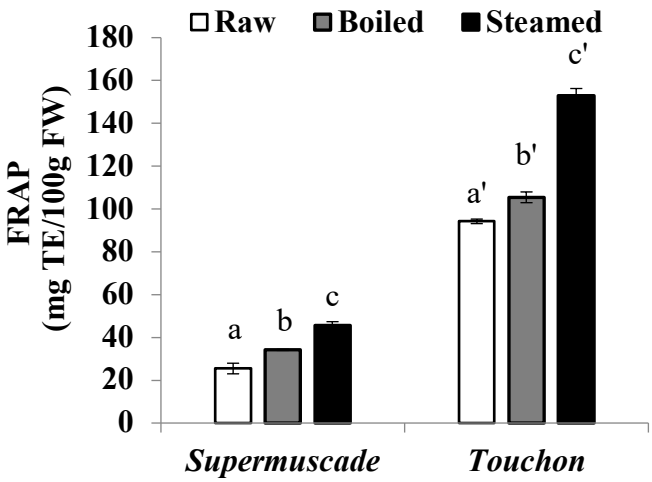


Figure 5. Ferric reducing antioxidant power of raw and cooked carrots.

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; c>b>a and c'>b'>a'.

radical-scavenging antioxidants through thermal chemical reaction; suppression of the oxidation capacity of antioxidants by thermal inactivation of oxidative enzymes; or the formation of novel compounds with antioxidant activity. To our knowledge, there is no published data regarding the effect of both tested method on the reducing power of the examined varieties.

3.5. Water cooking analysis

Since some of the bioactive components are water soluble and there is a possibility that these could also be dissolved in the cooking water, the remaining carrot cooking water were analyzed for TPC, TFC and TCC. The Table 1 shows that the high TPC was observed in boiling water of *Supermuscade* variety reaching a concentration of 90.15 ± 0.95 mg GAE/100 mL. However, in steaming water the highest level of TPC was registered in *Touchon* steamed water (63.45 ± 0.56 mg GAE/100 mL). Similarly, to the TPC,

the TFC of *Supermuscade* boiling water (9.49 ± 0.01 mg QE/100mL) was significantly ($p<0.05$) higher than that obtained in *Touchon* boiling water (8.82 ± 0.56 mg QE/100mL).

Nevertheless, no significant differences ($p<0.05$) were observed between the steaming waters of both varieties. These results were in agreement with those found by Oghbaei & Prakash (2021) in processed chickpea.

Moreover, the TCC in boiling and steaming waters ranged from 0.127 ± 0.001 mg βCE/100 mL to 0.167 ± 0.002 mg βCE/100 mL and 0.013 ± 0.000 mg βCE/100 mL to 0.016 ± 0.000 mg βCE/100 mL, respectively. Furthermore, results have shown that the TCC of boiling water was

10-fold higher than that registered in steaming water. In addition, results from the current study showed that boiling water contains more TPC, TFC and TCC then steaming water originated from leaching of phenolic compounds during cooking process and/or to the degradation of complex phenolics which generate simple compounds with high solubility in water.

Table 1. Antioxidant contents of carrots cooking water.

	TPC (mg GAE/100 mL)		TFC (mg QE/100 mL)		TCC (mg βCE/100 mL)	
	Boiling water	**Steaming water**	**Boiling water**	**Steaming water**	**Boiling water**	**Steaming water**
Supermuscade	90.15 ± 0.95^{b}	59.44 ± 0.98^{a}	9.49 ± 0.01^{b}	7.57 ± 0.65^{a}	0.167 ± 0.002^{b}	0.013 ± 0.000^{a}
Touchon	66.56 ± 0.69^{a}	63.45 ± 0.56^{b}	8.82 ± 0.56^{a}	7.67 ± 0.54^{a}	0.127 ± 0.001^{a}	0.016 ± 0.000^{b}

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; b>a.

The Table 2 shows that the cooking water exhibited good antioxidant activities where the best antiradical activity was registered in boiling and steaming waters of *Supermuscade* variety with a percentage of 53.07 ± 0.19 % and 28.52 ± 0.10 %, respectively. Furthermore, there is no significant difference ($p<0.05$) in the FRAP between the boiling waters of both varieties but the highest FRAP was noted in *Touchon* steaming water with a value of 39.63 ± 0.60 mg TE/100 mL. These antioxidant properties observed in cooking waters can be explained by the high content of phenolic compounds, which are released from carrots during the cooking process.

Table 2. Antioxidant activities of carrots cooking water.

	DPPH-FRSA (%)		FRAP (mg TE/100 mL)	
	Boiling water	**Steaming water**	**Boiling water**	**Steaming water**
Supermuscade	53.07 ± 0.19^{b}	28.52 ± 0.10^{b}	29.74 ± 0.23^{a}	25.76 ± 0.65^{a}
Touchon	48.70 ± 0.53^{a}	25.99 ± 0.13^{a}	29.53 ± 0.29^{a}	39.63 ± 0.60^{b}

Values are averages standard deviation of triplicate analysis; different letters indicate significant difference ($p<0.05$). Results are ranked in ascending order; b>a.

3.6. Pearson Correlation Analysis

Correlation analysis was used to determine the relationship between the different measured variables. The correlation matrix presented in Table 3 revealed a correlation between phytochemicals content and antioxidant activity of carrot extracts. A strong positive correlation was observed between TPC, TFC and TCC (r = 0.98 and r = 0.80). The antioxidant activity of carrot extracts was affected by the rate of antioxidant substances; DPPH-FRSA and FRAP activities were very highly and significantly correlated ($p<0.001$) with TPC (r = 0.98 and r = 0.82, respectively), TFC (r = 0.98 and r = 0.79, respectively), and TCC (r = 0.76 and r = 0.89,

respectively). This shows that these phytochemicals are the most bioactive molecules involved in the antioxidant activity of the raw and cooked carrot extracts.

Table 3. Correlation matrix between the phytochemical contents and antioxidant activity of raw and cooked carrots extract.

	TPC	**TFC**	**TCC**	**DPPH-FRSA**	**FRAP**
TPC	1.00				
TFC	0.98***	1.00			
TCC	0.80***	0.78***	1.00		
DPPH-FRSA	0.98***	0.98***	0.76***	1.00	
FRAP	0.82***	0.79***	0.89***	0.78***	1.00

TPC: *Total Phenolic Content;* ***TFC:*** *Total Flavonoid Content;* ***TCC:*** *Total Carotenoid Content;* ***DPPH-FRSA:*** *DPPH-Free Radical Scavenging Activity;* ***FRAP:*** *Ferric Reducing Antioxidant Power. ***Very highly significant at $p<0.001$*

Table 4. Correlation matrix between the phytochemicals content and antioxidant activity of cooking water.

	TPC	**TFC**	**TCC**	**DPPH-FRSA**	**FRAP**
TPC	1.00				
TFC	0.80**	1.00			
TCC	0.84***	0.89***	1.00		
DPPH-FRSA	0.78**	0.87***	0.99***	1.00	
FRAP	- 0.09	- 0.22	- 0.27	- 0.36	1.00

TPC: *Total Phenolic Content;* ***TFC:*** *Total Flavonoid Content;* ***TCC:*** *Total Carotenoid Content;* ***DPPH-FRSA:*** *DPPH-Free Radical Scavenging Activity;* ***FRAP:*** *Ferric Reducing Antioxidant Power. ***Very highly significant at $p<0.001$; ** Highly significant at $p<0.01$.*

Same results were noted by several researchers, who registered a good significant correlation between the phenolic compounds and antioxidant capacity of root vegetables (Carrillo *et al.,* 2017; Koley & Singh, 2019; Arkoub-Djermoune *et al.,* 2020). Moreover, a positive and significant correlation was observed between the antioxidant activities DPPH-FRSA and FRAP (r = 0.78). This correlation is probably due to the presence of phenolic substances displaying both antiradical activity and reducing power. These findings align with the results reported by Koley & Singh (2019) and Arkoub-Djermoune *et al.* (2020).

Similar to the carrot extracts, the residual cooking water from carrots present a highly significant correlation at $p<0.001$ (Table 4) between TFC, TCC and the antiradical activity of DPPH radical (r = 0.87 r = 0.99). Additionally, a highly significant correlation ($p<0.01$) was observed between TPC and the antiradical activity of DPPH (r = 0.78). This suggests that these antioxidants are the main compounds contributing to the antioxidant activity of cooking water. However, no significant ($p<0.05$) correlation was found between TPC, TFC, TCC and DPPH-FRSA as well as the ferric reducing power, which is in line with the result reported by Arkoub-Djermoune *et al.* (2019). This explained by Sroka & Cisowski (2003), that the antioxidant power depends not only on the concentration of polyphenols but also their chemical structure (the number and the position of the hydroxyl groups). Furthermore, other highly significant correlations have been observed between certain phytochemicals showing a synergistic effect between these antioxidants involved in the antioxidant activity of carrot water cooking.

4. Conclusions

This study has importance for culinary science in terms of bioactive properties exhibited by various cooked carrots varieties. In the current study, the impact of traditional or domestic cooking (boiling and steaming) on the antioxidant contents and antioxidant activities of two Algerian cooked carrots cultivars (*Supermuscade* and *Touchon*) were investigated. The results of the present study confirmed that the tested cooking method (boiling and steaming) caused an increase in the content of bioactive substances (phenolics, flavonoids and carotenoids) and an enhancement in the antioxidant power of carrot extracts. The findings indicated that the steaming was the best method with a positive effect. The increase in phytochemicals content can be attributed to thermal treatment which causes the denaturation of enzymes that are involved in the degradation of nutrients and bioactive compounds and/or resulting in a softening effect, which increases the extractability of bioactive compounds, resulting in a higher level in cooked products as compared to the raw material. Moreover, a good correlation was noted between the phytochemicals and the antioxidant capacity which indicated that these compounds are the main substances responsible of the antioxidant activity of carrots. Furthermore, the analysis of cooking water shows that the traditional cooking methods tested promote the release of some antioxidant compounds into the cooking water, contributing to their antioxidant activity.

In conclusion, the traditional or domestic cooking methods tested affect positively the phytochemical contents of orange carrots as well as their antioxidant properties, depending on the specific cooking way employed.

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Acknowledgment

The authors thank the Algerian Ministry of Higher Education and Scientific Research.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **https://chimie-biologie.ubm.ro/carpathian_journal/index.html**

ENHANCEMENT OF COMPOSITION AND OXIDATIVE STABILITY OF SUNFLOWER AND SOYBEAN OIL BY BLENDING WITH PALM OIL

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https://doi.org/10.34302/crpjfst/2024.16.2.2

Article history:
Received: January 10th, 2024
Accepted: March 12th , 2024

Keywords:
Palm oil,
Soybean oil,
Sunflower oil,
Oxidative stability,
Peroxide value,
Saponification value.

ABSTRACT

The objective of this research was to create vegetable oil blends for food that had higher oxidative stability than pure sunflower and soybean oil. The following proportions of sunflower, soybean, and palm oil were evaluated when they were blended: 50:50 (v/v). A 45-day, 60 °C, accelerated storage test was performed. We calculated the free fatty acid level, fatty acid composition, induction time, and primary and secondary oxidation products. In comparison to sunflower and soybean oil, the blends showed higher oxidative stability indicators. The findings imply that combining palm oil with sunflower, soybean, and other oils is a suitable substitute for obtaining oils with greater oxidative stability indices.

1.Introduction

Edible oils may deteriorate as a result of lipid oxidation while being stored, handled, or prepared. Oil quality is primarily lost due to oxidation, which also reduces oil's nutritional content and produces unfavourable off-flavors that make oils containing food less appealing to customers. Additionally, the oxidation of lipids results in the production of some toxic by-products such reactive carbonyl compounds (RCCs), which have the potential to produce advanced lipid peroxidation end products (ALEs) that could be dangerous to human health (Guillén et al, 2005). The degree of lipid oxidation is determined by both the internal characteristics of the oil, such as its degree of unsaturation, the presence of antioxidant compounds or metals like copper and iron, and the exterior characteristics of the oil, such as the oxygen content and temperature. Oils that come into touch with oxygen trigger chain reactions that advance more quickly at higher temperatures and with higher lipid unsaturation levels (Fadda et al, 2022).

Due to the rising trend of replacing hydrogenated oils with saturated fatty acids (SFAs) with monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs), which are regarded as healthier by consumers but are much more prone to oxidation than SFAs, lipid peroxidation is a major concern for the food industry. Additionally, when heated to deep-frying or cooking temperatures, oils high in MUFAs and PUFAs may produce RCCs, which give rise to ALEs, offering major health hazards. Even though it is highly recommended to consume MUFA and PUFA-rich oils, these oils must first be "preserved" before being used in cooking. Some oils, like olive oil, are inherently preserved by their high levels of endogenous antioxidants (polyphenols and tocopherols), but others, including soybean, sunflower, and peanut oils, must be fortified with exogenous antioxidants during manufacturing because of the refining process they go through (Viana da Silva et al, 2021). By delaying or preventing the breakdown of lipids, synthetic antioxidants like butylated hydroxyanisole (BHA), butylated

hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) have been able to modulate oxidation processes thus far (Taghvaei et al, 2015,Metzner Ungureanu et al, 2020: Odeh et al, 2021).

Hydrogenation, interesterification, fractionation, and blending are methods that can change an oil's nutritional properties, quality, and stability. Blending oils with diverse compositions and attributes is the most straightforward way to get the required oil characteristics because interesterification and fractionation require specialised and expensive equipment, while interesterification leads to the development of trans isomers (Hashempour-Baltork et al., 2016).When two or more oils from various vegetable species are combined (in a ratio greater than 5%), an edible oil blend is created (Guiotto et al., 2014). The fatty acid profile is altered by combining various vegetable oils, which may also increase their nutritional and practical usefulness. Because omega-3 and omega-9 fatty acids have anti-inflammatory characteristics, increasing consumption of MUFA and PUFA helps to lower the risk of coronary heart disease (Ramsden et al., 2013).The protection of cardiovascular illnesses, however, appears to be significantly influenced by merely ω -3, according to earlier research (Griffin, 2008). Additionally, high MUFA and PUFA content unsaturated oils are more susceptible to oxidation; hence, appropriately balancing MUFA and PUFA may result in oil blends with greater nutritional value, high storage stability, and even suitability for frying (Adhvaryu et al., 2000; Ramsden et al., 2013).

Fundamental yet distinct functions are played by the omega-3 and omega-6 fatty acids in the development of the cell membrane. The relationship between -3:-6 fatty acids in the diet is crucial because the varied numbers and positions of double bonds in the chain provide the fatty acids various physiological qualities. Since eicosapentaenoic acid and docosahexaenoic acid use the same metabolic pathways and compete for the same elongase and desaturase enzymes, the -3:-6 balances in the diet is crucial (Huerta-Yépez et al., 2016). Linoleic acid is converted to arachidonic acid, while -linolenic acid produces eicosapenta. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) should make up an even 1:1:1 ratio of the calories consumed by adults, according to the international nutrition and food committees established by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (FAO, 2012). Simopoulos (2002) suggested a 3:1:6 ratio that was less than 1:4 to lower the risk of chronic diseases. When Gomes et al. (2019) examined the relationships between erythrocyte and plasma n-3:n-6 fatty acids and a variety of oxidative stress biomarkers in breast cancer patients, they discovered that plasma n-3:n-6 ratio was related to the anti-inflammatory factor.

Tallow, lard, fish oil, and milk fat are examples of animal-derived fats and oils (butter). Oils of vegetable origin are often made from plant seeds or beans (Oils and Fats in the Market Place, 2021). The most popular major vegetable oils are palm oil, soybean oil, rapeseed oil, and sunflower oil (Woodgate et al, 2014: The Four Major Vegetable Oils, 2021).Less than 25% of the total fatty acids in the majority of vegetable oils are saturated fatty acids (Kim et al, 2010). However, it's been noted that the saturated fatty acid content of palm, palm kernel, and coconut oils is higher, at 49%, 80%, and 90%, respectively. Mono- and poly-unsaturated fatty acids, predominantly oleic (18:1) and linoleic (18:2), make up the remaining portion of the fatty acid makeup (Kim et al, 2010). The most widely used edible oil worldwide is palm oil (Akoh, 2017). Palm oil and palm kernel oil, which have differing fatty acid contents, can be extracted from palm fruit (Li et al, 2012). Both are extracted, with the former coming from the mesocarp (which is high in Palmitic and oleic acids) and the latter coming from the kernel (which is high in Lauric acid) (Akoh, 2017).

The predominant fat used in Egypt today is palm oil. It is a common and often used food ingredient in the global food industry. Due to the amount of saturated fat that provides stability

and increased resistance to oxidation when heating at high temperatures, the food processing industries favour palm oil more than other types of oil (Azrina et al.,2009) .Polyunsaturated fatty acids (PUFA), which are more susceptible to oxidative alterations, are present in significant concentrations in the majority of vegetable oils. Trans fatty acids are one type of oxidation product created by the oxidative modifications in PUFA (TFA) .These TFA have negative metabolic side effects that can change cell function and metabolism (Holohan, 1997). One of the most popular oils in use today is soybean oil. Because it contains a wide range of the essential fatty acids and sterols required by the body to maintain health, soybean oil is also healthier than the majority of other plant oils (Kailas et al., 2013).The physical and chemical composition of blended oils as well as blood lipids have previously been claimed to be improved by vegetable oils when blending (St-Onge et al., 2003).

Approximately 40–50% of sunflower seeds are made up of oil, which is a significant source of the polyunsaturated fatty acid (linoleic acid) with possible health advantages (Lopez et al,. 2000 and Monotti, 2004.) .Sunflower oil is a crucial oil to use for cooking due to the fatty acid structure . Due to its high linoleic acid content (48.3 to 74.0%), moderate oleic acid content (14.0 to 39.4%), and low level of saturated fatty acid content (12%), it is regarded as highly polyunsaturated oil. Because the body is unable to produce these fatty acids, they are vital to life (Gunstone, 2002). The widely used Rancimat method is an international standard that is carried out under accelerated storage circumstances at high temperatures (AOCS, 1998) and is accurate, repeatable, doesn't require the use of reagents, and its readings may be easily automated (Heidarpour and Farhoosh, 2018). The main objective of this works to study and evaluate the physico-chemical properties of blended palm oil with soybean and sunflower oils. Additionally, shelf life of the blends was computed using the oxidative stability index (OSI) values and extrapolating the results to standard storage temperatures.

2. Materials and methods

2.1. Materials and reagents

All chemicals used in the study, were purchased from Sigma–Aldrich (St. Louis, MO). All chemicals and solvents were analytical reagent grade. Refined, bleached and deodorized (RBD) sunflower oil (SFO), RBD soybean oil (SBO) and RBD palm oil were obtained from Tanta Oil and Soap Co, Tanta, Egypt.

2.2. Preparation of oil and its blends containing antioxidants

2.2.1. Selection of oils

The selection of test oils was based on the presence of varying polyunsaturated fatty acids (PUFA) composition. Thus, PO which includes SFO, SBO and its blending PO 50%: SFO: 50%, PO 50%: SBO: 50% and SMO 50%: SFO: 50% were used in this investigation.

2.2.2. Samples preparation

Three blends of palm oil, soybean oil and sunflower oil were prepared using a mechanical stirrer at 180 rpm for 15min (**Kumar *et al.* 2009**). These blends were prepared in the ratio of 50:50. These three blends named as:

Sample A=50% sunflower oil & 50% palm oil.
Sample B= 50% palm oil & 50% soybean oil.
Sample C= 50% soybean oil & 50% sunflower oil. All oil Blends were treated with and without 36 ppm TBHQ in a series of transparent glass bottles having a volume of 200 ml each, to examine their antioxidative activity. Schaal oven test was conducted to evaluate the effect of antioxidants against oxidation during the accelerated oxidative storage of oils.

2.2.3. Oxidative stability test:

2.2.3.1. Free fatty acid

Free fatty acid content (FFAC) was determined according to standard methods of **AOCS (2000).**

2.2.3.2. Peroxide value (PV)

The peroxide value (PV) was conducted by referring to the AOAC method 965.33 described by (**AOAC, 2011)**. 5.00 g of oil samples were dissolved in 30 mL of a 3:2 acetic acid to chloroform solution 1mL of saturated KI was

then added after that. The mixture was maintained in the dark for five minutes after being allowed to stand for a minute with intermittent shaking. Following this, 30 mL of distilled water was added. Sodium thiosulfate (0.002 M) was used to titrate the mixture until the yellow color practically vanished. Then a 0.5mL solution of 1% starch was added. The titration was carried out until the blue color vanished. The identical conditions were used for the analysis of a blank. The following equation was used to get the peroxide value:

Peroxide value (PV) = (S × M × 1000)/sample weight (g)

Where, S is the value of Na2S2O3 used (blank corrected); M is the molarity of Na2S2O3.

2.2.4. Fatty acid composition

2.2.4.1. Methyl Esterification

Prepared fatty acid methyl esters (FAME**)** 100µl of fat in 10ml tube with Teflon cap and 200µl methanolic KOH (11.2g KOH in 100ml methanol HPLC grade) Then vortex (witag, Germany) for 1 min and add10 ml hexane HPLC grade Close tube and centrifuge by(Bouch,R144.Italy) for 10 min 2500 rpm .

2.2.4.2. GC/MS Measurements

Thermo Ultra Trace GC series gas chromatography and Thermo DSQ mass spectrometer from Thermo Fisher Scientific were used to analyse the fatty acid methyl esters (Waltham, MA, USA) .Used was an SGE BP x 70 column with a 25 m x 0.25 mm, 0.25 m film thickness and a 65% methyl, 35% phenyl silicone composition. Helium (99.999%) was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature programme was modified as follows while the injection block temperature was maintained at 250 °C: Initial temperature ramping from 60 °C to 180 °C at 10 °C/min for 2 min, then ramping from 240 °C to 5 °C/min for 10 min. The source temperature was 220°C, while the injection temperature was 250°C. The MS interface was 240°C in temperature. The split ratio for the 0.5 L injection was 1:30. Ionization energy for the EI-MS measurements was 70 eV. The mass range was between 50 and 650 amu.With 0.1 interscan delays, the scan time is 0.5 seconds. NIST and Wiley (Gas Chromatography-Mass Spectrometry) GC-MS libraries were used for the library search (Sathianathan et al., 2014**).** The discovered fatty acids were quantified by comparing the area beneath each fatty acid peak to the sum of all fatty acid peaks.Results are given as grams of fatty acids per 100 grams of total fatty acids (**Lutterodt et al., 2011**). Each sample was examined three times.

2.2.5. Shelf-Life Testing

A forced-draft air oven with a temperature setting of 60 °C was used to speed up the oxidation of oil samples (30 g) in a series of 100 mL glass bottles. Over the course of the storage period, oxidation was observed every two weeks, and changes in PV and FFA were studied.

2.2.6. Accelerated Oxidation Storage Experiments (Rancimat Test)

Rancimat was used to measure storage of oils and blends in accordance with Official Method Cd12b-92 (AOCS, 1997, 1998). Using the Rancimat 743 equipment (Metrohm) and 3 g of oil sample that had been heated to 110°C with an air flow of 20 L/h, stability was expressed as the induction time (h).

2.3. Statistical analysis

All data were statistically analyzed using the general linear models procedure of the statistical analysis system SAS (1998).Significances of differences were defined at $p < 0.05$.All experiments as well as related analysis results were repeated three times and all obtained data are expressed as an average.

3.Results and discussions

3.1. Fatty acid composition for sunflower oil, palm oil and Soybean oil

The fatty acid composition of polyunsaturated and saturated fatty acid ratio of pure oils used in this studied are presented in Table (1). Palm oil contained (40.0%) oleic acid (C18:1) and 46.08 % Palmitic acid (C16:0), these results are in agreement with Naghshineh et al. (2010) and El-gazzar et al, (2021). whereas sunflower and soybean oils contained a lower

level of oleic acid was found to be (23.11%), (20.70 %), respectively. On the other hand Linoleic acid values were (67.79 %) and (59.0 %) for sunflower and soybean oils respectively. From the Table (3) it noted that samples of soybean oil content of Linolenic acid (6.26 %), while this fatty acid no detect for sunflower and palm oils. According to Juárez et al. (2011), fresh soybean oil included 10.3% of Palmitic acid (C16:0), 4.9% of stearic acid (C18:0), 21.5% of oleic acid (C18:1), 53.4% of linoleic acid (C18:2), and 5.1% of Linolenic acid. These findings are in agreement with their findings (C18:3). Ferrari et al. (1996) also discovered that the refined soybean oil's fatty acid content was 11.2% C16:0, 0.1% C17:0, 3.5% C18:0, 24.9% C18:1, 50.2% C18:2, 3.5% C18:3, 0.4% C20:0, 0.4% C20:1, 0.5% C22:0, and 0.2% C24:0. El-gazzar et al. (2021) also discovered that palm oil included (49.70%) oleic acid (C18:1) and (34.90% Palmitic acid (C16:0), whereas sunflower and soybean oils contained a lower level of oleic acid, which was found to be (18.80%) and (23.18%), respectively. On the other hand Linoleic acid values were (67.80 %) and (49.56 %) for sunflower and soybean oils respectively, soybean oil content of Linolenic acid (3.50 %), while the percentage of this fatty acid was (0.30%) and (0.30%) for sunflower and palm oils, respectively.

Table (1): Fatty acid composition for RBD sunflower oil, palm oil and Soya bean oil

Fatty acid composition		**Sunflower oil**	**Palm oil**	**Soybean oil**
Caprylic	**C 8:0**	0.00	0.00	0.00
Capric	**C10:0**	0.00	0.00	0.00
Lauric	**C12:0**	0.00 ± 0.00^{b}	0.31 ± 0.02^{a}	0.00 ± 0.00^{b}
Myristic	**C14:0**	0.00 ± 0.00^{b}	0.92 ± 0.05^{a}	0.00 ± 0.00^{b}
Palmitic	**C16:0**	5.30 ± 0.9^{c}	46.08 ± 2.3^{a}	10.29 ± 1.12^{b}
Stearic	**C18:0**	2.70 ± 0.1^{b}	3.5 ± 0.06^{ab}	3.65 ± 0.04^{a}
Arachidic	**C20:0**	0.10 ± 0.01^{b}	0.2 ± 0.02^{a}	0.10 ± 0.01^{b}
SFA		8.10 ± 0.85^{c}	51.00 ± 1.2^{a}	14.04 ± 1.05^{b}
Oleic	**C18:1**	23.11 ± 1.5^{b}	40.0 ± 2.4^{a}	20.70 ± 1.3^{c}
Linoleic	**C18:2**	67.79 ± 2.6^{a}	9.00 ± 1.2^{c}	59.00 ± 2.3^{b}
Linolenic	**C18:3**	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	6.26 ± 0.02^{a}
Archidonic	**C20:1**	0.00	0.00	0.00
USFA		91.40 ± 3.4^{a}	49.0 ± 1.8^{c}	85.96 ± 3.6^{b}

*** Values (means ±SD) with different superscript letters are statistically significantly different ($P \leq 0.05$).**

Table (2). Fatty acid composition for RBD sunflower oil, palm oil and Soya bean blends

Fatty acid composition		50% sunflower oil 50% palm oil	50 %palm oil 50% soybean	50% soybean 50% sunflower oil
Caprylic	**C 8:0**	0.00	0.00	0.00
Capric	**C10:0**	0.00	0.00	0.00
Lauric	**C12:0**	0.00	0.00	0.00
Myristic	**C14:0**	0.51 ± 0.04^{a}	0.45 ± 0.03^{b}	0.00 ± 0.02^{c}
Palmitic	**C16:0**	26.11 ± 1.3^{ab}	27.47 ± 1.1^{a}	7.58 ± 1.2^{c}
Stearic	**C18:0**	2.66 ± 0.12^{b}	3.15 ± 0.16^{a}	2.65 ± 0.14^{b}
Arachidic	**C20:0**	0.16 ± 0.02^{a}	0.17 ± 0.03^{a}	0.07 ± 0.02^{b}
SFA		29.44 ± 0.85^{b}	30.79 ± 1.0^{a}	10.30 ± 1.2^{c}
Oleic	**C18:1**	34.44 ± 1.6^{a}	32.79 ± 1.8^{b}	25.00 ± 1.5^{c}
Linoleic	**C18:2**	35.84 ± 2.00^{b}	33.72 ± 2.04^{c}	61.96 ± 3.22^{a}
Linolenic	**C18:3**	0.00 ± 0.02^{c}	2.05 ± 0.04^{b}	2.28 ± 0.05^{a}
Archidonic	**C20:1**	0.00	0.00	0.00
USFA		70.56 ± 2.6^{b}	69.21 ± 2.9^{c}	89.70 ± 3.2^{a}

*** Values (means ±SD) with different superscript letters are statistically significantly different ($P \leq 0.05$).**

Table (3). Shelf life for sunflower oil, palm oil and Soybean oils (by antioxidants and without antioxidant) After 45 days

par		sunflower oil By anti +ve	sunflower oil without anti -ve	palm oil By anti +ve	palm oil without anti -ve	soybean oil By anti +ve	soybean without anti -ve
15 days	**FFA**	0.04 ± 0.00^{d}	0.06 ± 0.00^{b}	0.07 ± 0.00^{a}	0.058 ± 0.002^{b}	0.048 ± 0.001^{c}	0.04 ± 0.00^{d}
	PV	0.8 ± 0.05^{f}	3.5 ± 0.14^{a}	1 ± 0.04^{e}	3 ± 0.22^{b}	1.5 ± 0.18^{d}	1.6 ± 0.20^{c}
30 days	**FFA**	0.03 ± 0.02^{e}	0.055 ± 0.00^{c}	0.095 ± 0.002^{b}	0.1 ± 0.001^{a}	0.035 ± 0.002^{e}	0.048 ± 0.001^{d}
	PV	2.2 ± 0.05^{b}	2.6 ± 0.08^{a}	0.6 ± 0.03^{f}	0.89 ± 0.05^{e}	1.4 ± 0.04^{d}	1.9 ± 0.08^{c}
45 days	**FFA**	0.038 ± 0.00^{d}	0.06 ± 0.002^{b}	0.12 ± 0.001^{a}	0.1 ± 0.002^{a}	0.04 ± 0.00^{c}	0.038 ± 0.001^{c}
	PV	1.2 ± 0.05^{e}	3.3 ± 0.12^{a}	0.8 ± 0.04^{f}	2 ± 0.08^{c}	1.3 ± 0.06^{d}	3.2 ± 0.08^{b}

* Values (means ±SD) with different superscript letters are statistically significantly different ($P \leq 0.05$).

Table (4). shelf life for sunflower oil, palm oil and Soybean blends (by antioxidants and without antioxidant)

par		50% sunflower oil 50% palm oil By anti +ve	50% sunflower oil 50% palm oil without anti -ve	50 %palm oil 50% soybean By anti +ve	50 %palm oil 50% soybean without anti -ve	50% soybean 50% sunflower oil By anti +ve	50% soybean 50% sunflower oil without anti -ve
15 days	FFA	0.05±0.001^{b}	0.06±0.00^{a}	0.06±0.001^{a}	0.065±0.002^{a}	0.016±0.001^{c}	0.05±0.00^{b}
	PV	0.6±0.03^{c}	0. 16±0.01^{e}	0. 7±0.02^{b}	1. 2±0.05^{a}	0. 16±0.01^{e}	0. 5±0.02^{d}
30 days	FFA	0.058±0.00^{d}	0.09±0.002^{a}	0.065±0.001^{c}	0.076±0.002^{b}	0.03±0.00^{e}	0.052±0.00^{d}
	PV	0.25±0.03^{f}	1.6±0.08^{b}	0.65±0.03^{e}	2±0.04^{a}	1±0.01^{d}	1.4±0.02^{c}
45 days	FFA	0.06±0.001^{b}	0.074±0.002^{a}	0.074±0.002^{a}	0.078±0.001^{a}	0.039±0.00^{d}	0.052±0.002^{c}
	PV	0.75±0.05^{f}	1.7±0.09^{b}	1.0±0.05^{e}	1.5±0.07^{c}	1.3±0.04^{d}	3.2±0.12^{a}

*** Values (means ±SD) with different superscript letters are statistically significantly different ($P \leq 0.05$)**

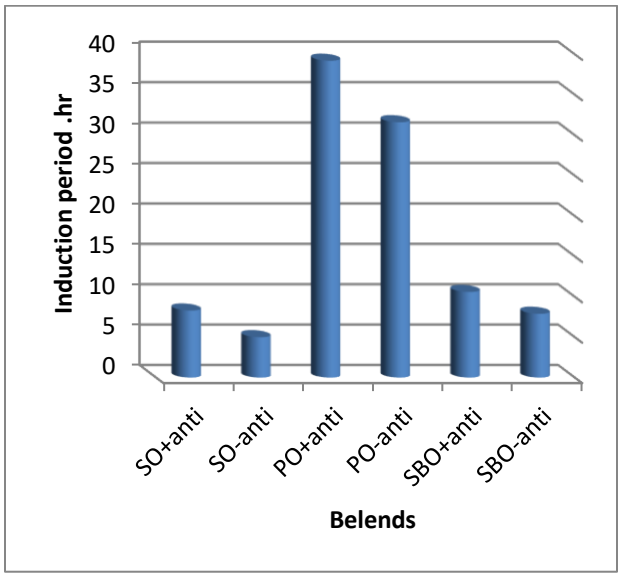

Figure 1. Thermophilic stability (Rancimat) for sunflower oil, palm oil and Soya bean oils (by antioxidants and without antioxidant) at 110˚c.

3.2. Thermophilic stability for sunflower oil, palm oil and Soya bean oils (by antioxidants and without antioxidant) at 110 ˚C

The antioxidative potential of the employed antioxidants was determined by the induction duration. The time needed to reach an end point of oxidation corresponding to either a degree of observable rancidity or a rapid change in the rate of oxidation is known as the induction period (IP), also known as the oxidative stability index (OSI) (Presa and Lopez, 1995). In general, raw vegetable oils are more oxidatively stable than their refined and processed counterparts. In addition to the fatty acid composition, the presence of minor bioactive elements such tocols, sterols, metal ions, polar lipids, and the initial concentration of hydro peroxides affects the oxidative stability of a substance (Madhujith & Sivakanthan ,2019: Cai et al, 2021). The results of induction periods at 11^{0}C for sunflower oil by antioxidant, sunflower oil without antioxidant, palm oil by antioxidant, palm oil without antioxidant, soybean oil by antioxidant and soybean oil without antioxidant were 8.3, 5.0, 5.23, 39.2, 31.6, 10.6 and 7.9 h, respectively (figure 1). Results indicated that the induction period of palm oil was the highest followed by soybean oil and finally sunflower oil. However, the differences between samples were significant. Also, the addition of industrial antioxidants to the oils increased the induction period for the used oils as a result of the effect of antioxidants which preserve fats and oils from deterioration, rancidity/discoloration (Mujeeda and Prasad, 2016) by acting as chain-breaking radical scavengers and peroxide decomposers (De Souza et al, 2011: Mujeeda and Prasad, 2016). These results are in agreement with El-gazzar et al, (2021).

3.3. Thermophilic stability for sunflower oil, palm oil and Soybean blends

The results of induction periods at 11°C for 50% sunflower oil 50% palm oil by antioxidant, 50% sunflower oil 50% palm oil without antioxidant, 50 %palm oil 50% soybean by antioxidant, 50 %palm oil 50% soybean without antioxidant, 50% soybean 50% sunflower oil by antioxidant, and 50% soybean 50% sunflower oil without antioxidant were 14.5, 9.9, 15.3, 11.7, 9.8, and 6.25 and respectively. (Figure 2).Results indicated that the induction period of palm oil was the highest followed by soybean oil and finally sunflower oil. Also, the addition of industrial antioxidants to the oils increased the induction period for the used oils As mentioned earlier .However, blending of different vegetable oil can also improve the content of antioxidant and bioactive lipids and these antioxidants and bioactive components are also improving the stability of vegetable oils (Abdel-Razek et al, 2011: Dhyani et al, 2018). Garg et al. (2021), González-Gamallo et al, (2021) and El-gazzar et al, (2021) who found that blending of different vegetable oil improved the stability of vegetable oils.

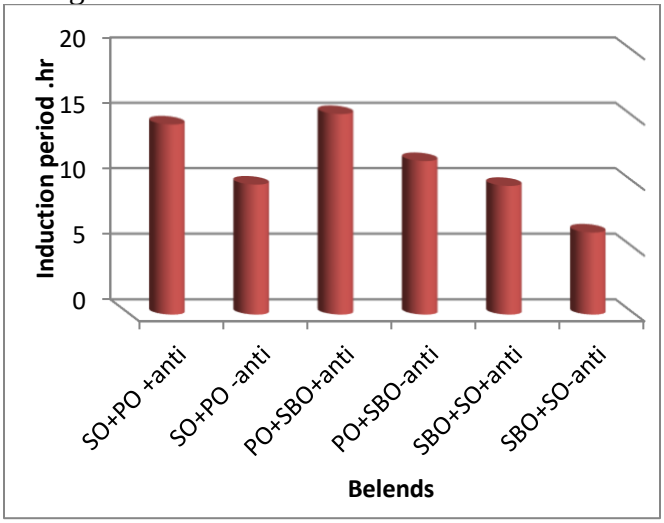

Figure (2). Thermophilic stability (Rancimat) for sunflower oil, palm oil and Soya bean blends oils (by antioxidants and without antioxidant) at 110 ˚C

3.4. Shelf life for sunflower oil, palm oil and soybean oils

The assessment of oxidative oxidation in oils and fats is frequently done using the PV test. Since the main byproduct of lipid oxidation is hydrogen peroxide, measuring PV or FFA can be utilized as an oxidative index in the early stages or lipid oxidation (Ramadan and Moersel 2004; Mohdaly et al. 2010).Table 3 presents the PV changes in oils and blends during storage for 45 days at 60°C. The final PV of sunflower oil had the highest value with 3.3 meq O2/kg among oils followed by soybean oil and finally sunflower oil. While the initial FFA of palm oil had the highest value with0.12 followed by soybean oil and finally sunflower oil. Addition of Antioxidants decreased the PV and FFA contents of oils ,which preserve fats and oils from deterioration, rancidity/discoloration (Mujeeda and Prasad, 2016) by acting as chain-breaking radical scavengers and peroxide decomposers (De Souza et al, 2011: Mujeeda and Prasad, 2016).

3.5. Shelf life for sunflower oil, palm oil and Soybean blends

PUFA concentration is a significant element determining the oxidative stability of particular oils and oil blends, in addition to the inherent natural antioxidants in oil

The oxidative stability of high PUFA oil can be increased by blending with high MUFA oil because the oxidative stability index (OSI) is inversely proportional to PUFA concentration (Chu et al., 1998; Kumar et al., 2009**)**. In other words, if oil's PUFA concentration is decreased by blending it with MUFA, MCFA, or SFA, the blend's oxidative stability will rise. The similar idea has been tried with the addition of palm oil to other vegetable oils. . The oxidative stability of palm oil and its blends is shown in Table (4). In the order of sunflower, soybean, and palm oil, peroxide generation rates were higher in sunflower oil and soybean than in palm oil. The peroxide values of the sunflower: palm oil and soybean: palm oil blends, on the other hand, dropped when palm oil was added to the blends, demonstrating that palm oil contributed to the

oxidative stability of the blends. In the oil mixes, palm oil partially prevented peroxide production. In contrast to the individual soybean oil, the soybean: palm oil blends significantly reduced the development of peroxide by 46.87% over a 45-day period, while the sunflower oil: palm oil blends significantly reduced the formation of peroxide by 51.1% over the same period

Compared to palm oil, TBHQ showed a stronger inhibitory effect on the production of peroxide. Therefore, it can be said that the lower rate of peroxide generation was caused by the inclusion of palm oil in the oil blends. FFA Follow the peroxide number in the same direction BO stabilized the similar behavior seen in SO and SBO (Kumar et al., 2009).

4. Conclusions

Blending of palm oil with sunflower oil and soybean oil to form binary blends led to the enhancement of oxidative stability of sunflower and soybean oil. The best binary blend was the blend which consists of 50% palm oil: 50% soybean

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Availability of data and materials

The current manuscript includes all of the data produced and examined for the study, and the associated authors have no objections regarding the data's and materials' accessibility.

Competing interests (Conflict of interest)

The authors assert that they have no competing interests.

Authors' contributions

N.A. R.: analyzed and interpreted the data of the work; **M. A. E.:** performed lab experiments, analyzed and interpreted the data of the work, and prepared the original manuscript; **S. M. A** supervised and reviewed the manuscript; **A. A. E.:** performed lab experiments, analyzed and interpreted the data of the work, and prepared the original manuscript; **M. F. A.:** performed lab experiments, analyzed and interpreted the data of the work, and prepared the original manuscript.

Funding

The authors received no external financial support for this study's practical part, authorship, and publication.

Acknowledgment

The authors gratefully thank Professor Doctor Valentina Pavlova - Professor of Chemistry and Biochemistry, Faculty of Technology and Technical Sciences, University St. Kliment Ohridski Bitola (UKLO), Republic of North Macedonia, Veles. Also, doctor Vesna Karapetkovska Hristova, Associate Professor of Biotechnology, Faculty of Biotechnical Sciences (FBS) – University "St. Kliment Ohridski" Bitola, Macedonia, for their help in the reviewing of the paper. Special thanks are also for the reviewers and the academic editor office for their exact reading and insightful comments that led to an improved manuscript.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **https://chimie-biologie.ubm.ro/carpathian_journal/index.html**

IMPACT OF USING SOME FOOD INDUSTRY WASTES ON COMPOSITION AND QUALITY OF PROCESSED CHEESE SPREAD

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https://doi.org/10.34302/crpjfst/2024.16.2.3

Article history:
Received: January 10th, 2024
Accepted: March 12th, 2024

Keywords:
Food wastes;
Processed cheese spread;
Composition;
Quality properties.

ABSTRACT

This study investigated the effect of using some food industry wastes such as broken rice (BR), broken pasta (BP), and broken faba bean (BFB) on the physicochemical, textural, microstructure, and sensory properties of processed cheese spread (PCS). The BR, BP, and BFB were converted into flour (BRF, BPF, and BFBF, respectively) and added to processed cheese formulas at levels of 5, 10 and 15%. The results showed an increase in the values of total solids, fat, protein, ash, carbohydrates, fiber, and acidity in the processed cheese by adding the obtained flours, and these values were increased with increasing the addition level. The PCS containing BFBF had the highest values of protein, ash, and fiber compared to all the other treatments. The texture parameters (hardness, adhesiveness, cohesiveness, gumminess and chewiness) were increased by increasing the rate of addition of BRF, BPF, and BFBF compared to the control treatment. Also, it was found that the BRF and BPF improved the microstructure properties of the PCS samples. The sensory evaluation results showed that the highest degree of acceptance was with samples made using BPF at levels of 10% and 15%., while with BRF and BFBF the most acceptable values were observed with an addition level of 10% compared to the control.

1.Introduction

Processed cheese (PC), in the most generic terms, is a blend of one or more natural cheeses of different ages, emulsifying salts, water, and other dairy and nondairy ingredients. The mixture undergoes heating and continuous agitation to produce a pasteurized product that is homogeneous and has an extended shelf life (Meyer 1973; Thomas 1973; Kapoor and Metzger 2008). It may be of interest to note That PC was invented in 1911 in Switzerland by Walter Gerber and Fritz Stelter and developed in the USA in 1916 by J. L. Kraft (Kapoor and Metzger, 2008). The earlier studies carried out between 1958 and 2015 on factors affecting the properties and quality of PC was recently reviewed by Abd El-Kader (2017).

In addition to the nutritional considerations of the components of these wastes, their functional properties, such as oil and water absorption, solubility, emulsification properties, and stability, and increases in yield also contribute significantly to the final quality of processed food products. The utilization of other non-dairy ingredients in processed cheese production has opened up a wide range of food products with enhanced functional properties. A wide range of processed cheeses with diverse textures and flavors can be produced thanks to the wide variety of ingredients that can be added to processed cheese blends. As a result, processed cheese can be eaten alone or used to make other dishes, such as snacks. These characteristics and features make processed cheese among the most innovative products in the dairy sector. Therefore, the processed cheese market is always in need of more innovations aimed at improving the nutritional value and

health benefits of the final product (Aly *et al.*, 2016) . All these substitutes including protein, fat and carbohydrate-based materials will lead to decrease production cost, provide flavour or texture, or improve the shelf life (Kapoor and Metzger, 2008). Relatively recent studies were done in Egypt to improve quality of market PC as well as to reduce cost of production via producing imitation or PC analogues. Such studies were reviewed-in details by Mehanna *et al.* (2016) and Dawoud (2021).

Recently, this trend has grown to provide nontraditional food additives that meet the need to fill the deficit in the quantities of food available for human consumption, and provide more diversity to the prevailing food dishes, and, on the other hand, contribute to maximizing the use of agricultural production residues (Rozan & Boriy, 2022) such as broken rice, broken pasta and broken faba bean (*Vicia faba*). However, more details about non-dairy ingredients from variety of plants and their use in dairy products were recently reviewed by Makinen *et al.* (2016); Tangyu *et al.* (2019).

In this context, the use of food waste plays an important role in achieving sustainable development. Exploiting these wastes provides opportunities to reduce waste, improve resource utilization, and promote the circular economy (Zhu *et al.* 2022). Food waste is materials resulting from some food production processes. These wastes are often neglected or used as animal feed. The types of food industry waste vary depending on the type of food industry involved in the process. The food industry generates large amounts of wastes, which is often overlooked as a valuable resource. There is a growing interest in the importance of recycling these wastes and reintegrating them into the value chain to achieve sustainable development principles. Among these wastes are broken rice, broken pasta, and broken beans.

Broken or ground rice refers to the fragments of rice grain obtained by milling. This product is separated after the polishing phase and has the same chemical composition of white rice, and its use is common in animal fodder. Broken rice is rejected by the consumer market and, for the most part, is intended for animal feed, for brewing industry, fertilizer etc. Forms of use of this product generated during rice processing, aiming to add value to it, is necessary since the incorporation of these in food formulations could solve the great waste arising from rice processing, and become an alternative form of income. According to Tavares *et al.* (2016).

Rice like cereals is carbohydrate-rich food. Carbohydrates of rice are predominantly starch with small portions of pentosans, hemicelluloses and sugars. The second most abundant constituent is protein and the major protein fractions are glutenin, albumin, globulin, and prolamin. Rice protein has one of the highest nutritive values among cereal proteins because of its lysine content (Bandyopadhyay & Roy, 1992). The nutritional level of rice is high among cereals and other grains and it has a comparatively high content of essential amino acids with high total digestibility of protein (Pillaiyer, 1988).

Broken pasta refers to the fragments of pasta obtained during the manufacturing process. This product is separated before the packing process phase and has the same chemical composition as Pasta. Pasta is one of the most important foods consumed around the world due to its relatively low cost and desirable organoleptic properties (Oyeyinka *et al.*, 2021) . Pasta is a rich source of carbohydrates and an acceptable source of vegetable protein (Oyeyinka *et al.*, 2021).

Broken Faba beans are known to be a potent resource of protein, and are commonly used in animal feed. They are widely known as 'Poor man's meat', the main plant source of proteins in the human diet. They are also generally rich in dietary fiber and carbohydrates (Rochfort & Panozzo., 2007).

Furthermore, grain legumes contain antioxidants and other bioactive compounds that can contribute to human health (Ganesan & Xu.,2017). Several health benefits have been proposed in relation to the consumption of grain legumes, including reduced risk of colorectal cancer (Aune *et al.*, 2011), improvement of gut health, and reduced blood cholesterol levels (Clemente & Olias, 2017).

The objective of the present study was to use broken rice, broken pasta and broken faba bean

in making processed cheese spread. The composition and quality of the prepared processed cheese were taken into consideration hoping to reduce the cost of the production by replacing part of the natural cheese by the above-mentioned materials.

2. Materials and methods

2.1. Raw Materials

Ras cheese was manufactured using fresh cow's milk as the procedure of Hofi *et al.*, (1970) in Food Technology Research Institute, Agricultural Research Center, Giza, Egypt. While matured Cheddar cheese (8 months old), was purchased from the local market in Giza, Egypt. Broken rice (BR), broken pasta (BP), and broken faba bean (BFB) were also purchased from the local market and converted into flour form according the procedure of Awad (2007) using laboratory mill (National, Japan), and then sieved through a 0.1mm mesh sieve. The resultant flours were packed in polyethylene bags and stored in the refrigerator (4±1°C) until use. The chemical composition of raw materials is shown in Table (1).

Table 1. Chemical composition of raw materials used in the manufacture of processed cheese spreads

Composition (%)	Ras Cheese	Cheddar Cheese	Butter	(BRF)	(BPF)	(BFBF)
Total solids	54.22	65.70	85.00	90.38	92.29	97.08
Fat	25.56	34.80	82.22	0.20	1.52	1.68
Protein	22.12[1]	25.77[1]	ND[2]	7.78[3]	10.69[4]	29.12[5]
Ash	4.73	5.03	ND[2]	0.66	0.99	3.47
Carbohydrate	1.81	0.10	ND[2]	81.53	78.56	50.07
Fiber	ND[2]	ND[2]	ND[2]	0.21	0.50	3.90

BRF: broken rice flour; BPF: broken pasta flour; BFBF: broken faba bean flour.
1: Protein% = N × 6.38 2: Not determined 3: Protein% = N × 5.17
4: Protein% = N × 5.33 5: Protein% = N × 5.52.

2.2. Methods

2.2.1. Manufacture of processed cheese spreads (PCSs)

Processed cheeses spread (PCSs) were manufactured according to the method of Meyer (1973). Control treatment samples were prepared using fresh Ras cheese and mature Cheddar cheese as a base blend. Meanwhile, other processed cheese treatments (BRF, BPF, and BFBF) were manufactured by replacing the natural cheese in the base blend with broken rice flour, broken pasta flour and broken faba beans flour at ratios of 5, 10 and 15%. The different formulations used to prepare processed cheese are shown in Table 2. Three replicates were carried out from each treatment. The composition of each batch of final processed cheese treatments was adjusted to 55-58 % moisture, 45-50 % fat /dry matter, and the pH value was between 5.6 – 5.9. Simultaneously required amount of emulsifying salts (2.5 %), butter and water were added and mixed using ultra turrax homogenizer for 5 – 10 min on 10 par and heated for a final temperature of 82°C in approximately 4 min then filled into plastic containers (120g) and rapidly cooled at 7 ±1°C. The final products were stored in refrigerator at 4±1°C for 3 months, and all treatments samples were analyzed for physicochemical composition, texture properties, and sensory evaluation at 0, 1, 2, and 3 months of cold storage.

2.2.2. Physicochemical analysis.

The chemical analysis (total solids (%), protein (%), fat (%) and ash (%)) of PCSs samples were tested for and was carryout according to the AOAC procedure (AOAC 2005) Total carbohydrates were calculated by differences as described, whereas fiber content was also determined (AOAC 1990). The acidity (%) of cheese was determined according to the method of Ling (1963), and pH values were measured using a digital laboratory pH meter

(HI 93 1400, Hanna instruments) with a glass electrode.

Table 2. Composition of different blend formulas (Kg/100Kg) used in manufacture of spreads processed cheese (PCSs) with different ratios of broken rice flour, broken pasta flour and broken faba bean flour in the base bland

Ingredients (%)	Control	BRF			BPF			BFBF		
		5 %	10 %	15 %	5 %	10 %	15 %	5 %	10 %	15 %
Ras cheese	38.44	37.44	35.32	33.56	37.44	35.32	33.56	37.44	35.32	33.56
Cheddar cheese	12.80	11.25	10.80	10.00	11.25	10.80	10.00	11.25	10.80	10.00
Butter	10.26	10.26	10.26	10.26	10.26	10.26	10.26	10.26	10.26	10.26
Emulsifying salts	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Food wastes	-	2.55	5.12	7.68	2.55	5.12	7.68	2.55	5.12	7.68
Salt	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Water	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00
Total	100	100	100	100	100	100	100	100	100	100

BRF: broken rice flour; BPF: broken pasta flour; BFBF: broken faba bean flour.

2.2.3. Oil separation index

Oil separation was determined according to the method outlined by Thomas (1973) as follow:

A cork borer was used to obtain cylindrical sample of processed cheese approximately 17.0 × 17.0 mm. the sample was pressed gently between tow sheets of Whatman No. 41 filter paper and incubated at 45 ºC for two hours. The diameter of the spread oil was measured in mm and was used as an index of oil separation according to the following equation

$$OSI = (D2- D1) / D1 \times 100$$

Where:

OSI: Oil separation index

D1: Diameter of cheese fat before heating

D2: Diameter of cheese fat after heating

2.2.4. Meltability

Meltability of cheese was measured in duplicate by using the melting test as described by Olson and Price (1958) with the modification of Rayan *et al.* (1980). A cylinder of cheese sample (15±0.2g) was put in a Pyrex glass tube, 30 mm in diameter and 250 mm long and a reference line was marked on the tube aligned with the front edge of the cheese sample. The tube was immediately placed in horizontal position in an oven at 110ºC for 30 mins. The distance of flow from the reference line to the leading edge of the melted cheese was quickly measured and recorded in mm as cheese flow or as cheese meltability.

2.2.5. Texture profile analysis (TPA)

Texture properties of PCSs samples were determined at 23°C as described by Bourne (1982) using an Instron Universal Testing Machine model 1195, Stable Micro System (SMS) Ltd., Godalming, UK, loaded with Dimension Software SMS program.

2.2.6. Microstructure of Processed Cheese

The microstructure of the processed cheese was evaluated by scanning electron microscopy according to the method of Tahmasebi *et al.*, (2015). Small pieces of fresh specimens of processed cheese samples were removed and fixed by immersing them immediately in 4F1G (fixative, phosphate buffer solution), pH 7.4 at 4°C for 3 hours. Specimens were then postfixed in 2% OsO_4 in the same buffer at 4°C for 2 hours. Samples were washed in the buffer and dehydrate at 4°C through a graded series of ethanol. Samples of processed cheese were dried by means of a critical point method, mounted using carbon paste on an AL-stub and coated

with gold up to a thickness of 400 A in a sputter-coating unit (JFC-1100E). The observation of processed cheese morphology in the coded specimens was performed in a JEOL JSM-IT200 scanning electron microscope operated between 15 and 20 KeV, and an irradiation current of 10 μm.

2.2.7. Organoleptic assessment

The organoleptic properties of PCS samples were evaluated by 15 regular panelists of the staff members at the Dairy Department, Al-Azhar University, and Dairy Department, Food Technology Research Institute, Agricultural Research Center according to the scheme of Meyer (1973).

2.2.8. Statistical analysis

All the data were statistically analyzed by the SPSS statistical software using one-way ANOVA. Analysis of variance and Duncan's test as well as average were carried out using SPSS computer program (SPSS, 2016; version 24) at $P \leq 0.05$.

3.Results and discussions

3.1. Chemical composition of PCSs

The changes in the gross chemical composition of PCSs made with replacement of the natural cheese in the base blend with BRF, BPF and BFBF (at 5, 10 and 15%) are shown in Table (3). The results showed that there were noticeable differences in the total solids and fat contents of PCSs due to applied replacements. These differences may be due to differences in the quantity and chemical composition of the materials used in the manufacture. The contents of total protein, ash and fiber of the treated PCSs with BFBF were significantly higher comparing with the other treatments and the control cheese. As well known, the dairy ingredients used never contain any dietary fiber, so the added food waste (FW) is considered a source of dietary fiber therefore thus a gradual increase in the proportion of (FW) was associated with a significantly proportional increase in the proportion of fiber in the treated PCSs Omar *et al.* (2012).

The contents of total protein, ash and fiber of the PCSs were significantly higher in PCS with BFBF compared with the other treatments and the control, and increased with increasing the proportion of replacement BFBF in the blend. This was expected and could be due to the chemical composition of broken BFBF this result agrees with Omar *et al.* (2012).

On the other hand, carbohydrate content in PCS was increased with an increase in the proportion of substituted BRF in the mixture compared with the other treatments and the control cheese due to the chemical composition of broken rice and its high carbohydrate content (Bandyopadhyay & Roy, 1992).

3.2 Acidity and pH values:

Fig. (1) shows that the acidity values of the control cheese samples were almost less than those of the treated samples. This was true in fresh and stored cheese. However, slightly higher values were recorded in case of using broken pasta flour in fresh cheese comparing with the control or the other food wastes used, but in all cases the higher was the ratio of the food waste used, the lower was the acidity value. Such changes were almost insignificant ($P>0.05$), while storage of cheese samples had significant impact ($P \leq 0.05$) on increasing the acidity values.

Data of pH shown in Fig. (2) revealed that the control sample had almost higher pH value than the treated cheese and this was recorded in case of fresh and stored cheese samples. On the other hand, the higher was the amount of the food waste, the lower were the pH values. Relatively lower pH values were recorded in case of using the broken beans. However, significant higher values were noticed in fresh cheese samples suggesting cold storage had decreasing impact in this respect. Such impact was significant in many cases and could be attributed to decomposition of protein and lactose during storage. The attained results are in general with those given in the literature by Tamime *et al.* (1990); Younis *et al.* (1991); Aly *et al.* (1995), Chambre and Daurelles (2000), Abdel-Hamid *et al.* (2000, a); Awad (2003); Awad *et al.* (2003) and Tohamy *et al.* (2018).

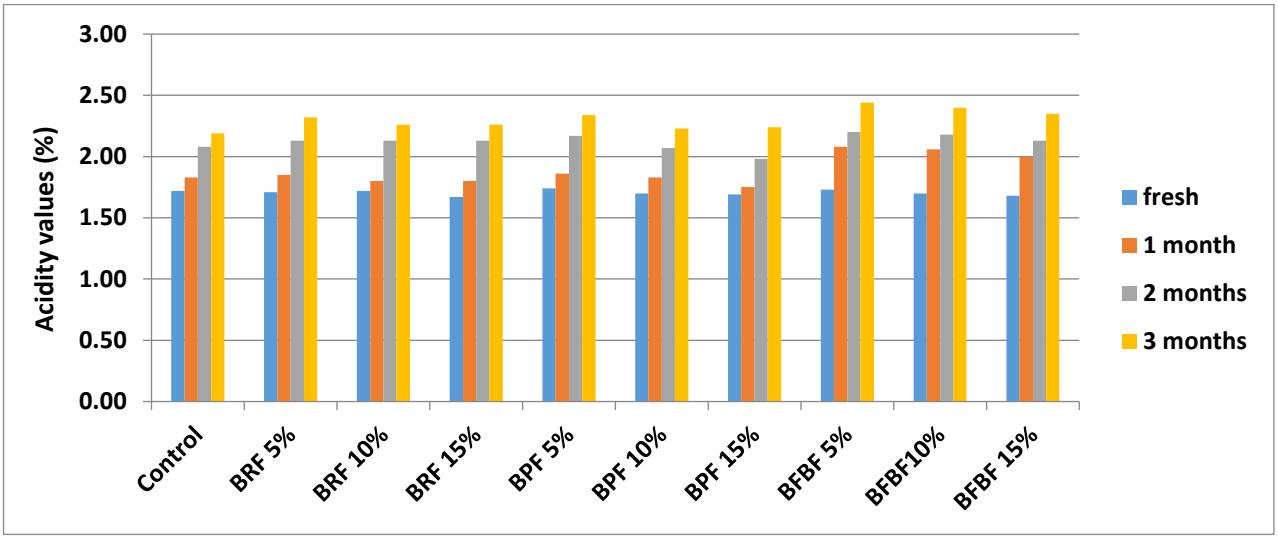


Figure 1. Changes in the acidity (%) values of processed cheese spreads supplemented with different ratios of BRF, BPF, and BFBF during cold storage (4°C) for 3 months.

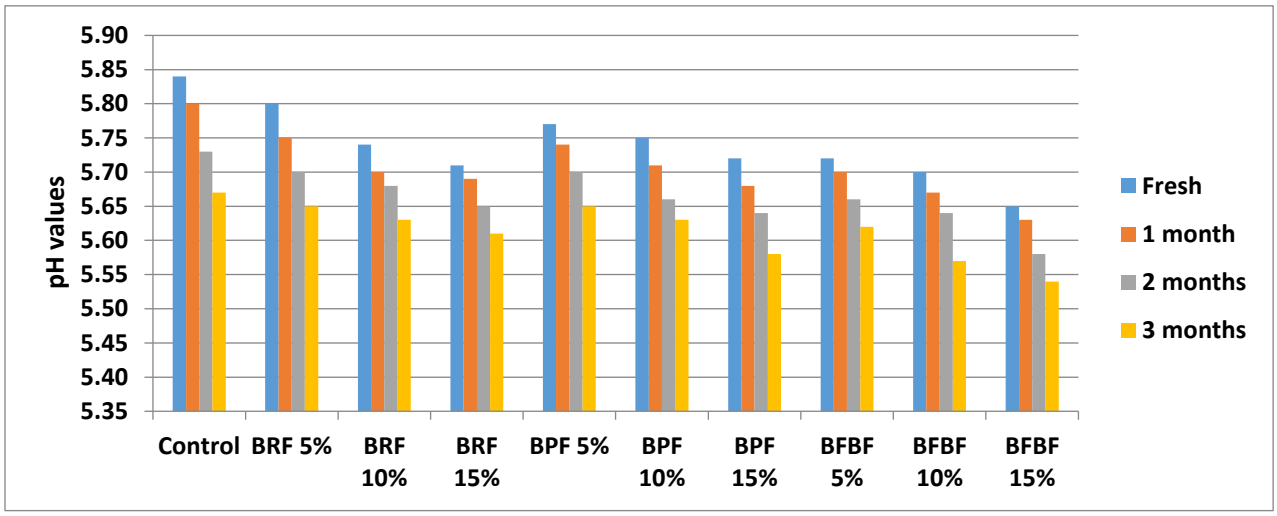


Figure 2. Changes in the pH values of processed cheese spreads supplemented with different ratios of BRF, BPF, and BFBF during cold storage (4°C) for 3 months.

Table (3). Chemical composition of processed cheese spreads (PCSs) made with different ratios of BRF, BPF, and BFBF during cold storage at 4 °C for 3 months.

Parameters	Storage (month)	Control	BRF			BPF			BFBF		
			5%	10%	15%	5%	10%	15%	5%	10%	15%
Total solids (%)	0	45.55±0.126^{fD}	46.02±0.185^{deC}	46.21±0.210^{cdC}	46.84±0.165^{bD}	45.76±0.185^{efC}	46.35±0.095^{cC}	46.99±0.100^{bD}	47.37±0.135^{aC}	47.46±0.135^{aC}	47.56±0.220^{aC}
	1	45.71±0.010^{fC}	46.19±0.055^{dC}	46.32±0.045^{dC}	47.04±0.080^{bC}	45.92±0.020^{eC}	46.56±0.13^{cB}	47.19±0.035^{bC}	47.48±0.240^{aC}	47.58±0.035^{aC}	47.65±0.060^{aC}
	2	46.27±0.040^{fB}	46.64±0.040^{eB}	46.70±0.075^{eB}	47.31±0.030^{dB}	46.30±0.055^{fB}	46.71±0.030^{eB}	47.61±0.045^{cB}	47.930.045±^{bB}	48.00±0.020^{bB}	48.11±0.045^{aB}
	3	46.67±0.015^{gA}	47.10±0.010^{fA}	47.14±0.005^{fA}	47.77±0.025^{eA}	46.70±0.020^{gA}	47.10±0.010^{fA}	48.06±0.020^{dA}	48.28±0.045^{cA}	48.38±0.020^{bA}	48.45±0.015^{aA}
Fat (%)	0	23.51±0.010^{bD}	23.48±0.040^{bC}	23.49±0.035^{bC}	23.51±0.040^{bC}	23.58±0.015^{aC}	23.60±0.005^{aD}	23.62±0.005^{aD}	23.50±0.040^{bC}	23.51±0.035^{bC}	23.52±0.060^{bC}
	1	23.56±0.010^{bC}	23.52±0.040^{bC}	23.54±0.035^{bC}	23.54±0.040^{bC}	23.63±0.015^{aC}	23.67±0.005^{aC}	23.68±0.005^{aC}	23.54±0.040^{bC}	23.55±0.035^{bC}	23.57±0.060^{bC}
	2	23.62±0.035^{dB}	23.62±0.045^{dB}	23.63±0.040^{cdB}	23.68±0.040^{bcdB}	23.70±0.045^{bcB}	23.74±0.025^{bB}	23.82±0.040^{aB}	23.64±0.020^{cdB}	23.68±0.045^{bcdB}	23.70±0.045^{bcB}
	3	23.78±0.025^{eA}	23.89±0.010^{cA}	23.90±0.015^{cA}	23.94±0.010^{bA}	23.93±0.020^{bcA}	23.96±0.030^{bA}	24.12±0.015^{aA}	23.81±0.020^{deA}	23.83±0.025^{dA}	23.85±0.045^{dA}
Protein (%)	0	14.87±0.020^{bC}	13.68±0.035^{dB}	13.71±0.040^{dB}	13.73±0.040^{dC}	13.78±0.015^{cC}	13.80±0.010^{cC}	13.82±0.010^{cB}	15.34±0.045^{aC}	15.37±0.025^{aC}	15.39±0.030^{aC}
	1	14.94±0.045^{bC}	13.70±0.055^{dB}	13.73±0.035^{dB}	13.79±0.040^{dC}	13.85±0.040^{cBC}	13.87±0.55^{cBC}	13.89±0.45^{cB}	15.37±0.025^{aC}	15.39±0.070^{aC}	15.42±0.060^{aC}
	2	15.20±0.055^{bB}	13.83±0.065^{eA}	13.85±0.040^{deA}	13.87±0.030^{deB}	13.89±0.055^{deAB}	13.92±0.045^{dAB}	14.10±0.060^{cA}	15.57±0.010^{aB}	15.58±0.045^{aB}	15.62±0.040^{aB}
	3	15.30±0.020^{cA}	13.89±0.020^{gA}	13.91±0.015^{fgA}	13.97±0.025^{eA}	13.95±0.025^{efA}	13.98±0.015^{eA}	14.05±0.040^{dA}	15.68±0.025^{bA}	15.70±0.035^{abA}	15.73±0.025^{aA}
Ash (%)	0	3.77±0.045^{cB}	4.02±0.040^{bB}	4.09±0.010^{aB}	4.10±0.030^{aB}	4.02±0.045^{bB}	4.10±0.020^{aA}	4.12±0.035^{aB}	4.13±0.035^{aB}	4.15±0.030^{aB}	4.15±0.045^{aB}
	1	3.79±0.035^{dB}	4.04±0.065^{cB}	4.10±0.55^{bdB}	4.12±0.040^{abcB}	4.04±0.025^{cB}	4.12±0.55^{abcA}	4.15±0.045^{abB}	4.14±0.025^{abB}	4.18±0.025^{abAB}	4.19±0.035^{aAB}
	2	3.90±0.040^{dA}	4.13±0.020^{abcA}	4.14±0.020^{abcA}	4.14±0.020^{abcB}	4.10±0.085^{cAB}	4.12±0.025^{bcA}	4.18±0.010^{abB}	4.17±0.025^{abAB}	4.18±0.025^{abAB}	4.20±0.025^{aAB}
	3	3.96±0.030^{eA}	4.18±0.025^{cdA}	4.18±0.020^{cdA}	4.20±0.025^{bcA}	4.15±0.020^{dA}	4.17±0.025^{cdA}	4.24±0.025^{aA}	4.20±0.025^{bcA}	4.23±0.025^{abA}	4.25±0.020^{aA}
Carbohydrate (%)	0	3.40±0.060^{dB}	4.72±0.040^{bC}	4.78±0.090^{bB}	5.30±0.030^{aB}	4.24±0.085^{cB}	4.70±0.020^{bB}	5.22±0.005^{aB}	4.17±0.010^{cB}	4.19±0.020^{cB}	4.26±0.040^{cB}
	1	3.42±0.100^{dB}	4.78±0.105^{bBC}	4.80±0.07^{bAB}	5.38±0.040^{aB}	4.25±0.040^{cB}	4.74±0.055^{bAB}	5.25±0.075^{aB}	4.19±0.025^{cB}	4.20±0.075^{cB}	4.28±0.035^{cAB}
	2	3.55±0.035^{gA}	4.87±0.055^{cAB}	4.89±0.065^{cAB}	5.40±0.005^{aA}	4.45±0.020^{eA}	4.75±0.045^{dAB}	5.28±0.010^{bB}	4.27±0.025^{fA}	4.28±0.015^{fA}	4.30±0.025^{fAB}
	3	3.63±0.035^{fA}	4.92±0.20^{bA}	4.93±0.20^{bA}	5.41±0.40^{aA}	4.47±0.20^{dA}	4.79±0.30^{cA}	5.42±0.40^{aA}	4.29±0.40^{eA}	4.31±0.30^{eA}	4.33±0.25^{eA}
Fiber (%)	0	0.00	0.12±0.030^{bC}	0.14±0.035^{bB}	0.20±0.025^{aA}	0.14±0.025^{bB}	0.15±0.040^{bA}	0.21±0.045^{aA}	0.23±0.005^{aB}	0.24±0.025^{aA}	0.24±0.045^{aA}
	1	0.00	0.15±0.015^{bBC}	0.15±0.020^{bB}	0.22±0.015^{abA}	0.15±0.005^{bB}	0.16±0.030^{bA}	0.22±0.040^{abA}	0.24±0.055^{aB}	0.26±0.065^{aA}	0.28±0.080^{aA}
	2	0.00	0.19±0.045^{cdAB}	0.19±0.045^{cdAB}	0.21±0.025^{cdA}	0.16±0.015^{dB}	0.18±0.025^{cdA}	0.23±0.035^{bcA}	0.28±0.010^{abAB}	0.28±0.010^{abA}	0.29±0.055^{aA}
	3	0.00	0.22±0.030^{bcA}	0.22±0.010^{bcA}	0.25±0.025^{bA}	0.20±0.020^{dA}	0.20±0.005^{dA}	0.23±0.35^{bcA}	0.30±0.20^{aA}	0.31±0.040^{aA}	0.31±0.005^{aA}

* Means ±SD, means with the different small letters within the same row and means with different capital letters within the same column are significantly different (P≤ 0.05).
BRF: broken rice flour; BPF: broken pasta flour; BFBF: broken faba bean flour.

Table (4). Oil separation index (%) values of processed cheese spreads made with different ratios of the broken materials used

Storage period	Control	BRF			BPF			BFBF		
		5 %	10 %	15 %	5 %	10 %	15 %	5 %	10 %	15 %
Fresh	39.89±0.60^{cC}	40.80±0.51^{cD}	40.80±1.27^{cC}	42.55±0.66^{bC}	42.55±0.02^{bD}	43.20±0.66^{bD}	46.23±0.33^{aD}	40.73±0.03^{cD}	40.51±0.72^{cD}	43.21±0.11^{bC}
1 month	43.11±0.11^{dB}	42.93±0.52^{dC}	44.38±0.04^{cB}	45.42±0.01^{bB}	44.18±0.08^{cC}	45.32±0.02^{bC}	48.29±0.02^{aC}	43.31±0.03^{dC}	43.31±0.60^{cC}	45.20±0.03^{bB}
2 months	43.8±0.61^{dB}	45.12±0.04^{cB}	45.50±0.06^{cAB}	46.56±0.08^{bA}	46.67±0.01^{bB}	46.64±0.53^{bB}	49.31±0.03^{aB}	44.21±0.02^{dB}	45.27±0.04^{cB}	46.95±0.61^{bA}
3 months	46.28±0.04^{eA}	46.17±0.04^{eA}	46.52±0.02^{eA}	46.58±0.53^{eA}	47.72±0.61^{cA}	48.84±0.04^{bA}	52.20±0.02^{aA}	46.75±0.04^{deA}	47.60±0.51^{cdA}	47.60±0.52^{cA}

* Means ±SD, means with the different small letters within the same row and means with different capital letters within the same column are significantly different (P≤ 0.05). BRF: broken rice flour; BPF: broken pasta flour; BFBF: broken faba bean flour.

Table (5). Meltability (mm) values of processed cheese spreads made with different ratios of the broken materials used

Storage period	Control	BRF			BPF			BFBF		
		5 %	10 %	15 %	5 %	10 %	15 %	5 %	10 %	15 %
Fresh	128±1.00^{aA}	126±0.00^{aA}	126±2.00^{aA}	125±3.00^{abA}	126±1.00^{aA}	125±2.00^{abA}	120±1.00^{cA}	121±2.00^{bcA}	118±4.00^{cA}	117±4.00^{cA}
1 month	125±1.00^{aB}	124±1.00^{aA}	120±1.00^{bcB}	120±1.00^{bcB}	121±1.00^{bB}	120±1.00^{bcB}	119±1.00^{cA}	120±1.0^{bcAB}	117±1.00^{dAB}	116±1.00^{dA}
2 months	123±1.00^{aC}	123±3.00^{aA}	120±1.00^{abB}	117±3.00^{bB}	118±1.00^{bC}	113±1.00^{cC}	113±1.00^{cB}	118±1.00^{bB}	113±2.00^{cB}	111±1.00^{cB}
3 months	120±1.00^{aD}	119±1.00^{aB}	113±1.00^{bC}	111±1.00^{bcC}	112±2.00^{bcD}	110±1.00^{cD}	107±2.00^{dC}	113±1.00^{bC}	107±2.00^{dC}	104±1.00^{eC}

* Means ±SD, means with the different small letters within the same row and means with different capital letters within the same column are significantly different (P≤ 0.05). BRF: broken rice flour; BPF: broken pasta flour; BFBF: broken faba bean flour.

3.3. Oil separation index and Meltability

The oil separation indicator is a defect described for PCS. Separated oil deteriorates quickly due to exposure to oxidation The data presented in Table (4) indicate that the control cheese sample had the lowest fat separation index 39.89 %when it was fresh compared to the treatments containing broken (rice, pasta, and faba beans). While, samples of processed cheese treated with (BPF) at a level of 15% showed the highest index of oil separation 46.23 and 52.20% respectively, when it was fresh and at the end of the storage period. While samples of PCS treated with (BFBF) at a level of 5% recorded the lowest index of fat separation 40.73 and 44.21% respectively, when it was fresh and at the end of the storage period. That can be mainly attributed to the protein nature of those residues which may affect the degree of emulsification of the product.

The value of the oil index depends on the fat and protein status of the resulting processed cheese emulsion. In general, the values of the oil separation index gradually increased in all treatments including control cheese with the increasing replacement ratio and also with the progress storage period of processed cheese. This may be due to the changes in pH and soluble nitrogen content along with the melting salt-protein interactions could be the main contributors to increasing the oil separation. Similar results were obtained by Awad and Salama (2010 a,b) and Tawfek (2018).

Meltability is the capacity of cheese particles to flow together and form a uniform continuous melt. Melting is an important character which use to determine the resistance of processed cheese against changes of temperature during transportation and storing. Therefore, as meltability decreased the quality of processed cheese improved (Abbas, 2003). The results in Table (5) showed that the meltability values of the treated PCS decreased in proportion to the increase the percentages of using BRF, BPF and BFBF compared to the control cheese samples. Reducing the meltability by adding these broken materials in PCS can be attributed to increasing the hardness of the resulting cheese samples, which is consistent with El-Shibiny *et al.* (2013). Combined with the cold storage period.

After 60 days of cold storage the meltability values were significantly ($P \leq 0.05$) decreased in all treatments comparing with the fresh and the control cheese samples. Cold storage led to decrease in meltability of PCS because of the changes occurred in chemical properties of processed cheese such as pH, protein state, and product setting (Olson and Price, (1958); Abd El-Salam *et al.*, (1996); Abd El-Hamid *et al.*, (2000 b); Awad *et al.*, (2003, 2004); Mohamed (2004); Awad and Salama, (2010 a, b).

3.4. Texture profile analysis (TPA)

Texture profile analysis (TPA) is affected by several factors such as pH value, state of protein network, fat and moisture content. The results given in Table (6) showed that the firmness values of all treatments significantly ($P \leq 0.05$) increased during the storage period (4±1°C for 3 months). These findings concur with those of El-Sayed, et al (2020) who discovered that 120 days of storage at 4 °C resulted in a significant ($P<0.05$) increase in the hardness of UHT-processed cheese. The increase in firmness of processed cheese is associated with a decrease in its moisture content. Cheese made with broken faba beans had higher values for hardness, adhesiveness, cohesiveness and chewiness than control. This was true in fresh and stored cheese samples and the values gradually increased with increasing the amount of the BFBF added. The results illustrated that the values of all the rheological properties tested except springiness in all processed cheese samples were significantly ($P \leq 0.05$) increased during cold storage, these results are consistent with the observations of Cunha, *et al.* (2010).

This means that in all processed cheese samples springiness values gradually decreased with increasing the amount of broken food wastes added from one side and with advancing storage period from the other-side. The decrease in moisture content and increase of DM% especially protein content may by the reason for the increase in the hardness of cheese. This result agrees with Awad *et al.*, (2006).

Moreover, the increase in hardness of stored cheese may be due to the decrease in moisture content and less availability of water during storage, and consequently change of texture properties.

The functional and physical properties of cheese samples are significantly impacted by the composition of the processed cheese, which can change depending on differences in moisture content, pH, and protein content (Lee and Anema 2009).

In this respect, Mehanna, et al (2020) mentioned that the rheological properties of PCS were affected by the composition of the blends used since the lowest values of hardness, gumminess, springiness, cohesiveness, chewiness, and adhesiveness were recorded when PCS was made from blends containing the highest amount of Ras cheese (20%) and the lowest amount of SMP (5.5%).

3.5. Microstructure of Processed Cheese

Scanning electron micrographs (Fig. 3) show the protein network structure and fat globules sizes of the processed cheese samples containing rice (BRF), pasta (BPF), and beans (BFBF) flour in comparison with the control sample. The results showed that there were significant differences in protein network structure and the distribution of fat globules in processed cheese containing rice, pasta, and beans flour, comparing with the control.

In the BRF treatment samples, the protein and fat network appeared swollen, and densely packed, and the texture was more homogeneous than the control, and there were some fat-free globules. This swelling and appeared homogeneity may be due to the presence of a large amount of starch in the rice flour, which swells and increases in size after absorbing water.

Starch also plays role as stabilizer in processed cheese, which improves the texture characteristics. In the case of processed cheese containing pasta flour (BPF), the results showed good emulsification of fat similar to that in the treatment BRF, but the protein network structure was different, as it appeared in the form of inhomogeneous threads and agglomerates. As for the processed cheese samples containing bean flour (BFBF), there was the most difference compared to the control or the rest of the other treatments. It was found that the microstructure of cheese

It may be important to reveal that in our study we used SEM in the microstructure of processed cheese made with 10% of the aforementioned food waste because this Ratio improved the sensory properties of the processed cheese as well as slightly improving the chemical composition without any adverse effect on the quality characteristics of PCSs, which was recorded with a ratio of 15%. Also, there were no significant differences between the PCSs made with a ratio of 5% and the control sample (C) in terms of the sensory acceptance characteristics for the cheese product.

3.6. Sensory evaluation of PCS samples:

Sensory evaluation is a scientific method used to evoke, measure, analyze, and interpret those responses to products as perceived through the senses of sight, hearing, touch, smell and taste (Stone and Sidel 1993; IFT 2007).

Scores of the different sensory attributes of PCS samples are shown in Fig.4. It seems from the attained results that the use of 5% of each of BRF, BPF and BFBF did not affect the flavour of PCS compared to the control sample, while an improvement in the flavor of the cheese was recorded when all food waste was used at levels of 10% and 15% except the BFBF the scoring points significantly decreased at level of 15% in fresh PCS and also during storage. Advancing cold storage period had no effect in this respect and this was true in the control and all treated PCS samples, except for the treatment with BFBF.

Table 6. Texture profile analysis (TPA) of processed cheese spreads made with different ratios of BRF, BPF, and BFBF during cold storage at 4 °C for 3 months.

Parameters	Storage Period (Month)	Control	BRF			BPF			BFBF		
			5%	10%	15%	5%	10%	15%	5%	10%	15%
Hardness (N)	0	2.16±0.045^{cD}	2.22±0.005^{cD}	2.33±0.020^{cC}	2.59±0.010^{bB}	2.18±0.015^{cD}	2.29±0.010^{cD}	2.53±0.010^{bD}	2.61±0.295^{bC}	3.34±0.120^{aC}	3.42±0.130^{aC}
	1	2.28±0.070^{cC}	2.31±0.055^{cC}	2.36±0.025^{cC}	2.71±0.045^{bB}	2.35±0.050^{cC}	2.37±0.020^{cC}	2.74±0.100^{bC}	2.82±0.160^{bB}	3.56±0.110^{aB}	3.66±0.115^{aB}
	2	2.40±0.060^{dB}	2.45±0.020^{dB}	2.51±0.025^{dB}	2.76±0.190^{cB}	2.51±0.035^{dB}	2.47±0.015^{dB}	3.08±0.105^{bB}	3.12±0.020^{bB}	3.69±0.005^{aB}	4.75±0.025^{aB}
	3	3.03±0.010^{dA}	3.07±0.025^{dA}	3.13±0.010^{cdA}	3.25±0.065^{cA}	3.11±0.010^{dA}	3.16±0.045^{cdA}	3.26±0.050^{cA}	3.65±0.205^{bA}	4.16±0.055^{aA}	4.20±0.015^{aA}
Adhesiveness (mJ)	0	1.105±0.010^{dD}	1.099±0.025^{dD}	1.113±0.011^{dD}	1.219±0.014^{bD}	1.123±0.020^{dD}	1.226±0.030^{bD}	1.239±0.035^{bD}	1.164±0.015^{cD}	1.244±0.040^{bD}	1.291±0.005^{aD}
	1	1.267±0.045^{eC}	1.282±0.006^{deC}	1.298±0.005^{deC}	1.311±0.010^{dC}	1.356±0.005^{cC}	1.372±0.015^{cC}	1.387±0.035^{cC}	1.316±0.005^{dC}	1.649±0.005^{bC}	1.716±0.025^{aC}
	2	1.200±0.020^{dB}	1.426±0.041^{dB}	1.463±0.020^{dB}	1.671±0.020^{cB}	1.459±0.030^{dB}	1.471±0.047^{dB}	1.667±0.058^{cB}	1.494±0.021^{dB}	1.825±0.030^{bB}	1.958±0.047^{aB}
	3	2.105±0.036^{eA}	2.250±0.052^{deA}	2.426±0.025^{cA}	2.936±0.041^{bA}	2.284±0.025^{dA}	2.263±0.047^{cA}	2.946±0.058^{bA}	2.283±0.030^{dA}	3.100±0.020^{aA}	3.163±0.015^{aA}
Cohesiveness (Ratio)	0	0.32±0.015^{fD}	0.44±0.015^{eD}	0.63±0.010^{cD}	0.69±0.010^{bD}	0.45±0.025^{eC}	0.62±0.010^{cC}	0.66±0.005^{bC}	0.59±0.015^{dD}	0.68±0.015^{bD}	0.77±0.015^{aD}
	1	0.47±0.015^{eC}	0.49±0.010^{eC}	0.66±0.20^{cC}	0.71±0.015^{bC}	0.50±0.040^{eC}	0.64±0.025^{cC}	0.66±0.040^{cC}	0.60±0.010^{cC}	0.71±0.010^{bC}	0.84±0.020^{aC}
	2	0.67±0.005^{eB}	0.68±0.005^{eB}	0.74±0.005^{dB}	0.85±0.010^{cB}	0.69±0.010^{eB}	0.72±0.025^{dB}	0.84±0.030^{cB}	0.73±0.005^{dB}	0.89±0.005^{bB}	0.94±0.015^{aB}
	3	0.76±0.010^{fA}	0.79±0.005^{eA}	0.87±0.020^{dA}	0.95±0.015^{bA}	0.82±0.020^{eA}	0.85±0.035^{dA}	0.91±0.040^{cA}	0.86±0.010^{dA}	0.95±0.005^{bA}	1.08±0.091^{aA}
Springiness (mm)	0	20.32±0.020^{aA}	20.29±0.010^{aA}	20.08±0.030^{bA}	19.99±0.100^{cA}	20.27±0.050^{aA}	20.05±0.040^{bcA}	19.98±0.010^{cA}	20.11±0.035^{bA}	19.84±0.025^{dA}	19.17±0.055^{eA}
	1	19.87±0.010^{aB}	19.82±0.030^{aB}	19.74±0.045^{bB}	19.60±0.045^{cB}	19.82±0.010^{aB}	19.71±0.045^{bB}	19.59±0.055^{cB}	19.57±0.045^{cB}	19.37±0.055^{dB}	18.88±0.035^{eB}
	2	19.31±0.010^{aC}	19.32±0.040^{aC}	19.29±0.025^{aC}	19.12±0.010^{bC}	19.28±0.025^{aC}	19.16±0.025^{bC}	19.12±0.015^{bC}	19.11±0.015^{bC}	18.96±0.020^{dC}	18.54±0.095^{eC}
	3	18.90±0.025^{aD}	18.88±0.035^{aD}	18.81±0.035^{aD}	18.54±0.090^{cD}	18.91±0.065^{aD}	18.65±0.110^{bD}	18.32±0.010^{dD}	18.58±0.055^{bcD}	18.26±0.055^{dD}	18.09±0.020^{eD}
Gumminess (N)	0	0.69±0.045^{eD}	0.97±0.025^{dD}	1.46±0.030^{cC}	1.78±0.040^{bC}	0.98±0.060^{dD}	1.41±0.060^{cC}	1.71±0.020^{bC}	1.43±0.035^{cC}	2.32±0.115^{aC}	2.34±0.035^{aC}
	1	1.03±0.015^{dC}	1.13±0.020^{dC}	1.55±0.120^{cC}	1.92±0.240^{bC}	1.17±0.020^{dC}	1.50±0.085^{cBC}	1.80±0.040^{bC}	1.58±0.055^{cC}	2.52±0.070^{aC}	2.64±0.025^{aC}
	2	1.60±0.025^{dB}	1.66±0.070^{dB}	2.24±0.030^{cB}	2.41±0.155^{cB}	1.73±0.130^{dB}	1.76±0.165^{dB}	2.78±0.200^{bB}	2.20±0.345^{cB}	3.28±0.410^{aB}	3.58±0.145^{aB}
	3	2.30±0.005^{eA}	2.42±0.110^{deA}	2.72±0.110^{dA}	3.07±0.115^{cA}	2.55±0.205^{deA}	2.68±0.225^{dA}	3.10±0.450^{cA}	3.13±0.080^{cA}	3.95±0.270^{bA}	4.53±0.305^{aA}
Chewiness (mJ)	0	2.21±0.010^{eD}	2.24±0.010^{eD}	2.33±0.025^{cdD}	2.40±0.005^{bD}	2.24±0.010^{eD}	2.32±0.010^{dD}	2.37±0.015^{bcD}	2.32±0.025^{dD}	2.51±0.020^{aD}	2.55±0.060^{aD}
	1	2.43±0.020^{eC}	2.44±0.025^{eC}	2.55±0.010^{dC}	2.61±0.040^{cC}	2.45±0.010^{eC}	2.55±0.020^{dC}	2.64±0.020^{cC}	2.51±0.025^{dC}	2.71±0.020^{bC}	2.84±0.010^{aC}
	2	2.27±0.020^{eB}	2.77±0.015^{eB}	2.87±0.020^{cdB}	2.89±0.020^{cB}	2.76±0.030^{eB}	2.89±0.010^{cB}	2.94±0.010^{bB}	2.83±0.005^{dB}	2.96±0.010^{bB}	3.05±0.060^{aB}
	3	2.94±0.015^{fA}	2.94±0.030^{fA}	3.07±0.005^{eA}	3.16±0.015^{dA}	2.96±0.010^{fA}	3.09±0.005^{eA}	3.21±0.010^{cA}	3.09±0.015^{eA}	3.28±0.010^{bA}	3.38±0.045^{aA}

* Means with the different small letters within the same row and means with different capital letters within the same column are significantly different ($P \leq 0.05$).
BRF: broken rice flour; BPF: broken pasta flour; BFBF: broken faba bean flour.

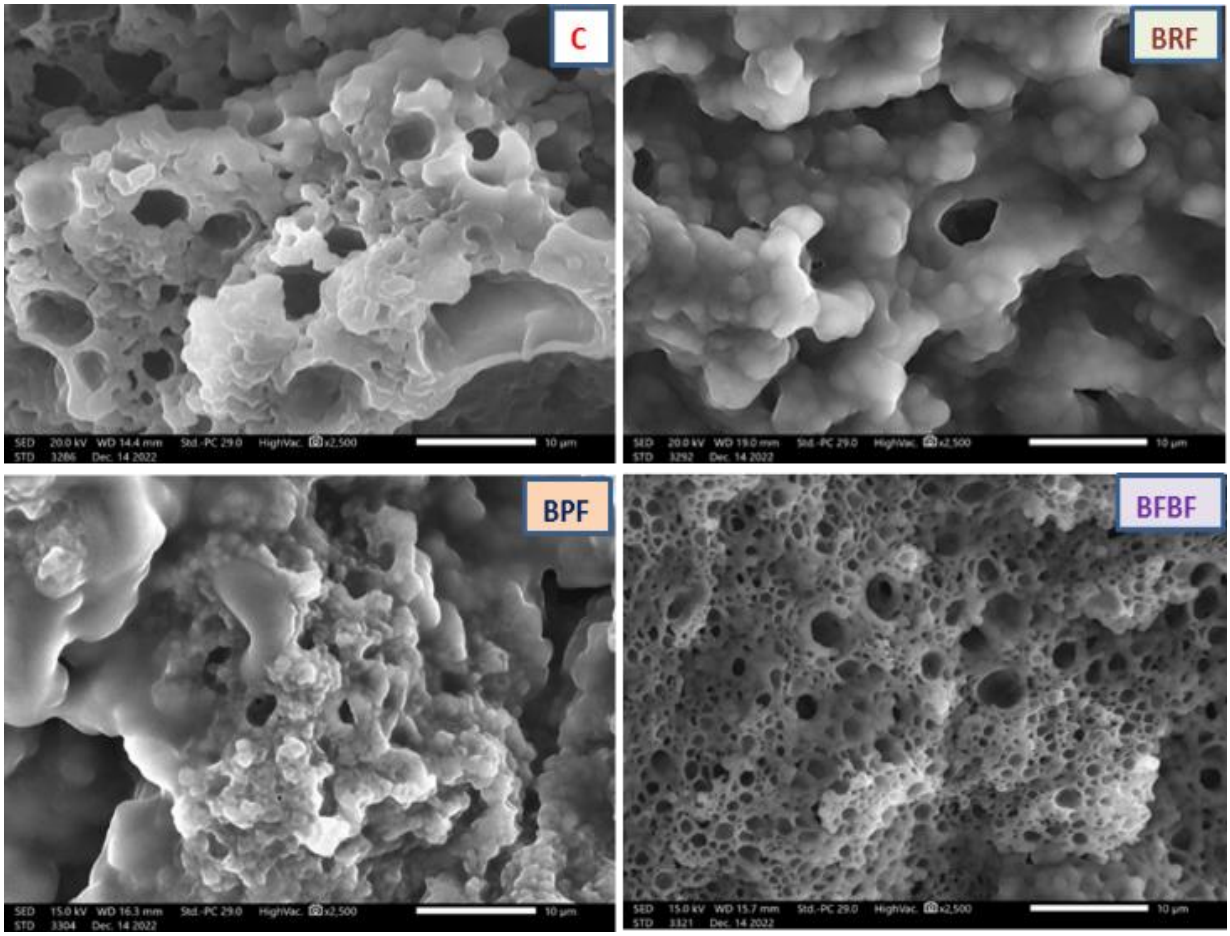
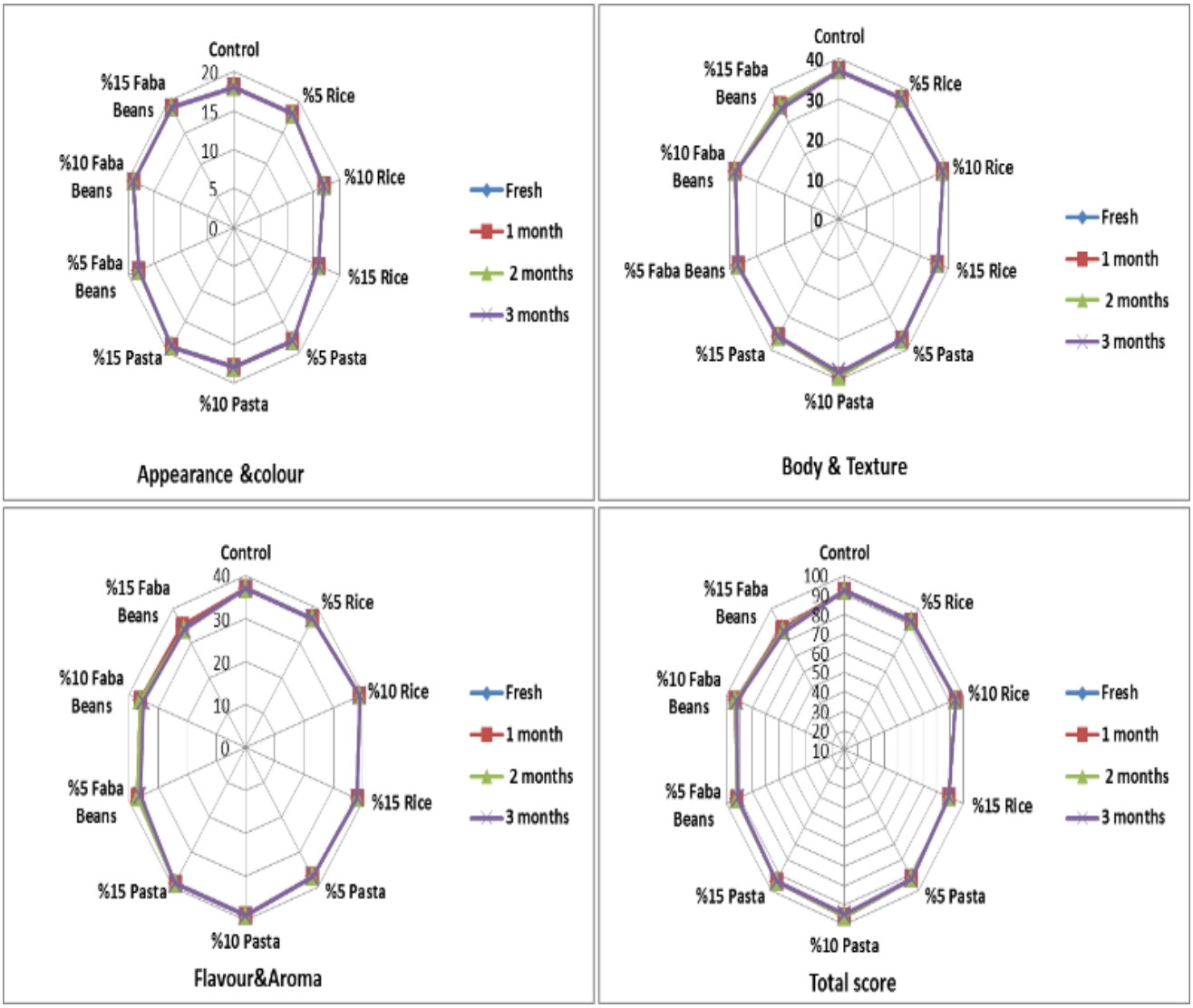


Figure 3. SEM image of microstructure of processed cheese. Control fresh processed cheese of young Ras and matured Cheddar cheeses as a base blend. C: Control; BRF: Processed cheese containing of 10 % broken rice flour; BPF: Processed cheese containing of 10 % broken pasta flour; BFBF: Processed cheese containing of 10 % broken faba bean flour.

Body and texture of fresh and stored PCS samples were no affected (P>0.05) by using all food wastes at level of 5%. Improvements in body and texture scores were recorded when all food waste was used at level of 10%. On the other hand, the body and texture properties were negatively affected for all samples of cheese produced using all food waste at the 15% level, recorded a score lower than the control PCS. There were no significant differences (P>0.05) in the appearance and color scores of the PCS samples from different treatments at level of 5%, and the control sample in fresh and stored cheese. The treated PCS with BFBF and BPF had the highest scores for appearance and colour being 19 out of 20 points at level of 10% and 15%, this was true in fresh and during storage period. Whereas the minimum points were given for the BRF treated PCS samples. The use of the BRF negatively (P≤0.05) affected the appearance and colour of cheese at levels above 5% and this was true both in fresh and also during three months of cold storage.

Figure 4. Organoleptic properties of processed cheese spreads made with different ratios of BRF, BPF, and BFBF during cold storage for 3 months.



4. Conclusion

Finally, this study cleared that processed cheese can be manufactured by replacing natural cheese in the processed cheese formula with BRF, BPF, and BFBF at ratios of 5 and 10 %. The utilization of BRF, BPF, and BFBF in the preparation of PCS caused significant changes in the physicochemical, textural, and microstructural properties of the final product. An addition ratio of 15% reduced the acceptability of some sensory properties in the resulting cheese, with the exception of BPF. This study recommended the use of BRF, BPF, and BFBF in the preparation of PCS at a ratio of 10 %. The decrease in the amount of natural cheese used in making processed cheese will in turn decrease the cost of production. This is quite important from an economic point of view. Moreover, the use of food industry waste in new products offers numerous benefits in terms of resource conservation, innovation, and consumer acceptance. By adopting these practices, the food industry can contribute significantly to achieving the Sustainable Development Goals.

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FATTY ACID PROFILE AND PROXIMATE COMPOSITION OF SIRLOIN AND CHUCK OF SELECTED ETHIOPIA CATTLE TYPES

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https://doi.org/10.34302/crpjfst/2024.16.2.4

Article history:
Received: January 22nd, 2024
Accepted: June 2nd, 2024

Keywords:
Fatty Acid Profile;
Beef;
Sirloin;
Chuck;
Principal component analysis.

ABSTRACT

The fatty acid composition of beef meat is important for nutrition and human health. This study examined the composition of sirloin and chuck of beef from three cattle breeds in Ethiopia (Boran, Senga, and Sheko). Twelve bulls aged 18 to 24 months were used, and standard methods were employed to measure various parameters. The results showed that moisture content ranged from 64.32±0.29% in Boran sirloin to 66.47±0.13% in Sheko sirloin, crude fat ranged from 10.79±0.36% in Sheko chuck to 13.25±0.38% in Boran chuck, and crude protein ranged from 21.65±0.50% in Senga sirloin to 26.83±0.78% in Boran chuck. The color evaluation revealed L* values of28.20±3.09 to 32.52±1.70 for senga chuck and Boran chuck, a* values of 5.18±0.88 to 9.35±2.96 for Boran sirloin and Senga chuck, and b* values of2.24±1.47 to 4.33±1.05 for Sheko sirloin and Senga sirloin. The dominant fatty acid was Palmitic acid (C16:0), comprising 24.64% to 31.60% of the total. The study found that the sirloin cut had significantly higher levels of monounsaturated fatty acids (42.38%) and lower levels of polyunsaturated fatty acids compared to the chuck cut. In conclusion, Sheko beef had higher moisture content, while Boran beef had higher levels of crude protein and fat compared to Senga and Sheko. Principal component analysis (PCA) identified fatty acid profiles as the main factors influencing variation among cattle breeds. This research provides valuable information for cattle breeding and meat quality improvement efforts in Ethiopia and beyond.

1.Introduction

Beef meat is known to contain a wide range of fatty acids, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) are only a few of the many fatty acids that are known to be exist in beef meat. The nutritional value of meat is significantly influenced by the amount and composition of fatty acids, which are a significant component of animal muscle (Xin Zhang, 2022). The quantity and kind of intramuscular fat and fatty acids have a considerable impact on the eating quality, sensory qualities (such as taste, tenderness, and flavor), as well as meat color, shelf life, and fat hardness in beef (Bhuiyan, 2018).

The fatty acid profile of beef meat plays a substantial role in the nutritional composition and has implications for human health. To evaluate the nutritional value and potential health impacts of beef meat, it is crucial to comprehend the composition and distribution of its fatty acids (Wood, 2008). Polyunsaturated fatty acids, comprising omega-3 (n-3) and omega-6 (n-6) fatty acids are essential for the body and cannot be produced internally, so they must be obtained through dietary sources

(Micha, 2012). Triglycerides from dietary sources and fatty acids make up the majority of the fat in meat animals (Dinh, 2021).

Additionally, about 30% of the fatty acid content in conventionally formed beef is made up of oleic acid (C18:1), a monounsaturated fatty acid (MUFA) that lowers cholesterol levels and other health benefits like lowering the risk of stroke and significantly lowering together systolic and diastolic blood pressure in vulnerable populations (Daley1, 2010). Due to their contribution to the odors of cooked meat, fatty acid content and the roles of each fatty acid in thermal oxidation during cooking are of interest (Dinh et al. 2021). Beef meat contains two important fatty acids called α- linolenic acid (α-LA) and linoleic acid (LA).

In comparison to beef from grain-fed cattle, grass-fed beef typically contains higher levels of monounsaturated fatty acids and polyunsaturated fatty acids, specifically omega-3 fatty acids (Daley1, 2010).

The fatty acid profile of beef meat has imperative implications for human health. Excessive intake of saturated fatty acids, particularly long-chain saturated fatty acids, has been related with an increased risk of cardiovascular diseases, such as coronary heart disease. While fatty fish and some plant sources are better suppliers of n-3 fatty acids than beef, grain-fed cattle may not have as much of these healthy fats as grass-fed beef (Simopoulos, 2002).

To the extent that we are aware, only a small number of publications have been found to evaluate beef from the Harar, Arsi, and Bale cattle breeds in Oromia, Ethiopia focusing on eating quality, as well as the instrumental tenderness of the meat. The proximate compositions and fatty acid profile of sirloin and chuck cuts have not yet been researched. Therefore, the purpose of this study was to evaluate the proximate composition and the fatty acid profile of sirloin and chuck meat cuts.

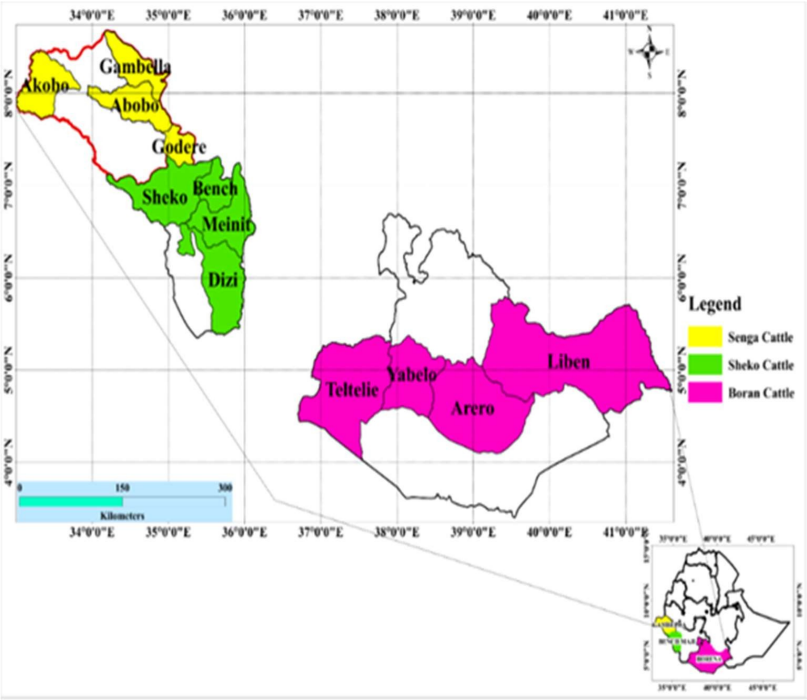

Figure 1. The Study Are

2.Materials and methods

2.1. Meat samples

A total of two cuts (sirloin and chuck) of 48 meat samples from three different cattle types namely Boran, Senga and Sheko (aged 18–24 months) were used in this study.

2.2. Study area

The Sheko and Senga cattle were gathered from south-western Ethiopia and the Akobo region of Gambella, respectively, while the Boran cattle originate from the Borana zone in the southern rangelands of Ethiopia (Figure 1). The southern rangelands of Ethiopia, particularly those in the Liben, Mega, and Arero plains, are the primary home of the Ethiopian Boran. Cattle are essentially huge, broad-framed cattle animals (Abdurehman, 2019). According to Coppock, (1994), 27% of the annual precipitation falls between September and November and 59% between March and May. Gambella is located in the southwest Ethiopian plains (Figure 1). The study area is divided into the four regional habitats of Akobo, Gambella, Abobo, and Godere (Kassie, 2020).

The height of the Bench Maji Zone, which is in the tropical area of the planet, ranges from 500 meters above sea level in the lowlands to more than 2,800 meters above sea level in the highlands. It is between 7.5° and 9.5° north latitude (Wubie, 2015).

2.3. Sample collection and preparation

From the study area, male indigenous breed cattle types were chosen. The samples were kept chilled until analysis after being collected and put in an ice box. Each animal's flesh sample, which was taken from three distinct native breeds, was examined. Sirloin and chuck were both have all visible fat removed (FAO, 2005).

2.4. Proximate composition

The proximate composition of the beef samples was evaluated in triplicate. Total moisture, total protein, and fat (ether extract) were calculated using the Association of Official Analytical Chemistry technique (AOAC, 2000).

2.4.1. Moisture determination

Based on AOAC 2000, method 101/1, the moisture content was determined. Two separate slices of fresh muscle samples (5g each) were obtained and baked at 1000°C for 24 hours. Following cooling in desiccators, the samples' weights were determined.

2.4.2. Determination of crude protein

Nitrogen was determined using the Kjeldahl technique, and crude protein was determined by multiplying the result by 6.25 (AOAC, 2000). The fresh meat sample was ground, and 1 g of it was used as the substrate for the digestion of 25 ml of concentrated H2SO4 and mercury tablets in a Kjeldahl flask. Nitrogen was distilled from the flask and then dissolved in 4% boric acid in a solution of 40% NaOH. The combination was titrated against solutions of 0.1 N HCl.

mg Nitrogen =VxNx14. (Hall et al.2013) (1)

2.4.3. Fat content determination

The petroleum ether extract was used to extract crude fat. From the sample, 15 grams were brought to the Soxhlet device. The samples underwent continuous ether extraction for six hours. The extract was then removed from the extractor and dried in the oven for two hours (AOAC, 2000). After cooling, the sample was weighed to determine the percentage of ether extraction.

2.5. Meat color

Four non-frozen meat samples from the LD were utilized in order to determine the meat color using the L*, a*, and b* standard CIE reference system. Using the MinoltaCR-400 colorimeter (Konica Minolta, Osaka, Japan) at 20C, in anaerobic and dark circumstances, the color was assessed. Each cut underwent a total of six scans, with the average measurements being utilized for statistical analysis. A standard white was used to calibrate L*, a*, and b*. A* ranges from green (-) to red (+), b* ranges from blue (-) to yellow (+), and L* is a brightness indicator (0 = black, 100 = white) (Lazăr et al, 2014). Chroma index (C*) measurements were made in accordance with (Purslow et al. 2016) by applying the following formula equation:

C* = ((a*)2+(b*)2)0.5. (Neto et al. 2015) (2)

2.6. Fatty acid profile analysis

Fatty acids were extracted from meat samples using 600µl of hexane. The resulting supernatant was collected in a vial for further analysis. The fatty acid analysis was conducted using the GC-FID method FAME 100 M SUPELCO and followed the AOAC 996.06 20th Ed.2016 met and test method. In the analysis, C23:0 (Sigma-Aldrich, Darmstadt, Germany) was used as the internal standard for measuring the total fatty acids in a 15 gm sample. For additional identification, the individual standards cis- 11-octadecenoic acid methyl ester, hexacosanoic acid methyl ester, 14-methyl- pentadecanoate, 14-methyl-hexadecanoate, and 16-methyl-heptadecanoate were all purchased. Every single chemical was of the analytical quality (Pleadin, 2021).

2.7. Statistical analysis

Statistical analysis was carried out using SPSS program version 25. The means of the beef meats that were put to the test were compared using one-way and two-way ANOVA testing as well as Tukey's post hoc test. To find out if there was a significant difference, a significance level of 0.05 was used. To analyze the fatty acid profile and proximate composition dataset, principal component analysis (PCA) was conducted using the XLSTAT 2022.4.1.1382 OS

Version software and SPSS version 20.

3. Results and discussions

3.1. Proximate composition of sirloin and chuck cuts of Boran, Senga and Sheko cattle

Table 1 shows percentage mean proximate composition and color of raw beef cuts from different cattle types. The high percentage mean moisture content of beef was 66.47±0.13 which represents the sirloin cuts of Sheko cattle and the lower values was from the sirloin of Boran cattle (64.32±0.29) and Boran chuck was significantly different ($p<0.05$) from Senga and Sheko cuts. The report of (Li, 2017) 66.63 ± 1.85 was in line with the current study particularly to the Sheko cuts. The water activity (aw) of the meat is the standard unit used by microbiologists to characterize the water needs of microorganisms. The present study's moisture content was lower than and inconsistent with the finding of (Alamin, 2019) that showed 70.54%.

The percent mean protein composition ranged from 21.57±0.45 to 26.83±0.7 in different animals, with the Boran chuck displaying the greatest protein content (26.83±0.78). The protein content of Boran sirloin and chuck was significantly different ($p<0.05$) from the protein contents of Senga and Sheko cattle. The protein contents reported by Karakok, (2010) ranged from 18–22%, and the report by Timketa, (Dagne T et al,.2021) ranged from 18.46±0.35 to 22.76 ± 1.04 was in line with the present study. The percentages mean aw content was ranged from 0.83±0.78 to 1.65±0.57. The Sheko chuck 1.65±0.57 was significantly different ($p<0.05$) from Senga and Boran cuts. The a_w result in the current study was similar to the 0.992 ± 0.001 result reported by (Li, 2017).

The percentage mean of fat content of the three cattle were ranged 10.79±0.36 (Sheko chuck) to 13.25±0.38 (Boran chuck) and Boran chuck contains high fat percentage. The report of Belhaj (Belhaj, 2021) the fat content was 5.50 ± 1.30 which was lower than the current study. These findings were corroborated those of (Oz, F and Celik, 2015) or this study's settings and moisture content support the work of (Hammuel, 2019).

3.2. The principal component analysis

The meat of three different types of cattle, namely Boran, Senga, and Sheko species, was analyzed for its composition and color using Principal Component Analysis (PCA), which breaks down the original data into different sets of scores and component loadings (Nkansah et al. 2021). Based on Eigenvalues greater than 1, primary components were chosen to keep in the analysis. Poor, moderate, and high loadings are denoted by component loadings below 0.5, between 0.5 and 1, and over 0.5, respectively. Figure 2 displays the findings of the PCA analysis for the chosen cattle types and meat slices.

Table1. The mean percentage values for proximate composition and color values of the Boran, Senga and Sheko cattle types and cuts

Cuttle cuts types	Moisture	aw	Crude protein	Crude fat	L*	a*	b*
Boran sirloin	64.32±0.29^{a}	0.95±0.70^{a}	24.95±0.70^{a}	12.97±0.87^{a}	30.79±4.06^{a}	5.18±0.88^{a}	2.92±0.68^{a}
Chuck	65.06±o.93^{c}	0.83±0.78^{b}	26.83±0.78^{b}	13.25±0.38^{b}	32.52±1.70^{a}	5.38±1.45^{a}	4.11±1.41^{b}
Senga sirloin	64.75±0.54^{ab}	0.99±0.01^{a}	21.65±0.50^{c}	11.89±0.09^{c}	32.05±5.12^{a}	6.38±3.65^{b}	4.33±1.05^{b}
Chuck	65.06±0.87^{b}	0.98±0.02^{a}	21.57±0.45^{c}	11.76±0.36^{c}	28.20±3.09^{a}	9.35±2.96^{c}	2.90±2.42^{ac}
Sheko sirloin	66.47±0.13^{b}	0.99±0.01^{a}	23.49±0.32^{d}	11.21±0.38^{c}	30.17±2.41^{a}	8.36±3.71^{c}	2.24±1.47^{ac}
Chuck	65.31±0.23^{b}	1.65±0.57^{c}	22.14±0.24^{c}	10.79±0.36^{c}	29.12±1.60^{a}	6.14±1.30^{ab}	2.43±1.13^{ac}

*Means with different superscripts within a column were significantly different at P<0.05
Abbreviations: aw=water activity, L=, brightness, a=, red, b= yellow.

Table 2. The loadings of the important principal components (PCs) for the proximatecomposition and color were analyzed using Eigen values

Parameters	F1	F2	F3	F4	F5	F6	F7
Eigenvalue	2.619	1.644	1.342	0.494	0.363	0.339	0.201
Variability (%)	37.409	23.480	19.165	7.051	5.185	4.837	2.874
Cumulative %	37.409	60.888	80.053	87.104	92.289	97.126	100.000

Table 5. Fatty acid content (%) of various meat cuts from Boran, Senga, and Sheko Cattle types.

Cattle types	**Boran**		**Senga**		**Sheko**	
Beef cuts	**Sirloin**	**Chuck**	**Sirloin**	**Chuck**	**Sirloin**	**Chuck**
Saturated	60.08±0.02^{c}	59.36±0.56^{b}	56.66±0.18^{a}	61.21±0.06^{e}	60.48±0.06^{d}	66.65±0.19^{f}
MUFA	25.65±0.13^{a}	35.76±0.67^{d}	42.38±0.55^{f}	37.49±034^{e}	34.85±0.11^{c}	29.20±0.26^{b}
PUFA	14.26±0.07^{g}	4.92±0.10^{e}	1.70±0.34^{c}	1.32±0.33^{b}	4.81±0.18^{d}	5.22±0.16^{f}
Trans	2.07±0.02^{c}	2.45±0.23^{ef}	1.52±0.23^{ab}	1.45±0.06^{ab}	4.36±0.18^{g}	4.38±0.19^{g}
Omega6	14.33±0.17^{g}	5.22±3.86^{c}	1.44±0.47^{a}	1.30±0.23^{a}	3.42±0.15^{b}	3.65±0.32^{b}
Omega3	1.94±0.05^{f}	1.85±0.04^{efg}	0.32±0.07^{a}	0.51±0.45^{ab}	0.37±0.03^{a}	0.82±0.33^{bc}
C14:0	4.47±0.21^{b}	4.26±0.26^{b}	5.34±0.18^{c}	6.16±0.33^{d}	3.67±0.19^{a}	4.45±0.21^{b}
C14:1	0.37±0.06^{ab}	0.81±0.15^{ab}	0.69±0.41^{abc}	0.90±0.09^{d}	0.57±0.15^{abc}	0.65±0.33^{abc}
C15:0	0.63±0.04d^{e}	0.31±0.02^{a}	0.60±0.11^{bcde}	0.49±0.07^{abcd}	1.16±0.11^{f}	0.81±0.22^{e}
C16:0	27.38±0.06^{d}	26.33±0.40^{c}	29.64±0.13^{e}	31.60±0.17^{f}	24.64±0.29^{a}	27.47±0.35^{d}
C16:1	1.08±0.93^{ab}	2.51±0.26^{g}	3.50±0.08^{h}	2.43±0.48^{g}	1.81±0.13^{d}	0.91±0.09^{b}
C17:0	0.71±0.13^{a}	0.80±0.13^{a}	1.57±0.26^{bc}	2.13±0.63^{d}	1.39±0.18^{b}	1.79±0.17^{bcd}
C17:1	0.00±0.00^{a}	0.00±0.00^{a}	0.84±0.21^{e}	0.76±0.10bc^{d}	0.49±0.32^{bcd}	0.00±0.00^{a}
C18:0	27.28±0.35^{def}	26.41±0.35^{bcd}	19.64±0.55^{a}	19.59±0.25^{a}	27.57±0.16^{bde}	31.56±0.34^{i}
C18:1n9t	2.16±0.14^{b}	2.30±0.12^{b}	1.68±0.30^{a}	1.71±0.25^{a}	4.32±0.11^{cd}	4.27±0.13^{cd}
C18:1n9c	21.64±0.09^{a}	31.03±0.03^{d}	35.59±0.03^{f}	32.25±0.23^{e}	27.87±0.09^{c}	22.82±0.14^{b}
C18:2n6c	9.76±0.03^{f}	3.80±0.30^{e}	1.51±0.41^{b}	1.07±0.06^{a}	2.79±0.13^{c}	3.39±0.25^{d}
C20:4n6	3.56±0.03^{e}	0.85±0.06^{d}	0.00±0.00^{a}	0.00±0.00^{a}	0.34±0.02^{bc}	0.00±0.00^{a}
C21:0	0.00±0.00^{a}	1.20±0.08^{cde}	0.00±0.00^{a}	2.11±0.12^{g}	1.71±0.05^{defg}	0.64±0.55^{b}
C18:3n3	0.00±00^{a}	0.00±0.00^{a}	0.37±0.15^{ab}	0.00±0.00^{a}	1.43±0.09^{d}	0.56±0.77^{ab}
C20:0	0.91±0.58b^{c}	1.21±0.01^{c}	0.44±0.39^{ab}	0.26±0.07^{a}	0.37±0.34^{a}	0.40±0.41^{ab}

** Means with various letters in a row differed significantly at P<0

Table 8. Correlation coefficients between different fatty acids found in beef need to

Variables	Saturated	MUFA	PUFA	Trans	Omega6	Omega3	Myristic	Myristic	Pentadecanoic	Palmitc	Palmit	Heptadeca	CisHeptadec	Stearic	TransElaidic	Linoleic	Arachidonic	ALinolenic	Arachidic
MUFA	**1**																		
PUFA	**-0.405***	**1**																	
Trans	0.006	**-0.892***	**1**																
Omega6	**0.662***	**-0.289***	0.032	**1**															
Omega3	0.014	**-0.712***	**0.742***	-0.041	**1**														
C14:0	**0.529***	-0.155	-0.056	**0.624**	-0.010	**1**													
C14:1	**-0.672***	**0.375***	-0.126	**-0.982**	-0.051	**-0.595***	**1**												
C15:0	**-0.428***	**0.761***	**-0.647***	**-0.623**	**-0.517***	**-0.318***	**0.682***	**1**											
C16:0	0.224	0.124	-0.223	**0.678**	-0.025	**0.372***	**-0.661***	**-0.347***	**1**										
C16:1	-0.168	**-0.794***	**0.918***	**-0.274**	**0.758***	**-0.242***	0.185	**-0.434***	**-0.404***	**1**									
C17:0	**-0.483***	**-0.274***	**0.472***	-0.162	0.163	-0.220	0.157	**-0.300***	-0.138	**0.555***	**1**								
C17:1	**0.718***	**-0.274***	0.016	**0.931**	-0.091	**0.603***	**-0.920***	**-0.530***	**0.540***	**-0.301***	**-0.283***	**1**							
C18:0	**-0.331***	**0.848***	**-0.769***	**-0.276**	**-0.597***	-0.096	**0.347***	**0.662***	0.159	**-0.653***	**-0.264***	-0.225	**1**						
C18:1n9t	**0.572***	**-0.767***	**0.593***	**0.771**	**0.472***	**0.401***	**-0.825***	**-0.861***	**0.377***	**0.319***	0.056	**0.736***	**-0.704***	**1**					
C18:1n9c	**0.658***	**-0.246***	0.001	**0.978**	-0.062	**0.612***	**-0.973***	**-0.595***	**0.697***	**-0.314***	-0.229	**0.937***	-0.217	**0.739***	**1**				
C18:2n6c	0.010	0.097	-0.123	-0.133	-0.093	-0.002	0.126	**0.254***	-0.063	-0.058	-0.100	-0.121	0.135	-0.168	-0.084				
C20:4n6	0.082	**-0.747***	**0.808***	0.003	**0.933***	-0.053	-0.102	**-0.565***	-0.035	**0.757***	0.121	-0.013	**-0.637***	**0.534***	-0.007	**1**			
C21:0	-0.109	**-0.631***	**0.765***	-0.178	**0.909***	-0.192	0.081	**-0.425***	-0.092	**0.779***	0.187	-0.207	**-0.506***	**0.342***	-0.171	**0.957***	**1**		
C18:3n3	0.082	-0.011	-0.076	0.020	-0.085	0.023	-0.058	-0.008	0.209	0.066	**0.273***	-0.085	0.137	-0.118	0.017	-0.134	-0.052		
C20:0	**0.487***	0.116	**-0.312***	**0.884**	**-0.285***	**0.536***	**-0.854***	**-0.344***	**0.784***	**-0.609***	**-0.338***	**0.828***	0.039	**0.497***	**0.900***	**-0.240***	**-0.389***	**1**	
MUFA	-0.120	**-0.726***	**0.832***	-0.097	**0.649***	-0.146	0.038	**-0.444***	-0.192	**0.845***	**0.482***	-0.100	**-0.502***	**0.383***	-0.119	**0.653***	**0.664***	**-0.406***	**1**

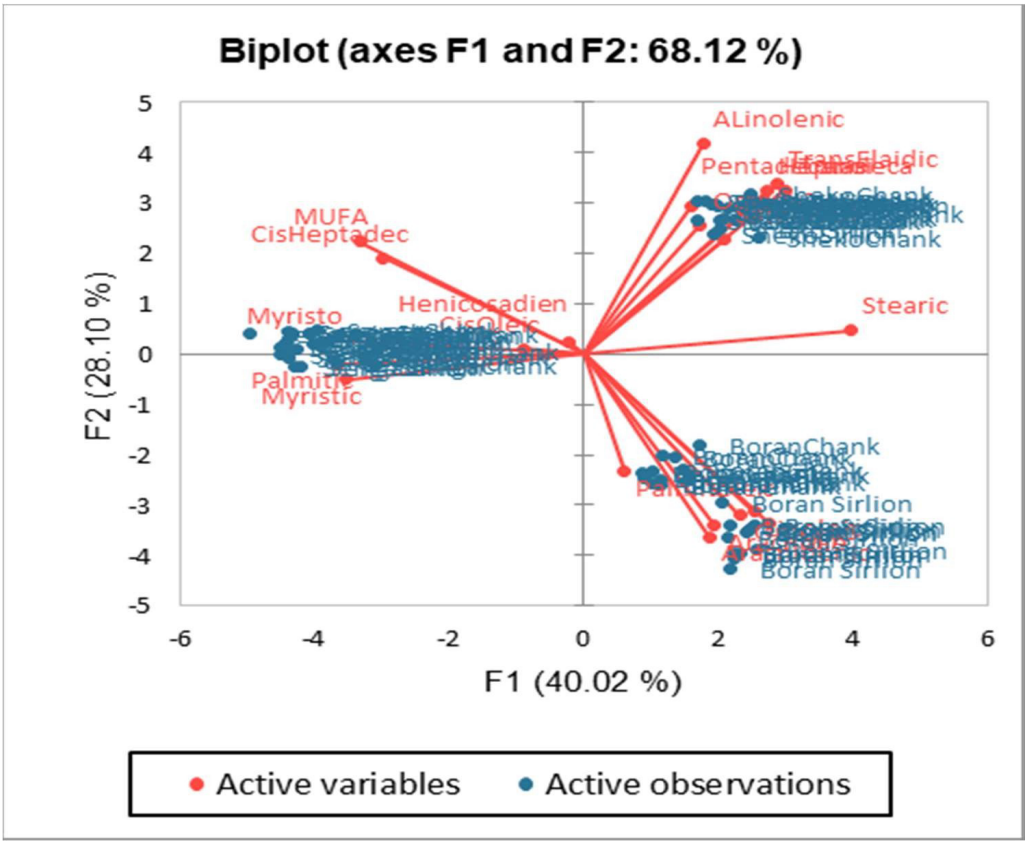

Figure 2. For the combined datasets of the proximate composition and color of meat cuts from theBoran, Senga, and Sheko breeds, a score plot of the principal component analysis (PCA) scores isused to visually display the results. Abbreviations: aw=water activity, L=, brightness, a=,red,b=yellow

The principal component (PC) analysis revealed that the first five PCs accounted for 100% of the variance among the two cuts of the three cattle types. The eigenvalues of PC1 to PC4 were 2.619, 1.644, 1.342, and 0.363, respectively, indicating their relative contributions to the total variance (Table 3). PC1 explained 37.409% of the variance in the dataset. Its loadings showed strong positive correlations with L*, a*, b*, and Fat%, suggesting that this PC represents the overall color and fat content characteristics of the beef samples. PC2 had high positive loadings for a_w, moisture%, and L*, a*, b*, while it had negative loadings for crude protein% and Fat%. This indicates that PC2 captures the relationship between water activity, moisture, and color, as well as the inverse relationship with protein and fat content. The top contributors to PC3 were crude protein%, moisture%, and a_w, suggesting that this PC reflects the protein and water-related properties of the beef samples. PC4 was primarily influenced by the L*, a*, b* color parameters, whereas PC5 was mainly associated with moisture content (Table 3).

The principal component loadings (Table 3) provided insights into the relationships and grouping patterns among the analyzed variables. The two-dimensional visualization of the loadings demonstrated the correlations between the variables and the positioning of the different cattle types and their cuts in the multivariate space.

Table 3. Principal component (PC) loadings for the significant cattle types and their cuts according to an Eigen analysis

Parameters	F1	F2	F3	F4	F5
moisture%	-0.625	0.143	0.635	0.008	0.402
Aw	-0.64	0.392	0.485	-0.147	-0.351
crude protein%	0.337	-0.501	0.742	-0.027	-0.101
Fat%	0.724	-0.535	0.232	-0.139	-0.072
L*	0.673	0.384	0.293	0.53	0.015
a*	0.429	0.787	0.095	-0.09	-0.13
b*	0.737	0.408	0.069	-0.404	0.214
Eigenvalue	2.619	1.644	1.342	0.363	0.201
Variability (%)	37.409	23.48	19.165	5.185	2.874
Cumulative %	37.409	60.888	80.053	92.289	100

Pearson's correlation coefficients between components of the proximate composition and color(L*, a*, b*) of meat

Table 4. Pearson's correlation between variables of proximate compositions and meat color of different cattle breeds and their meat cuts slices.

Cuttle cuts types	Moisture (%)	aw	Protein (%)	Fat (%)	L*	a*	b*
Moisture (%)	1						
aw	**0.606***	1					
Protein	0.127	-0.051	1				
Fat (%)	**-0.368**	**-0.502**	**0.614***	1			
L*	-0.186	-0.179	0.213	**0.277***	1		
a*	-0.100	0.050	-0.139	-0.036	**0.498***	1	
b*	**-0.314**	**-0.242**	0.085	**0.331***	**0.506***	**0.543***	1

*Values in bold differ from 0 with a significance difference level of alpha = 0.05.

In Quadrant 1, Figure 2, these variables are located in the lower right corner. Additionally, the second quadrant's area, represented by PC1 (37.31%), has a positive correlation with the color parameters (L*, a*, and b*). This quadrant includes cuts with the highest content of these color components (Figure 2), and the color parameter represented by Senga cattle (specifically sirloin cuts).

In principle component analysis (PCA), new axes known as principal components (PCs) are created by using the baseline data for crude protein, crude fat, water activity, moisture content, and color parameters L*, a*, and b* Table 2. These primary components, which are orthogonal to one another, show the most notable patterns of variation in the initial data. Table 2 displays the findings of the principal component analysis for each of the five main components. The results of the research show that the first principle component explains around 37.409% of the total variation, the first two principal components about 60.888%, and the first three main components about 80.053% of the total variation Table 2.

The first three PCs, with eigenvalues of 2.619, 1.644, and 1.342 in that order, explained 80% of the variance among the three species of cattle (Table 2). These figures represent significant contributions made by each PC to the overall variance. Crude protein, crude fat, and color L*, a*, and b* had high loadings on PC1, which explained 37.409% of the variance in the data set. Likewise, PC2 displayed top positive loadings for water activity (aw) and moisture levels.

Protein and moisture content did not significantly correlate, while there was a negative link between meat color and fat percentage and between protein and moisture content ($P<0.05$). Comparably, there was no association between protein content and color parameter L*. However, there was one between protein and color parameter a* that was negative. Conversely, there was a positive association ($P<0.05$) among protein and fat content. Additionally, no correlation was found between water activity and color parameter a*, and a negative association was observed among water activity and moisture content, fat, and protein. Likewise, a positive correlation ($P<0.05$) was observed among fat and color value, except for color parameter a*, which showed a negative association with fat. Furthermore, strong positive correlations ($P<0.05$) were found between meat color represented by L*, a*, and b* as shown in Table 4.

3.3. Fatty acid profiles

The percentage of intramuscular saturated fatty acids (SFAs) in sirloin and chuck slices of three different beef types Boran, Senga, and Sheko is shown in Table 5.

Each breed's total fatty acids were made up of about 60% SFAs, with Palmitic acid (C16:0), stearic acid (C18:0), and cis-oleic acid (C18:1n9c) accounting for more than 90% of the total saturated fatty acids. These results were in agreement with the results documented by Liu (2020) and (Kazala, 1999). Variations among breeds were observed in multiple fatty acids. In particular, the Senga cattle type exhibited significantly higher levels ($p<0.05$) of C16:0 in both the sirloin (29.64±0.13) and chuck cuts (31.60±0.17) compared to the Boran and Sheko breeds in both types of cuts(Acar et al,.2008). Conversely, C18:0 levels tended ($p<0.05$) to be higher in Sheko chuck (31.56±0.34) compared to the Boran and Sheko cuts of both sirloin and chuck (27.28±0.35, 26.41±0.35, 19.64±0.55, and 19.59±0.25, respectively). These findings align with the results reported by (Lisitsyn, 2017).

The presence of specific saturated fatty acids (SFAs) like C16:0 and C14:0 in meat is widely recognized to contribute to elevated levels of total cholesterol and low-density lipoprotein, increasing the threat of coronary heart illness (Barton, 2010). In contrast, the Senga breed demonstrated significantly higher levels ($p<0.05$) of heptadecanoic acid (C17:0) in both the chuck (2.13±0.63) and sirloin (1.57±0.26) cuts compared to the Boran breed, as well as higher levels ($p<0.05$) of myristic acid (C14:0) in the sirloin (5.34±0.18) and chuck (6.16±0.33) cuts compared to both Sheko and Boran breeds (4.47±0.21, 4.26±0.26, and 3.67±0.19, 4.45±0.21), as illustrated in the data Table 5. The result is in line with the study of (Dagne, 2021) In addition, Senga cattle tended to have lower ($p<0.05$) trans fatty acid in sirloin 1.52±0.23 and chuck 1.45±0.06 than Boran and sheko cattle in their both sirloin and chuck (2.07±0.02, 2.45±0.23 and4.36±0.18, 4.38±0.19) cuts of meat respectively, lower ($p<0.1$). Similarly, there was also high transelaidic acid (C18:1n9t) in Sheko cuts (sirloin and chuck) 4.32±0.11and 4.27±0.13 than in Boran and Senga (2.16±0.14, 2.30±0.12 and 1.68±0.3, 1.71±0.25) sirloin and chuck respectively (Tarricone, 2020).

Senga cattle type in sirloin 56.66±0.18 and chuck 61.21±0.06 constituted lower ($p<0.05$) total SFA than Boran and Sheko sirloin and chuck (60.08±0.02, 59.36±0.56 and 60.48±0.06, 66.65±0.19) respectively. These findings were similar with the study of Rennaa (Rennaa, 2019). Since SFA, particularly 12:0, 14:0, and 16:0, have historically been linked to higher levels of blood cholesterol and, as a

result, with coronary heart disease (CHD) and cardiovascular disease (CVD), saturated fatty acid (SFA) is recognized as a significant predisposing factor (Pighin et al. 2016). Table 4 details the sirloin and chuck muscles' intramuscular unsaturated fatty acid (MUFA) content in the three breeds of cattle. In sirloin muscle slices, total MUFA ranged from 25.65% to 42.38%, while in chuck muscle cuts, it ranged from 29.20% to 37.49% (Table 5). These outcomes align with the findings of Pleadin et, al. (Pleadin, 2021). MUFA constituted the largest proportion of UFA, and the most plentiful MUFA was oleic acid (C18:1n9c). There was a significant difference in the amount of cisoleic acid (C18:1n9c) among different breeds. Table 5 for showed more detailed information. These findings align with a previous study by Manuela et al. (Renna et al,. 2019). One particular polyunsaturated fatty acid (PUFA) which is considered beneficial for human health is C18:3n3 (Frank, 2016). The observed trend of higher C18:3n3 levels in Sheko cattle are consistent with previous investigations that have reported significantly higher C18:3n3 content (Barton, 2007). For sirloin cut, C16:1 had relatively ($p<0.05$) to be higher (3.50±0.08) in Senga cattle than in Boran and Sheko breed (1.08±0.93 and 1.81±0.13) respectively. These findings were consistent with the report of (Liul, 2020) showed that C16:1 of three different mussels had 0.91±0.36, 1.33±0.43 and 1.91±0.40.

According to (Ekine-Dzivenu et al. 2017), PUFAs possess certain preventive properties against cardiovascular disease and can potentially delay the progression of atherosclerosis. As a result, there have been active efforts to enhance the PUFA composition in beef in order to meet the preferences of consumers. Differences in breed have been noticed in the average proportions of saturated fatty acids (SFA) to unsaturated fatty acids (UFA) and monounsaturated fatty acids (MUFA) to polyunsaturated fatty acids (PUFA) in various beef cuts. Specifically, Boran cattle exhibited a significantly lower ($p<0.05$) SFA/UFA ratio than Sheko cattle, but they had greater amounts of saturated fatty acids when matched to Senga cattle. In contrast to Senga and Sheko cattle, the average MUFA content of Boran cattle was significantly lower ($p< 0.05$) than that of Senga and Sheko cattle, whereas the average PUFA content of Boran cattle was significantly greater (see Table 5). A high SFA/UFA ratio is widely established to be strongly associated with a number of pathological disorders in humans, including an elevated risk of vascular and coronary illnesses (Philip, 2003).

Principal component analysis (PCA) was utilized to analyze a combination of fatty acid profile, proximate composition, and color (L*, a*, b*) data of meat from three different types of cattle (Figures 3). When examining the fatty acid profile, the first and second principal constituents (PCs) accounted for 68.12% of the total variation, with PC1 explaining 40.02% and PC2 explaining 28.10% (Figure 3). Along the PC1 axis, three distinct groups were observed in the fatty acid profile, with Sheko cattle types being clearly separated from the other two types of cattle. In the first quadrat, Boran cattle contained linoleic acid (C18:2n6c), palmitoleic (C16:1), arachidic acid (C20:0), arachdonic acid (C20:4n6), and omega-6 PUFA. Alinolenic acid (C18:3n3), pentadecanoic acid (C15:0), omega-3 fatty acids, heptadecanoic acid (C17:0), and trans eliadic acid (C18:1n9t) were all present in Senga type, in contrast.

The research findings demonstrate that the first principal component explains approximately 40.020% of the total variation observed, the first two principal components collectively account for around 68.118% of the variation, and the first three major components combined explain approximately 76.001% of the overall variation (Table 6).

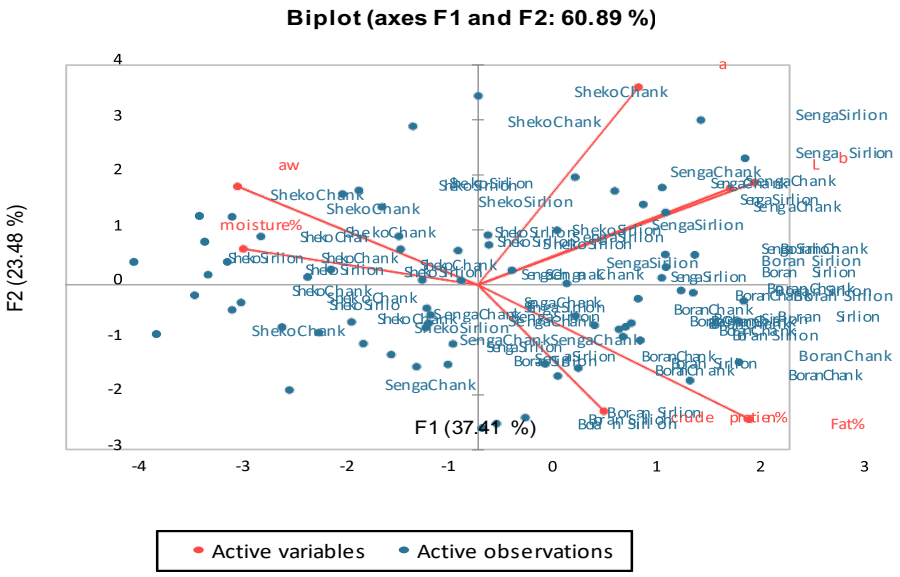

Figure 3. Presents a score plot obtained from a principal component analysis (PCA) using a comprehensive dataset that combines the fatty acid content of meat cuts from the Boran, Senga, and Sheko cattle breeds. The plot visually represents the PCA scores. The abbreviations used in the plot are as follows: saturated fatty acids = SFA, monounsaturated fatty acids = MUFA, polyunsaturated fatty acids = PUFA, and linoleic acid = LA

The first five PCs had eigenvalues of 8.404, 5.901, 1.655, 1.254, and 1.127 in that sequence, and they accounted 87.3% of the variation across the three species of cattle (Table 6). These figures represent significant contributions made by each PC to the overall variance. PC1 explained 40.020% of the variance in the data set, and its loadings indicated that it has high contributions from Myristoleic acid (C14:1;-0.318), Pentadecanoic acid (C15:0; -0.318,), Heptadecanoic acid (C17:0; 0.051, Stearic acid (C18:0; -0.251), cisOleic acid (C18:1n9c; -0.074), Heneicosanoic acid (C21:0; -0.018). Myristic acid (C14:0; - 0.265), palmitoleic acid (C16:1; 0.598), and Henicosadienoic acid (C21:0; 0.314) were the main donors to PC3. Contrarily, cis-oleic acid (C18:1n9c; 0.537), Henicosadienoic acid (C21:0; 0.666), pentadecanoic acid

(C15:0; 0.545), linoleic acid (C18:2n6c; 0.304), and arachidonic acid (C20:4n6; 0.389) were the major influences on PC4 and PC5, respectively.

Table 6. The loadings of the significant principal components (PCs) for the meat cuts of variouscattle types are subjected to Eigen analysis.

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**
Eigenvalue	8.404	5.901	1.655	1.254	1.127	0.814	0.571
Variability (%)	40.020	28.098	7.883	5.972	5.369	3.877	2.720
Cumulative %	40.020	68.118	76.001	81.974	87.342	91.220	93.939

3.4. Correlation of PCs with fatty acid composition parameters

The principal component loadings (Tables 7) showed how much the examined variables affected how the kinds were grouped as well as how strongly they were related to one another. The loading projections showed the location of the variables, the cuts in the two-dimensional plot, and their related correlations for each of the three species of cattle. Positive correlation exists between variables that are both near and far from the plot origin. In the cattle kinds, the results, for instance, reveal a favorable link between MUFA and Cis- Heptadec acid (C17:1) as well as between Palmitic acid (C16:0) and Myristoleic acid (C14:1) (Table 8). The PC scores were also computed for a correlation analysis with parameters for fatty acid composition. Table 8 displays the factor score coefficients that were utilized to determine the scores for each PC. After that, Pearson's correlation analysis was done to find the associations among PC and the parameters of the fatty acids profiles. In the current research, significant ($p<0.05$) positive correlations were obtained between saturated fatty acid and Omega6 fatty acid, Myristic C14:0), TransElaidic(C18:1n9t), cisOleic(C18:1n9c) and Arachidic (C20:0) (r=0.662, 0.529, 0.572, 0.658 and 0.487) which, in turn, were negatively correlated with PUFA, Myristoleic acid (C14:1), Pentadecanoic acid (C15:0) and Heptadeca acid (C17:0) (Table 8).

4.Conclusion

The nutritional value and health impact of beef are influenced by its fatty acid content and composition, which are determined by factors such as genetics, diet, and environmental conditions. Variations in fat levels can be found in different beef cuts, affecting both nutritional value and consumer health. In a recent study, we examined the fatty acid composition of sirloin and chuck cuts, and found that the composition is significantly influenced by the breeds of cattle. Specifically, the levels of saturated, monounsaturated, and polyunsaturated fatty acids differ among Boran, Sheko, and Senga cattle. There were also notable differences in the overall composition and fatty acid content between the three breeds and the beef cuts. Several factors, including feed, sex, origin, and genetics, likely contributed to the variations in composition and fatty acid profiles among the cattle breeds studied. The study further revealed that animals grazing on pasture until slaughter had higher fat content and increased levels of n-3 polyunsaturated fatty acids compared to those fed other types of feed. However, adult cattle had lower concentrations of polyunsaturated fatty acids than young cattle, despite benefiting from pasture. To enhance human diets and overall health, it is advisable to select beef cuts with a healthy balance of fatty acids. This data can inform consumer guidelines, influence dietary choices, and assist producers in tailoring beef cuts to meet consumer demands for specific fatty acid profiles. Additionally, resource mapping helps identify knowledge gaps and potential research areas, contributing to our understanding of beef's fatty acid profiles and their impact on human health.

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Acknowledgment

We would especially want to thank the Addis Abeba Science and Technology University's (AASTU) Department of Food Science and Applied Nutrition, Bioprocessing and Biotechnology Center of Excellence, for awarding the student the scholarship.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **https://chimie-biologie.ubm.ro/carpathian_journal/index.html**

INVESTIGATION OF TOTAL FLAVONOID, PHENOLIC AND ANTIOXIDANT ACTIVITY OF FERMENTATION BROTH OF FERMENTED CLIMBING SWAMP FERN (*Sthenochlaena palustris*)

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https://doi.org/10.34302/crpjfst/2024.16.2.5

Article history:
Received: June 20th, 2023
Accepted: May 3rd, 2024

Keywords:
Fermented fern;
Exogenous lactic acid bacteria;
Stenichaena palutis;
Antioxidant activity.

ABSTRACT

Fermented vegetable has become more popular as part of daily diet due to their increased shelf life and, more importantly, their beneficial effect for health. Climbing swamp fern (*Sthenochlaena palustris*) is an edible plant that known has medicinal properties. Fermentation can improve functionality of food, and therefore in the present study we prepare fermented fern and examine antioxidant activity, total phenolic and flavonoid content of the fermentation broth. The fronds of fern were collected from local farmer and fermented with different salt concentration (2, 4, 6% w/w), addition of exogenous lactic acid bacteria (none vs *Lactobacillus plantarum*) and time of fermentation (72 vs 144 hours). The experiment was performed using completely randomized design with factorial design approach. Broth formed during fermentation was collected and examined for their total flavonoid content (TFC), total phenolic content (TPC) and antioxidant activity, measured as percentage of DPPH inhibition. The result indicated that salt has significant effect on TFC, while all the three factors have significant effect TPC and DPPH inhibition. The highest TFC was observed when lowest salt was used, and the highest TPC was observed when the lowest salt concentration was used with the addition of *L. plantarum* and the lowest fermentation time. While the highest antioxidant activity was observed when the highest concentration of salt used with the addition of *L. plantarum* and the lowest fermentation time. TPC has significant and moderate correlation with TFC and DPPH inhibition, while TPC and TFC indicate no significant and no correlation. Further investigation is required to examine antioxidant compounds other than polyphenol and flavonoid in the fermentation broth. Furthermore, the solid fraction of the fermented fern fronds needs to be examined for its antioxidant property.

1. Introduction

Climbing swamp fern (*Stenichaena palustris*) is classified as a wild plant that can thrive on peatlands without special treatment (Jaelani *et al*., 2019) and easily found in Borneo Island, Indonesia. The Dayak tribes in Central Kalimantan use this plant to prevent anemia, increase breast milk production and apply it as antiaging (Zannah *et al*. 2015). The young leaf of the plant are greenish red and when it getting older the color become greener and become rich of antioxidant and nutritional compounds.

Some phytochemical that are found in the fern leaf include phenolic and flavonoid

compounds (Chai *et al*., 2012; Chear *et al*., 2016). Mature fronds tend to have higher antioxidant activity and can be used as exogenous antioxidant (Chai *et al*., 2012). Fronds of the fern have some important nutritional component including protein, iron, copper, vitamin C, β-carotene, and folic acid (Irawan *et al*., 2006). Iron in the fern fronds considered plays important roles to overcome anemia (Cahya *et al*., 2016). In addition, it also contains fatty acid, phytosterol, and kaempferol glycoside (Chaer *et al*., 2016). Like other vegetables, fern has low shelf life, due to high water and nutritional content which can promote food deterioration, therefore processing method to improve shelf life and, if possible, improve nutritional value and functional properties is required.

Fermentation of vegetables can increase shelf life and become source of probiotic microorganism (Torres *et al*., 2020), increase the quality of fermented product by increasing secondary metabolite that can promote antioxidant properties (Wu *et al*., 2015; Septembre-Malaterre *et al*., 2018). Fermentation process can increase folic acid and vitamin B12 content (Masuda *et al*., 2012) and remove anti nutritional component in food (Marco *et al*., 2017). The fermentation can be performed as a spontaneous process without or with addition of exogenous microorganism (Yang *et al*., 2020; Pejcz *et al*., 2021). Lactic acid bacteria (LAB) is the most widely used microorganism in vegetable fermentation (Gerardi *et al*., 2019; Lee *et al*., 2016; Wang *et al*., 2021).

Some important factors for optimal fermentation process include the microbes used, substrate and conducive environment for the microbial growth (Septembre-Malaterre *et al*., 2018). Different conditions applied during fermentation may affect the product quality and properties such as profile of volatile compounds and other chemical in the fermented products (Wang *et al*., 2021; Liang *et al*., 2020), which in turn may affect functionality of the food such as safety and stability, flavour and taste, nutrition, bioactivity and health, and texture and rheology (Terefe & Augustin, 2020). During vegetable fermentation, including climbing fern, liquid portion is usually accumulated known as fermentation broth (Gerardi *et al*., 2019; Kim *et al*., 2003). The fermentation broth is of our interest in the present study.

İn the present study we investigate whether salt concentration, exogenous LAB and fermentation time affecting total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity of fermentation broth of fermented climbing fern.

2. Materials and Methods

2.1. Materials

Fronds of climbing fern were purchased from farmers in Rasay Jata Umum III Village, Kubu Raya District, West Kalimantan Province. Salt (NaCl) used in the present study is purchased in traditional market in Kemuning market, Pontianak, West Kalimantan. Folin-Ciocalteu solution, sodium carbonate, gallic acid, aluminium chloride, potassium acetate, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were provided by the Agriculture Laboratory, Universitas Tanjung Pura, Pontianak with analytical grade. While ethanol used in the present experiment were technical grade.

2.2. Preparation of Climbing Fern Fronds

The fronds were collected early in the morning by picking the second and third fronds which aged 12-14 days. The age of the fronds was determined based on the appearance of leaf shoot until the age that usually consumed by local people according to initial survey. The collected fronds were then taken to the laboratory, sorted and washed with running water. It was cut to give around 4-5 cm fronds.

2.3. Preparation of *Lactobacillus plantarum* Culture

The culture was grown on MRSB broth and shaken at room temperature for 48 hours. The cell density was determined using haemocytometer by direct counting using light microscope with 400 × magnification.

2.4 Fermentation of Fern Fronds

Fermentation was performed with 3 independent variables, i.e. salt concentration (3 levels), exogenous LAB (2 levels), and time of fermentation (2 levels). The experiment was conducted as a completely randomized design. The independent variables and their levels are presented in Table 1. The responses (dependent variable) in the present study were TFC, TPC and antioxidant activity measured as percentage of DPPH inhibition. The experiments were run in triplicates.

The fermentation was performed in a plastic container. As much as 100 g of the fronds was put into the container and salt was added according to the concentration presented in Table 2. For exogenous microbe, 5 mL of the starter culture was added with the density of the cell was 10^7 cell/mL. The lid of the container was then closed and the fermentation was performed at room temperature for the designated time as indicated in Table 2.

After the fermentation concluded, the broth was separated from the solids, by filtering through a cheese cloth and the broth was collected and keeps in a dark vial. The sample was deposited in a freezer until analysis was conducted.

Table 1. Independent variables and their levels used in this experiment

Independent variables	Levels		
Salt concentration (%w/w)	2	4	6
Exogenous microbial	none	*L. plantarum*	-
Fermentation time (hours)	72	144	-

Table 2. Experiments run in the present study

Salt concentration (%w/w)	Exogenous LAB	Fermentation time (hours)
2	None	72
4	None	72
6	None	72
2	*L. Plantarum*	72
4	*L. Plantarum*	72
6	*L. Plantarum*	72
2	None	144
4	None	144
6	None	144
2	*L. Plantarum*	144
4	*L. Plantarum*	144
6	*L. Plantarum*	144

Note: "None" in exogenous microbial column indicate that the fermentation was spontaneous. The experiments were run in triplicate

2.5. Determination of Flavonoid Content

Flavonoid content was determined using method described by Dewi *et al*. (2020). Briefly, 500 µL of sample was mixed with 0.1 mL 10% w/v $AlCl_3$ solution, 1.5 mL methanol, 2.8 mL distilled water and 0.1 mL 1 M potassium acetate in a test tube. The mixture was then homogenized and incubated for 30 minutes for in dark room at room temperature. The absorbance of the mixture at 415 was then recorded. The total flavonoid content was indicated as percentage of quercetin equivalent per 1 g sample (mg QE/g).

2.6. Determination of Total Phenolic Compound

Total phenolic compound was determined using Folin-Ciocalteu reagent as described by

Dewi *et al*. (2020). As much as 200 µL samples was mixed with 1 mL of 10% v/v Folin-Ciocalteu reagent and 3 mL of 2% sodium carbonate solution. The mixture was then homogenized and leave stand for 30 minutes in dark room at room temperature. The absorbance at 765 nm was then recorded. The total phenolic compound was indicated as percentage of gallic acid equivalent per gram sample (mg GAE/g).

2.7. Determination of Antioxidant Activity

The antioxidant activity was determined using DPPH method as described by Safari *et al*. (2019). As much as 4 mL of sample was mixed with 1 mL 0,2 mM DPPH. The mixture was homogenized and incubated for 30 minutes at room temperature in dark room. The absorbance at 517 was recorded, and the inhibition of DPPH was calculated against control using equation (1), where control was prepared by substituting sample with ethanol.

$$\text{Percentage inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad \ldots (1)$$

2.8. Statistical Analysis

All collected data was tabulated to Minitab 17 statistical software and analyzed to give Anova table and factors considered to have significant effect when p-value < α, where in this study α = 0.05.

3. Results and Discussions

3.1. Experimental Data

The experiment was performed based on factorial design with completely randomized design run in triplicate. The data obtained is presented in Table 3.

Table 3. Experimental data of experiments run in the present study

Salt concentration (%w/w)	Exogenous LAB	Fermentation time (hours)	TFC (mg QE/g)			TPC (mg GAE/g)			DPPH inhibition (%)		
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
2	None	72	0.69	0.76	0.87	4.47	4.16	4.43	2.7	3.5	2.2
4	None	72	0.69	0.53	0.54	5.36	5.40	5.33	10.1	9.6	11.1
6	None	72	0.60	0.41	0.71	4.26	4.57	4.50	5.4	5.3	4.4
2	*L. Plantarum*	72	1.00	1.01	1.12	8.57	8.67	8.53	14.5	14.2	14.3
4	*L. Plantarum*	72	0.52	0.52	0.69	6.50	6.40	6.22	12.3	11.7	12.7
6	*L. Plantarum*	72	0.47	0.52	0.54	7.24	7.24	7.05	18.0	17.7	18.4
2	None	144	0.63	0.51	0.69	4.60	4.67	4.33	3.8	4.5	3.1
4	None	144	0.94	1.01	1.12	5.36	5.64	5.33	2.0	1.1	2.6
6	None	144	0.60	0.69	0.43	4.57	4.64	4.57	6.0	7.2	5.1
2	*L. Plantarum*	144	0.81	0.85	0.65	8.26	8.43	8.57	12.3	12.5	12.2
4	*L. Plantarum*	144	0.54	0.56	0.51	1.43	1.40	1.33	13.0	12.6	11.6
6	*L. Plantarum*	144	0.80	0.61	0.72	6.67	6.53	6.78	10.6	10.4	11.2

3.2. Total Flavonoid Content of Fermentation Broth

Fermentation may change chemical composition of the product, including flavonoid (Nazarni *et al*., 2016; Lee *at al*., 2016) due to the presence of enzymatic activity releasing free flavonoid (Lee *et al*., 2016). The result of the present study indicate that salt concentration has significant effect on TFC of fermentation broth ($p<0.001$) as shown in Anova table (Table 4). As can be seen on Figure 1, higher salt concentration led to lower TFC. This observation is in agreement with Lee *et al*. (2016) who found that higher salt concentration may inhibit β-glucosidase and therefore lowering the number of flavonoid aglycones, which eventually reduce the TFC. No significant effect detected when exogenous LAB incorporated into the fermentation process ($p=0.937$) and different fermentation time ($p=0.403$).

Table 4 also showed that there is significant effect of interaction of salt concentration vs exogenous LAB ($p<0.001$), salt concentration vs fermentation time ($p<0.001$) and exogenous LAB vs fermentation time ($p=0.049$). Table 4

also show that interaction of the three factors significantly affect TFC (p= 0.001). This result indicates that all factors contribute to the change of TFC when interact each other even though exogenous LAB and fermentation time do not individually affect TFC.

Table 4. Anova of total flavonoid content (TFC, mg QE/g) versus salt concentration (%w/w), exogenous LAB, and time (hours)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	1.09893	0.099902	11.22	0.000
Linear	4	0.26652	0.066631	7.48	0.000
Salt Conc. (%w/w)	2	0.26002	0.130008	14.60	0.000
Exo. LAB	1	0.00006	0.000057	0.01	0.937
Time (hours)	1	0.00645	0.006451	0.72	0.403
2-Way Interactions	5	0.65758	0.131516	14.77	0.000
Salt Conc. (%w/w)*Exo. LAB	2	0.33481	0.167405	18.80	0.000
Salt Conc. (%w/w)*Time (hours)	2	0.28457	0.142284	15.98	0.000
Exo. LAB*Time (hours)	1	0.03820	0.038201	4.29	0.049
3-Way Interactions	2	0.17482	0.087412	9.82	0.001
Salt Conc. (%w/w)*Exo. LAB*Time (hours)	2	0.17482	0.087412	9.82	0.001
Error	24	0.21367	0.008903		
Total	35	1.31260			

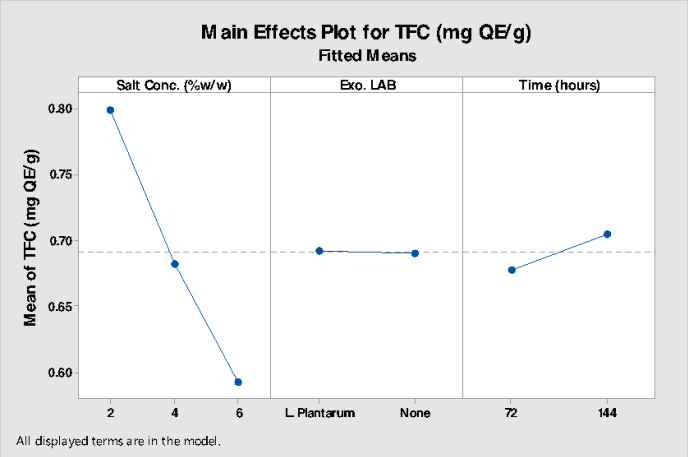

Figure 1. Main effect plot of salt concentration, exogenous LAB and fermentation time for total flavonoid content (TFC)

3.3. Total Phenolic Content of the Fermentation Broth

Some published results indicate that fermentation can change the TPC of fermented product, including the fermentation broth (Kim *et al*., 2003). The result of the present study indicates that all of the factors investigated in this study have significant effect on TPC. Anova table as shown in Table 5 indicate that all factors, either individual, interaction between 2 factors and interaction between 3 factors have p-value < 0.05. Study reported by Kim *et al*. (2003) indicate that TPC data tend to show inconsistencies trend either between different fermentation time for different type of vegetable. Some vegetable showed increased TPC up to 3-4 months of fermentation and decrease after 6 months, and even lower than the unfermented sample. However, in general increase in TPC was detected in all treatment even the increase appeared at different time point (Kim *et al*., 2003). Plot of main factors (Figure 2) showed that fermentation with the lowest salt concentration, addition of *L. plantarum* and shorter fermentation time (72 hours) give higher TPC. Therefore our present result is in agreement with the result reported by Kim *et al*. (2003) where in general fermentation increases the TPC of the fermentation product.

Table 5. Anova of total phenolic content (TPC, mg GAE/g) versus salt concentration (%w/w), exogenous LAB, and time (hours)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	132.059	12.0054	723.90	0.000
Linear	4	51.745	12.9363	780.03	0.000
Salt Conc. (%w/w)	2	20.375	10.1873	614.27	0.000
Exo. LAB	1	24.438	24.4375	1473.53	0.000
Time (hours)	1	6.933	6.9332	418.06	0.000
2-Way Interactions	5	69.896	13.9793	842.92	0.000
Salt Conc. (%w/w)*Exo. LAB	2	49.377	24.6884	1488.66	0.000
Salt Conc. (%w/w)*Time (hours)	2	11.236	5.6181	338.76	0.000
Exo. LAB*Time (hours)	1	9.283	9.2834	559.77	0.000
3-Way Interactions	2	10.418	5.2088	314.08	0.000
Salt Conc. (%w/w)*Exo. LAB*Time (hours)	2	10.418	5.2088	314.08	0.000
Error	24	0.398	0.0166		
Total	35	132.457			

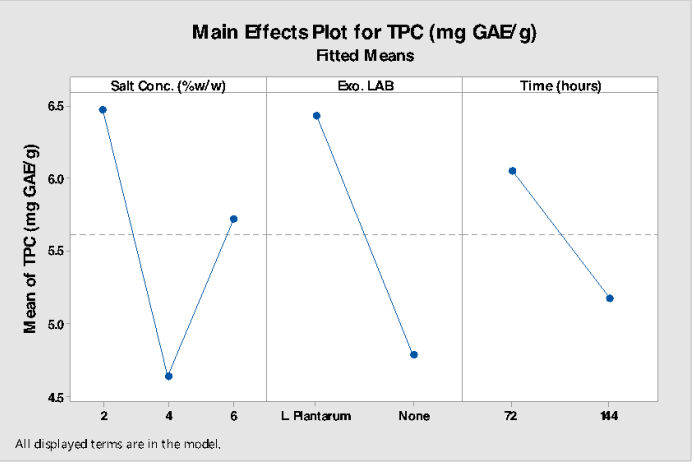

Figure 2. Main effect plot of salt concentration, exogenous LAB and fermentation time for total phenolic content (TPC)

3.4. Antioxidant Activity of the Fermentation Broth

Antioxidant activity is very important properties of food and one of factors that make functional properties of food. Fermentation is reported to increase antioxidant properties of fermented vegetables (Sayin & Alkan, 2015; Lee *et al*., 2016; Wang *et al*., 2021). Antioxidant activity was found either in the fermentation broth (Kim *et al*., 2003) or solid part (Wang *et al*., 2021; Lee *et al*., 2016) of the fermentation product. In the present report we focused on fermentation broth formed during the fermentation process.

Anova of DPPH inhibition is presented on Table 6. The results indicate that all factors affect the DPPH inhibition significantly. All of the individual factors and interaction of the factors has p-value < 0.05 as shown in Table 6. The highest antioxidant activity was found at the highest salt concentration, with addition of *L. plantarum* and fermented for 72 hours (Figure 3.). This result is in agreement with finding by Lee *et al*. (2016) who also found that fermentation and, even more, addition of LAB to the fermentation process increase antioxidant activity of the fermentation product. Kim *et al*. (2003) also found some increase in antioxidant activity of fermentation broth of some of the vegetable they used in their experiment.

The results of the present study indicate that most likely the antioxidant activity of the fermentation broth is predominantly determined by the TPC. However, according to Kaur &

Kapoor (2001) antioxidant activity not only given by the presence of phenolic and flavonoid compound but also the presence of other compounds such as vitamin E, coenzyme Q10, lycopene, β-carotene, α-carotene and vitamin C. Therefore, the presences of other antioxidant compounds in fermentation broth of fermented fern need to be elucidated.

3.5. Correlation Analysis between Factors

Correlation between factors can explain whether one factors correlate with the other factors in the experiment. The correlation analysis results are presented on Table 7. TPC has significant ($p<0.05$) and moderate association with TFC ($r=0.405$) and DPPH inhibition ($r = 0.452$). While TFC is not significant ($p=0.295$) and negative very week or no association ($r= -0.179$) (Fowler *et al*. 1998).

Table 6. Anova of DPPH inhibition (%) versus salt concentration (%w/w), exogenous LAB, and time (hours)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	860.028	78.184	205.36	0.000
Linear	4	703.221	175.805	461.78	0.000
Salt Conc. (%w/w)	2	16.235	8.117	21.32	0.000
Exo. LAB	1	627.611	627.611	1648.51	0.000
Time (hours)	1	59.375	59.375	155.96	0.000
2-Way Interactions	5	45.292	9.058	23.79	0.000
Salt Conc. (%w/w)*Exo. LAB	2	22.544	11.272	29.61	0.000
Salt Conc. (%w/w)*Time (hours)	2	20.681	10.340	27.16	0.000
Exo. LAB*Time (hours)	1	2.067	2.067	5.43	0.029
3-Way Interactions	2	111.515	55.758	146.46	0.000
Salt Conc. (%w/w)*Exo. LAB*Time (hours)	2	111.515	55.758	146.46	0.000
Error	24	9.137	0.381		
Total	35	869.165			

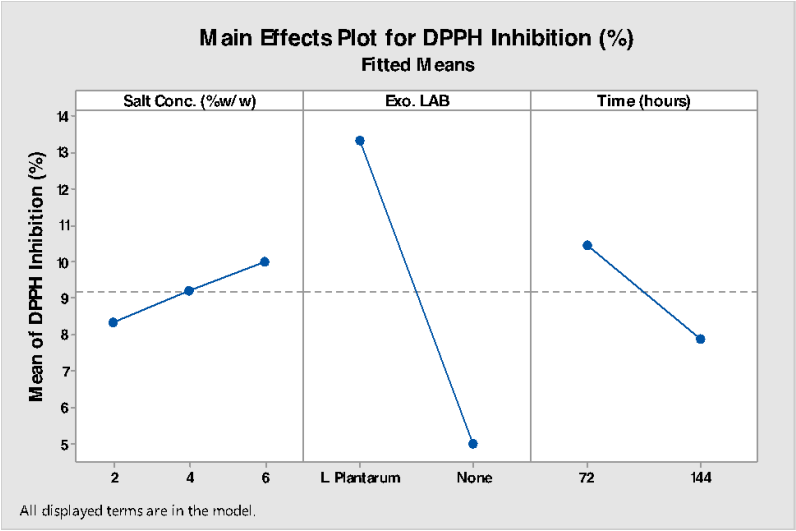

Figure 3. Main effect plot of salt concentration, exogenous LAB and fermentation time for DPPH inhibition

This result indicate that antioxidant activity of fermentation broth of fermented fern is most likely dictated by TPC which is in agreement with statement by Kaur & Kapoor (2001) who

stated that polyphenol compound account for the majority of antioxidant activity of a sample.

Table 7. Correlation between TFC, TPC and antioxidant activity

	TFC (mg QE/g)	TPC (mg GAE/g)
TPC (mg GAE/g)	r = 0.405 p = 0.014	
DPPH Inhibition (%)	r = -0.179 p = 0.295	r = 0.452 p = 0.006

4. Conclusions

As far as we are aware, our present work is the first to report fermentation of fern fronds. We investigated the effect of salt concentration, LAB and fermentation time on TFC, TPC and antioxidant activity of the fermentation broth formed during fermentation process of fern fronds. We found that only salt concentration has significant effect on TFC. While all factors investigated in the present study has significant effect on TPC and antioxidant activity. Correlation analysis showed that there is a moderate correlation between TPC vs TFC and DPPH inhibition vs TPC while no correlation for DPPH inhibition vs TFC. Further investigation of the antioxidant compounds in fermentation broth of fermented fern need to be performed.

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Acknowledgment

The authors would like to acknowledge the Ministry of Health, Republic of Indonesia for funding support with contract number HK.05.01/I1/939.7/2022.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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A COMPARATIVE STUDY ON ANTIOXIDANT AND INHIBITORY EFFECT OF RAW AND BOILED GINGER (*ZINGIBER OFFICINALE* ROSCOE) ON FERROUS SULPHATE INDUCED OXIDATIVE STRESS IN RAT'S TESTES – *IN VITRO*

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https://doi.org/10.34302/crpjfst/2024.16.2.6

Article history:
Received: May 24th, 2023
Accepted: June 23rd, 2024

Keywords:
Ginger;
Oxidative stress;
Antioxidant and thermal processing.

ABSTRACT

It is believed that oxidative stress in the male germ line affects male fertility and has an impact on typical embryonic development. So far, the comparative antioxidant potencies and inhibitory effect between raw and boiled ginger have not been studied in detail and reported. Accordingly, this study aimed at evaluating and comparing the effects of ginger rhizome extracts on pro-oxidant-induced oxidative stress in rat's testes whilst reflecting on the total phenolic content, total antioxidant capacity and total flavonoid content of the extracts. After preparing the raw and boiled ginger's aqueous extracts, the antioxidant activities of the extracts were assessed by means of a spectrophotometric method, and HPLC was used to characterize the extracts. The result of HPLC characterization of these extracts reveals that chlorogenic acid, coumarin, gallic acid, caffeic acid, catechin, shogaol, gingerol, gingerenone, quercitrin, quercetin, kaempferol, and rutin are the major constituents of these extracts. Also, the result revealed that both extracts of ginger rhizome investigated in this study brought about a concentration-dependent decrease in the level of malondialdehyde (MDA) associated with $FeSO_4$- stressed testes homogenates. In addition, the extracts exhibited concentration-dependent NO, OH, DPPH, and ABTS radicals' scavenging abilities. The result of this study also showed that compared to raw ginger extract, boiled ginger extract has a considerably ($p < 0.05$) higher total phenolic content. The high levels of quercetin in these ginger extracts may be the cause of their possible antioxidative effects, and their capacity to scavenge free radicals may be the mechanism by which these potentials are effected. Aqueous extracts of ginger rhizome could be considered as good antioxidant therapeutic candidates for oxidative stress linked with male infertility.

1.Introduction

Infertility, being one of the challenges faced by humans is impacted by behavioral, genetic, genotoxic and environmental variables that decrease spermatogenesis at different phases and result in male infertility (Kamiński *et al.,* 2020). Many chemical medications have been used to treat infertility, however some of them have quite a few negative effects. The search for alternative therapies with less side effects and toxicity is therefore necessary (Ghajari *et al.,* 2022). Because it is efficient, affordable, safe, and readily available, herbal medicine and medicinal plants are being employed to treat a variety of diseases. Additionally, they contain strong antioxidant properties that can scavenge free radicals and enhance spermatogenesis (Rehman *et al.,* 2022). Infertility affects one in five males in Nigeria. Lately, quite a number of researches have been done to investigate the role

that oxidative stress (OS), one of the main factors affecting reproductive status, plays in male infertility. Reactive oxygen species (ROS) are required to preserve standard cell activities at physiological levels since oxygen is crucial for life (Sies *et al.,* 2022). On the other hand, oxygen breakdown products like ROS can be harmful to cell survival and function (Napolitano *et al.,* 2022). Although, cytosolic and mitochondrial free iron has the potential to significantly impair cellular function and integrity by acting as a catalyst in the synthesis of ROS, which has the ability to annihilate cellular nucleic acids, lipids, carbohydrates, and proteins, iron is physiologically required as a constituent of proteins, part of which are enzymes (Carocci *et al.,* 2018). By participating in Fenton reaction, iron (II) produces hydroxyl radical (*OH) when it reacts with hydrogen peroxide (H_2O_2), whereas iron (III) can restore iron (II) that takes part in the Fenton reaction when in reaction with superoxide (Charkoudian *et al.,* 2006). An excessive amount of ROS can cause a direct damage to fatty acids in biological membranes and trigger peroxidation of lipid. Spermatozoa constantly struggle with oxygen paradox, much like other aerobic cells (Ogbuewu *et al.,* 2010) due to the fact that it is rich in fatty acids that are polyunsaturated in nature, which makes them vulnerable to ROS attack and causes a drop in sperm motility (Udipi *et al.,* 2012).

The byproduct of lipid peroxidation is malondialdehyde (MDA). ROS break down polyunsaturated fatty acids during this process. The synthesis of this electrophilic aldehyde is utilized as a biomarker to quantify an organism's level of oxidative onslaught (Murray *et al.,* 2000). Nevertheless, increasing the antioxidant status of the body through increased fruit and vegetable eating is the most feasible and likely method to combat degenerative diseases. A significant class of secondary metabolites produced by plants is phenolic chemicals.

Since ancient times, numerous plants and their parts have been utilized to treat a variety of ailments. A member of the *Zingiberaceae* plant family, ginger (*Zingiber officinale*) is regarded as one of the most popular culinary seasonings. It has also been used medicinally for a very long time in Chinese traditional medicine (Kumar *et al.,* 2011). Ginger is grown throughout West Africa, the Caribbean and south-east Asia, with India and China thought to be its primary sources (El-hameed *et al.,* 2019). Several bioactive substances that could potentially have biological and pharmacological effects are present in ginger (Tomaino *et al.,* 2005).

Gingerols, protodioscin, saponins, zingibrene, gingerdiol, and schogaols are the most significant components of ginger (Sakr and Badawy, 2011). Ginger, is frequently used to flavor food and treat a variety of illnesses. The presence of many bioactive chemicals in ginger may be related to its ability to promote health. According to Palatty *et al.* (2013), ginger has significant anti-inflammatory, antiemetic, antithrombotic, anti-hepatotoxic, and antioxidant properties that can help with nausea and vomiting (El-Morsy Ibrahim and Al-Shathly, 2015). Similar to natural antioxidants like retinol, ascorbic acid and tocopherol, which can shield DNA from damage and other essential biomolecules from oxidation, improve the quality of sperm and thus fertility in men, the antioxidant content of herbal medicines is crucial for enhancing antioxidant defense and reducing oxidative state (Rajeev *et al.,* 2006). The antioxidant (El-hameed *et al.,* 2019) and androgenic activity (Kamtchouing *et al.,* 2002) of ginger may be responsible for its beneficial impact on male fertility. Gingerol, which gives ginger its flavor (Semwal *et al.,* 2015), shogaols, and certain phenolic ketone derivatives are the major antioxidants in ginger (Pittler, 2004).

Although numerous studies claimed that ginger enhanced sperm motility, viability, and testosterone concentration while lowering malondialdehyde (MDA), which in turn reduced lipid peroxidation and increased reproductive success (Khaki et al., 2009; Memudu *et al.,* 2012; Ghlissi *et al.,* 2013); furthermore, Morakinyo *et al.* (2008) hypothesized that ginger's protodioscin and saponins could raise levels of libido, luteinizing hormone (LH), and testosterone, which was crucial for the traditional medical approach to treating sexual

dysfunction; in addition, Sabik and El-Rahman (2009) discovered that ginger could raise testosterone, estrogen, and pregnenolone levels in men as well as their sexual potency; the potential of ginger in the treatment and averting oxidative stress-related reproductive problems is yet poorly understood. Therefore, in this *in vitro* study, we evaluate and compare the effects of raw and boiled ginger rhizome extracts on pro-oxidant-induced oxidative stress in rat's testes.

2. Materials and Methods

2.1. Chemicals and reagents

The water utilized for this investigation was glass distilled, and all of the chemicals and reagents were procured from Sigma-Aldrich Chemie GmBH and Sigma-Aldrich Co.

2.2. Sample Collection

Fresh ginger rhizomes were purchased fresh from a local market in Ilorin metropolis, Kwara State, South Western Nigeria. The samples were authenticated in the Department of plant Science and Biotechnology, Ekiti State University, Ado Ekiti, Nigeria with voucher number UHAE 2021054.

2.3. Sample preparation

Fresh ginger rhizomes of the Roscoe kind were cleaned, peeled, and then cut into cubes. It was then divided into two equal pieces. The initial portion of ginger was air dried at room temperature (37 degrees Celsius) to make raw ginger powder, which was then made by grinding raw dried ginger into powder. The second portion of the prepared ginger was cooked at medium heat for one hour to make the boiled ginger powder. The ratio used is 1 gram of ginger rhizome to 100 milliliters of distilled water. After boiling, boiled ginger together with the water was blended in a blender to create a homogeneous mixture. Once blended, the mixture was lyophilized to obtain boiled dried ginger powder. The two ginger samples (raw and boiled) were stored in tightly closed plastic jars and kept in freezer until use.

2.4. Preparation of aqueous extract

Separately, the ground ginger samples were soaked in distill water for 48 hours before being filtered. The crude extract needed for HPLC-DAD characterization was then obtained by freeze drying and rotary evaporation of the filtrate.

2.5. Ethical clearance

The use of animals in this research was authorized by the Ekiti State University ethics committee (reference number: ORDI/AD/EAC/23/127). The animals were treated humanely at all times throughout the experiments, as outlined in the Guide for the Care and Use of Laboratory Animals published by the National Academies of Science and the National Institutes of Health.

2.6. Experimental animals

Male Wistar strain albino rats weighing 200–300 g were purchased from the breeding colony of Department of Veterinary Medicine, University of Ibadan, Nigeria. Rats were maintained at 25°C, on a 12 h light/12 h dark cycle, with free access to food and water. They were acclimatized under these conditions for 1–2 week before the experiment. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

2.7. Preparation of Tissue Homogenates

Under mild diethyl ether anaesthesia, the rats were decapitated, and the testes and liver tissues were separately swiftly removed, stored on ice, and weighed. This tissue was separately homogenized in cold saline (1/10 w/v) in a Teflon glass homogenizer (Mexxcare, mc14 362, Aayu-shi Design Pvt. Ltd., India) with around 10 strokes at roughly 1200 rev/min. The homogenate was centrifuged for 10 minutes at 3000 g (KX3400C Kenxin International Co. Hong Kong) to produce a pellet that was discarded and a low-speed supernatant (SI) that was preserved for the lipid peroxidation assay (Belle et al., 2004).

2.8. Lipid peroxidation and thiobarbituric acid reactions

The Ohkawa *et al.* (1979) method was used to conduct the lipid peroxidation assay. In a nutshell, one hundred milliliter of a fraction was combined with thirty milliliters of freshly generated 250 mM $FeSO_4$, thirty milliliters of zero-point one molar Tris-HCl buffer, and one hundred milliliter of ginger aqueous extract. Prior to incubation at thirty-seven degrees Celsius for an hour, the volume was filled to a capacity of 300 L with water. By adding three hundred liters of eight-point one percent sodium dodecylsulphate to the solution that contains the fraction, the colour reaction was developed. Following this, five hundred liter each of acetic acid/hydrochloric acid (with a pH of 3.4) and five hundred liters of zero-point eight percent TBA were added. For one hour, the resulting solution was incubated at one hundred degrees Celsius. At 532 nm, the reactive species of thiobarbituric acid were evaluated for absorbance. MDA output was measured as a percentage (%). Using non-linear regression analysis, the EC_{50} (concentration of extract needed to suppress 50% of malondialdehyde generated) value was determined.

2.9. DPPH free radical scavenging ability

The extract's capacity to scavenge DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical was assessed according to Gyamfi *et al.,* (1999). In a nutshell, the extracts were diluted appropriately (one milliliter) and combined with one milliliter of zero point four millimolar DPPH radical-containing methanolic solution. This reaction mix was then allowed to stand in the dark for thirty minutes, and the absorbance was taken at 516 nm. The test samples were not used in the control, which was performed using a 2 mL DPPH solution. DPPH free radical scavenging ability was calculated using the mathematical expression:

DPPH scavenging ability (%) = [(absorbance of control − absorbance of samples)/ absorbance of control] × 100. (1)

2.10. 2,2-azinobis(3-ethylbenzo-thiazoline-6-sulfonate) (ABTS*) scavenging ability

The ginger rhizome extracts' capacity to scavenge ABTS* was assessed using the Re *et al.* (1999) method. In the absence of light, an aqueous solution of ABTS (7 mmol L) was combined with 2.45 mmol L of $K_2S_2O_8$ to generate ABTS*.

2.11. Degradation of deoxyribose (Fenton's reaction)

Using the Halliwell and Gutteridge (1981) method, the ginger rhizome extracts' capacity to stampede iron (II)/hydrogen peroxide -triggered breakdown of deoxyribose was tested. In a nutshell, newly obtained extracts of ginger (zero – one hundred liter) was introduced to the reaction solution consisting of eight hundred liter of distilled water, one-hundred and twenty liters of twenty millimolar deoxyribose, four hundred liters of zero-point one molar phosphate buffer, forty liters of twenty millimolar hydrogen peroxide, and forty liters of five-hundred millimolar of Iron (II) tetraoxosulphate (VI). After 30 minutes of incubation at thirty-seven degrees Celsius, the reaction mixture was halted by introducing five hundred liters of two-point eight percent trichloroacetic acid solution, which was then followed by four hundred liters of zero-point six percent TBA solution. The tubes were then subjected to heat for 20 minutes in water bath at one hundred degrees Celsius. The extracts' % capacity to neutralize hydroxyl radicals was determined using the mathematical expression below whilst the absorbance was taken at five hundred and thirty-two nanometers:

[(Absref - Abssample) / Absref] x 100 (2)

2.12. Nitric oxide scavenging assay

The Igbinosa *et al.* (2011) method was used to assess the extracts' ability to scavenge nitric oxide. To (100-400 L) of plant extract, 25 mM sodium nitroprusside was added in one milliliter of zero-point five phosphate buffer saline (having a pH of 7.4) before being vortexed. The resulting solution was then incubated for two hours a thirty-seven degree Celsius before being combined with one milliliter of Griess reagent

(equivalent quantities of one percent sulphanilic acid produced in two percent orthophosphoric acid and 0.01% (w/v) naphthylenediamine dichloride) before being incubated for 30 minutes at twenty-five degrees Celsius. The absorbance was quantified at 546 nm, and the mathematical expression below was used to determine the amount of the inhibitory effect of the ginger rhizome extracts on NO radical:

NO scavenging activity (%) = [(Abs control – Abs sample)] / (Abs control) x 100

3. Results and Discussion

Peroxidation of lipids associated with cellular membranes is one of the main causes of cellular damage in living organisms exposed to oxidative onslaught (Repetto *et al.,* 2012). As demonstrated in this study, incubating rat testes homogenates in the presence of iron (II) tetraoxosulphate (VI) ($FeSO_4$) led to a noticeably higher MDA level (263.24 % Control) in the testes (Figure 1). These results are consistent with our past reports on how Fe^{2+} interacts with the testes, in which it was demonstrated that Fe^{2+} is a very strong instigator of lipid peroxidation in the testes (Akomolafe *et al.,* 2012). This finding is somewhat in-line with the earlier study by Akintunde *et al.* (2013), which found greater levels of MDA in rat testes subjected to repeated consumption of leachate samples containing higher quantities of mixed metals than allowed. Catalytically, iron (II) can drive one-electron transfer events that produce reactive hydroxyl radical (•OH), which is produced from hydrogen peroxide via the Fenton reaction; which may account for the enhanced peroxidation of lipid in the presence of $FeSO_4$ observed in figure 1. Additionally, iron breaks down lipid peroxides, producing radicals of peroxides and alkoxides, as a result, which encourages the progression of peroxidative damage of lipids (Zago *et al.,* 2000). The MDA level of the $FeSO_4$-stressed testes homogenate did, however, significantly decrease upon the addition of raw and boiled ginger aqueous extract, with the littlest MDA synthesis recorded at the highest concentration of all extracts (1.00 mg/ml) (Figure 1). The inherent chemicals may form complexes with iron (II) to prevent them from driving the peroxidation of lipids, which would explain the mode of inhibition of $FeSO_4$-induced peroxidation of lipid, or possibly the plant chemicals have sucked up radicals liberated in Fe^{2+}-driven reaction (Oboh *et al.,* 2007).

Table 1. Effective concentration causing 50% antioxidant ability (EC_{50} values) of 1,1-diphenyl-2 picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS*), hydroxyl (OH*), nitric oxide (NO*) radical scavenging abilities of and inhibition of FeSO4-Induced lipid peroxidation of aqueous extract by aqueous extract of raw and boiled ginger rhizome.

	EC_{50} values (µg/ml)		
Parameters	Raw	Boiled	BHT
DPPH Radical Scavenging Ability	96.78 ± 2.30^a	78.25 ± 1.20^b	51.70 ± 2.13^c
ABTS* Radical Scavenging Ability	81.96 ± 1.20^a	57.05 ± 2.01^b	42.90 ± 2.41^c
NO* Radical Scavenging Ability	66.74 ± 2.12^a	48.07 ± 1.14^b	36.78 ± 2.08^c
OH* Radical Scavenging Ability	77.11 ± 2.04^a	66.80 ± 1.11^b	49.85 ± 0.89^c
FeSO4-induced lipid peroxidation (Testis)	780.7 ± 1.18^a	592.7 ± 2.12^b	394.2 ± 2.13^c
FeSO4-induced lipid peroxidation (Liver)	564.0 ± 2.20^a	511.9 ± 1.32^b	493.5 ± 1.21^c

Values represent means ± standard deviation of triplicate readings. Superscripts with the same letters along the same column are not significantly different ($P > 0.05$). BHT = Butylated hydroxytoluene

According to the EC_{50} value (Table 1), the concentrations of the raw and boiled ginger rhizome extracts that will have a 50% inhibitory effect on $FeSO_4$-induced peroxidation of lipid in testes, are 780.7 µg/ml and 592.7 µg/ml respectively. This speculates that the boiled ginger rhizome extract has a greater potential to reduce lipid peroxidation. Our discoveries are in line with a related study by El-hameed *et al.* (2019), who demonstrated that aqueous ginger rhizome extract reduces lipid peroxidation and enhances male rabbit fecundity.

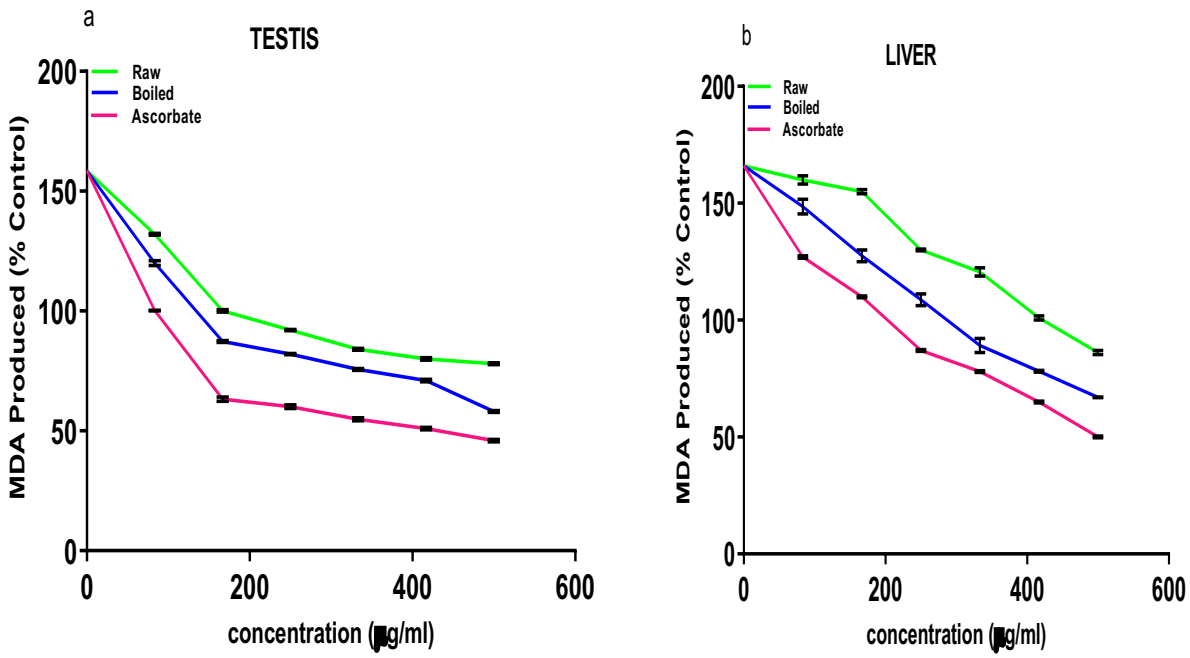

Figure 1. $FeSO_4$-induced MDA inhibition of aqueous extract of raw and boiled ginger rhizome in rat (a) testicular and (b) liver homogenates. Values represent means ± standard deviation of triplicate readings.

Table 2. Phenolic constituents of aqueous extract of raw and boiled ginger rhizome

Constituents	**Fresh ginger rhizome (mg/g)**	
	Raw	**Boiled**
Chlorogenic acid	12.96 ± 0.83^{a}	14.45 ± 0.32^{a}
Coumarin	29.05 ± 1.24^{b}	29.64 ± 0.07^{b}
Gallic acid	8.32 ± 0.67^{c}	8.72 ± 0.89^{c}
Caffeic acid	3.41 ± 0.43^{d}	3.72 ± 0.46^{d}
Catechin	2.17 ± 0.43^{e}	2.46 ± 0.12^{e}
Shogaol	0.66 ± 0.31^{f}	0.79 ± 0.14^{f}
Gingerol	0.72 ± 0.37^{f}	0.90 ± 0.29^{f}
Gingerenone	0.09 ± 0.02^{g}	0.69 ± 0.22^{f}
Quercitrin	0.73 ± 0.03^{f}	1.10 ± 0.03^{f}
Quercetin	95.14 ± 0.19^{h}	97.09 ± 0.04^{g}
Kaempferol	25.96 ± 0.64^{i}	28.42 ± 0.48^{b}
Rutin	7.20 ± 0.22^{c}	9.51 ± 0.33^{c}
Luteolin	2.02 ± 0.01^{e}	3.61 ± 0.31^{d}

Values represent means ± standard deviation of triplicate readings. Superscripts with the same letters along the same row are not significantly different ($P > 0.05$).

Many significant phenolic and non-phenolic plant chemicals have been linked to the antioxidant capabilities of plants (Cheplick *et al.,* 2007). In this study, the ginger extracts were characterized using HPLC-DAD, and the results are presented in table 2. The major components of the raw and boiled ginger rhizome aqueous extracts were found to be chlorogenic acid, coumarin, gallic acid, caffeic acid, catechin, shogaol, gingerol, gingerenone, quercitrin, quercetin, kaempferol, and rutin, with quercetin being the predominating phytochemical (95.14 ± 0.19 mg/g and 97.09 ± 0.04 mg/g for raw and boiled ginger rhizome extracts respectively). The extremely high quantity of quercetin in the ginger rhizome extracts may therefore be responsible for protecting testes tissue from $FeSO_4$-induced lipid peroxidation. Overall, the result shown in table 2 indicates that the boiled ginger extract contains more of each phytochemical constituent than the raw ginger extract does. This might have to do with the rupture of cells brought on by boiling them at a high temperature, which eventually causes the release of cellular components.

Table 3. Ferric Reducing Antioxidant Property (FRAP) and Total Antioxidant Capacity (TAC) of aqueous extract of raw and boiled ginger rhizome

	Ginger rhizome		
Parameters	Raw	Boiled	BHT
Ferric Reducing Antioxidant Property (mg/g)	0.50 ± 0.01^{a}	0.75 ± 0.01^{a}	1.04 ± 0.01^{b}
Total Antioxidant Capacity (mg/100mg)	9.46 ± 0.66^{a}	11.92 ± 0.86^{b}	15.65 ± 0.93^{c}

Values represent means ± standard deviation of triplicate readings. Super-scripts with the same letters along the same column are not significantly different (P > 0.05). BHT = Butylated hydroxytoluene.

Table 4. Total phenolic and total flavonoid contents of aqueous extract of raw and boiled ginger rhizome

	Ginger rhizome	
Parameters	Raw	Boiled
Total Phenolic content (mgGAE/g)	30.57 ± 1.59^{a}	35.97 ± 0.35^{b}
Total Flavoniod content (mgQE/g)	20.17 ± 0.71^{a}	29.58 ± 0.63^{b}

Values represent means ± standard deviation of triplicate readings. Superscript with the same letters along the same column are not significantly different (P > 0.05). GAE = Gallic acid equivalent. QE = Quercetin equivalent.

The ferric reducing antioxidant property, total antioxidant capacity, as well as the total flavonoid and phenolic contents of boiled and raw ginger rhizome extracts is presented in Tables 3 and 4. When compared to the raw ginger extract (30.57 mg/QE), the boiled ginger extract had a considerably ($p < 0.05$) higher phenolic content (35.97 mg/QE). The total flavonoid content, ferric reducing antioxidant property and total antioxidant capacity of these ginger extracts showed the same trend of results. Strong antioxidants, phenolic substances can neutralize free radicals, mask metal catalysts in form chelation, and activate antioxidant enzymes in addition to impeding oxidases (Cheplick *et al.,* 2007). According to Ozgen *et al.* (2016), quercetin, the bioflavonoid that predominates in these extracts, is a potent antioxidant whose effects have been attributed to its aromatic rings, which contains not less than one hydroxyl group each, linked by a bridge of three carbons to form a heterocyclic six-membered ring (Hanasaki *et al.,* 1994). The release of these compounds into water during boiling may account for the drop in total phenolic and total flavonoid concentrations seen in the boiled ginger extract.

In order to protect cells from damage, antioxidants can either stop the synthesis of free radicals, counterbalance or scavenge those synthesized naturally by the body, or lessen or chelate the transition metal content of food (Ami *et al.,* 2003; Kamdem *et al.,* 2013). The ability of these extracts to neutralize DPPH, ABTS, OH, and NO radicals was evaluated in an effort to identify the primary mechanism by which the aqueous extracts of raw and boiled ginger rhizome protect testes tissue against $FeSO_4$-induced lipid peroxidation. Figures 2, 3, 4 and 5 illustrate the concentration-dependent radical scavenging properties of ginger rhizome boiled and raw aqueous extracts.

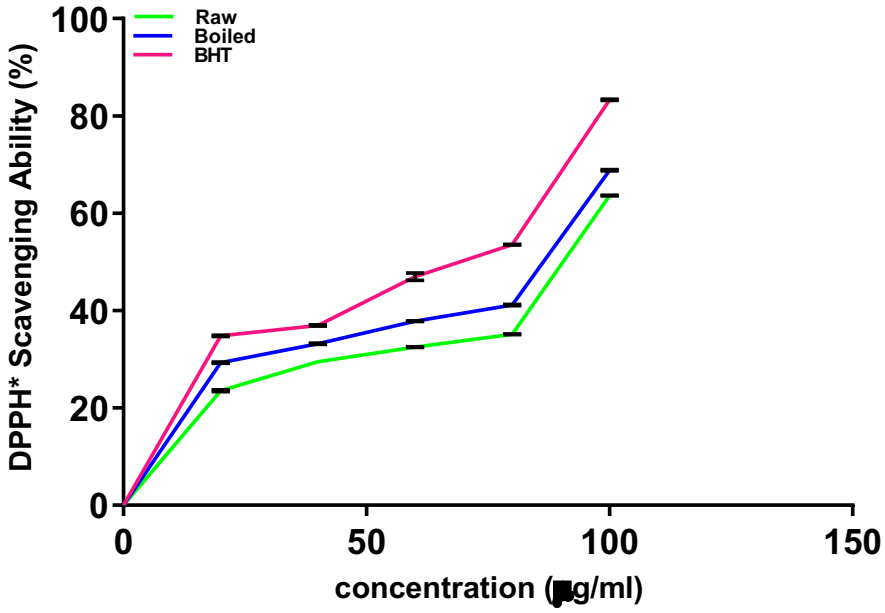

Figure 2. 1,1-diphenyl-2 picrylhydrazyl (DPPH) radical scavenging ability of aqueous extract of raw and boiled ginger rhizome. Values represent means ± standard deviation of triplicate readings.

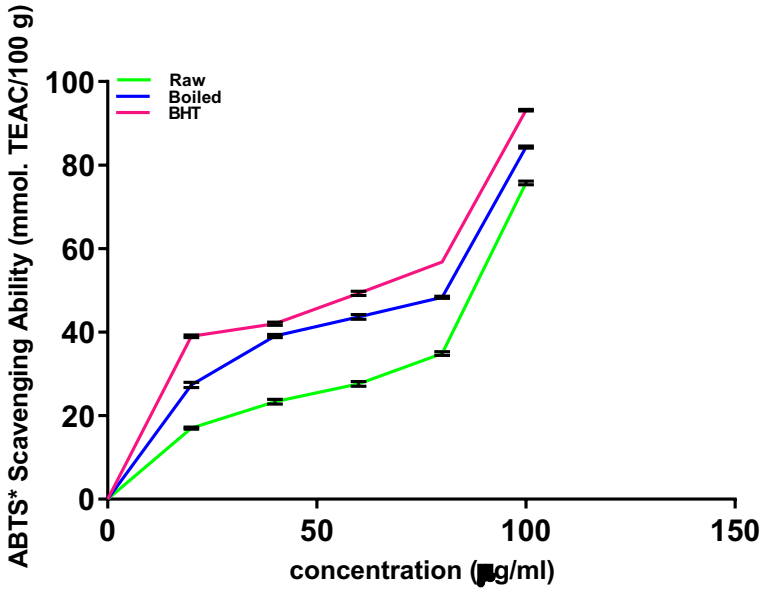

Figure 3. 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging ability of aqueous extract of raw and boiled ginger rhizome. Values represent means ± standard deviation of triplicate readings.

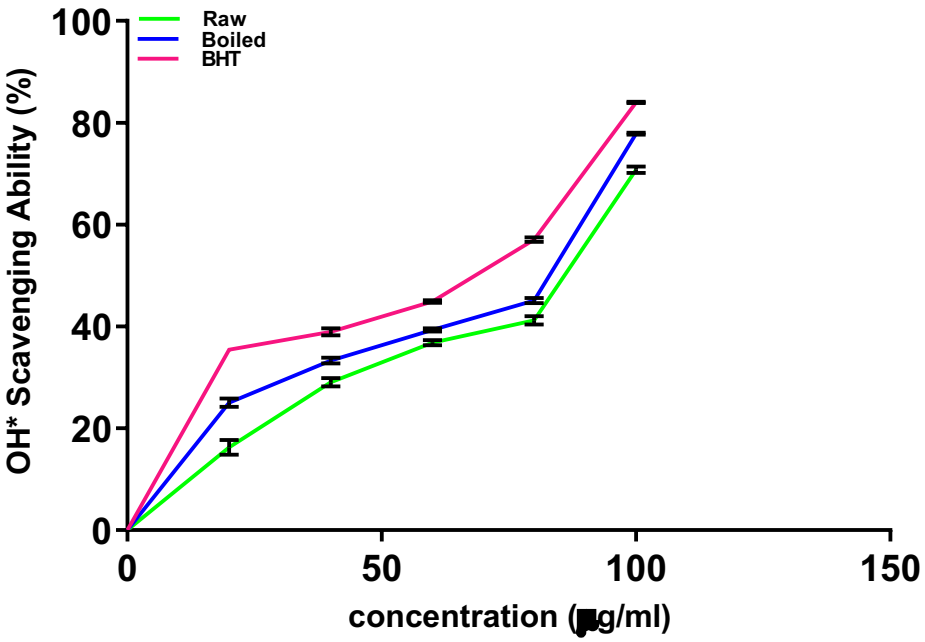

Figure 4. Hydroxyl (OH) radical scavenging ability of aqueous extract of raw and boiled ginger rhizome. Values represent means ± standard deviation of triplicate readings.

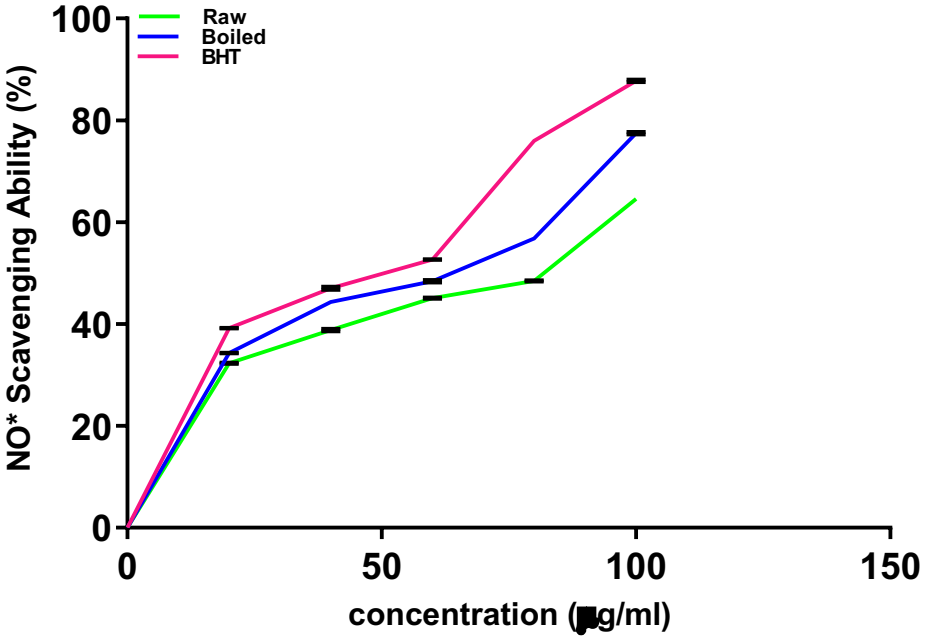

Figure 5. Nitric oxide (NO) radical scavenging ability of aqueous extract of raw and boiled ginger rhizome. Values represent means ± standard deviation of triplicate readings.

A key antioxidant method of action is thought to be the hinderance of the chain-starting step (Gulcin, 2020). DPPH, a type of free radical, changes to a diamagnetic molecule upon its acceptance of electron or hydrogen (Je *et al.,* 2009). When defining antioxidant action that has to do with free radical-scavenging, tendencies of electron or hydrogen donation are

crucial considerations (Hu *et al.,* 2000). Our research showed that ginger rhizome extracts, both boiled and raw, neutralized DPPH in a concentration-dependent fashion as presented in Figure 2. But boiled ginger extract is more effective at scavenging DPPH radicals than raw extract is (Table 1). Due to its unusual electron configuration, DPPH exhibits a prominent absorption band in visible spectroscopy at a wavelength of 517nm. The decolorization that results is stoichiometric with regard to the quantity of electrons accepted as the absorption disappears, as the odd electron pairs up in the presence of free radicals (Fauconneau *et al.,* 1997). The ability of both extracts to neutralize DPPH radicals can therefore be linked to their potential to donate hydrogen. The extracts' capacity to scavenge free radicals is what causes the DPPH radical to be bleached.

As in this work, the capability to neutralize DPPH radicals is frequently used in various experimental activities that examine the antioxidant potential of a test compound. However, sample solubility and color interference have been observed to limit its use as a measure of antioxidant capacity (Dorman *et al.,* 2004). Therefore, in order to assess the antioxidative capacities of the ginger extracts, we conducted the ABTS assay as a complementary assay for free radical scavenging ability. According to Shalaby and Shanab (2013), the blue-green dye, ABTS is predominantly reactive with sulfhydryl group-containing compounds, phenolics, and other antioxidants. ABTS is a comparatively stable free radical. Its test, which is essentially a decolorization assay, relies solely on the formation of the ABTS radical monocation. Instead of the free radical formation occurring continuously at the introduction of antioxidant compound, in this case, the radical cation is typically produced prior to the addition of the antioxidant test component. Table 1's EC_{50} values for the extracts demonstrate that the boiled ginger rhizome has more capacity to scavenge free radicals than its raw counterpart. The enhanced ability of the extracts to scavenge ABTS* radicals (Figure 3 and Table 1) may be linked to the polyphenolics therein's capacity to donate hydrogen, which finally stops or inhibits the generation of ABTS*. The higher ABTS* scavenging capability of the boiled extract could be due to a stronger capability of its inherent polyphenolics to donate hydrogen, which eventually prevent/inhibit the production of ABTS radical.

Additionally, this study's findings demonstrated that the extracts exhibited a concentration-dependent capacity to neutralize the OH* generated by the breakdown of deoxyribose in the Fenton reaction (Figure 4 and Table 1). This finding shows that the extract can be utilized in place of synthetic antioxidants to treat the hydroxyl radical's oxidative activity. However, as shown in table 1, the boiled ginger rhizome extract demonstrated a much better OH* radical scavenging ability than the raw ginger rhizome extract based on the EC_{50} values of these extracts. Nitric oxide (NO) radical, a highly reactive substance, produced from sodium nitroprusside at physiological pH has been shown to have the capability of altering the structural components of cell and thus its functionality (Ashokkumar *et al.,* 2008). The ginger rhizome's aqueous extracts reduced NO* production in a concentration-dependent fashion (Figure 5). The ability of the ginger rhizome extracts to scavenge reactive oxygen species and its derivatives is what causes them to have an inhibitory effect on NO radicals (Packer, 1997). The EC_{50} values in Table 1 show that boiled ginger extract has a considerably ($p < 0.05$) stronger NO radical scavenging activity than raw ginger extract.

4. Conclusions

Abundance of quercetin in these aqueous ginger extracts may be responsible for extracts' ability to protect testes tissues from $FeSO_4$-induced lipid peroxidation. This ability may be due to quercetin's capacity to scavenge free radicals. Reactive oxygen species-related reproductive cellular damage may be managed or treated with the help of this plant.

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Ethical clearance

The use of animals in this research was authorized by the Ekiti State University ethics committee (reference number: ORDI/AD/EAC/23/127). The animals were treated humanely at all times throughout the experiments, as outlined in the Guide for the Care and Use of Laboratory Animals published by the National Academies of Science and the National Institutes of Health.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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EXTRACTION OF ESSENTIAL OIL FROM AJWAIN SEED BY USING SOLVENT EXTRACTION METHOD AND ITS UTILIZATION IN VALUE ADDED BAKERY PRODUCT

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https://doi.org/10.34302/crpjfst/2024.16.2.7

Article history:
Received: January 1st, 2024
Accepted: June 3rd, 2024

Keywords:
Ajwain, essential oil;
Bioactive compound;
Nutritional;
Total phenolic content (TPC);
Total flavonoid content (TFC).

ABSTRACT

In the present study, nutritional composition of ajwain seed, bioactive components of different solvent extracted ajwain essential oil and nutritional composition, textural attributes and sensory properties of ajwain essential oil fortified cookies were investigated. Ajwain seed has shown to have higher amount of carbohydrate (55.38%), fat (10.08%) and protein (15.73%) and also significant ash content (7%) and crude fibre content (18.98%). Furthermore, it contains potential functional qualities, including TFC (2.0608 mg quercetin/gm), TPC (126.021 mg GAE/100 gm), and antioxidant content of 36.36 g/ml. Essential oil extracted by using solvent extraction method with the application of different solvents and its combination was analysed in terms of yield and characterization of bioactive constituents using GC-MS technique. Maximum yield was found in combination of n-hexane and petroleum ether (3.2%). Thymol was found as dominant bioactive constituents both by using petroleum ether (75.831%) and combination of n-hexane and petroleum ether (71.372%) whereas p-cymene showed the least by using combination of combination of n-hexane and petroleum ether (1.602%). Nutritional constituents in terms of carbohydrate (51.89%), fat (31.004%), protein (7.14%), crude fibre (3.14%), Ash (2.59%) and moisture content (4.23%) of 1 ml essential oil fortified ajwainjeera cookies were found as satisfactory. Moreover, fortified cookie was shown best sensory acceptability using 9-point hedonic rating test. Texture profile analysis of essential oil fortified cookie results satisfactory values. Experimental investigation revealed that antimicrobial potential of the essential oil and essential oil fortified cookie against *Staphylococcus aureus, Bacillus cereus,* and *E. coli* were highly satisfactory. (If we are reducing word count then some of part missed in abstract.)

1. Introduction

One of the herb and spice plants in this family is *Trachyspermumam*mi L. which is generally known as ajwain. Ajwain is an annual herbaceous plant that is widely distributed and harvested in Afghanistan, Egypt, India, and European countries. Ajwain seeds are oval in form, yellowish-brown to greyish in color, and wrinkled(Gaba et al., 2019). Ajwain seed is bitter and pungent, and it also contains anthelmintic, carminative, digestive, and anti - ulcer effects(Bairwa et al., 2012). One of the most active and beneficial components of medicinal plants that may be isolated from various parts is essential oil. The herbal cure ajwain is often used to treat a wide range of illnesses in both people and animals. chemicals that are antibacterial and antifungal. Moreover, it contains a significant quantity of fat, protein, ash, and carbohydrates. Many phytochemicals,

including as alkaloids, chalcones, coumarins, flavonoids, glycosides, saponins, steroids, and tannins, have been shown to be present in ajwain(Shahrajabian et al., 2021).Due to its distinctive odor and bitter flavor, ajwain is used as a seasoning in curries. Its seeds are used as food flavorings and preservatives (Awais Hanif et al., 2021).Ajwain is a well-liked spice that may also be used in medical practices to treat febrile illnesses, dyspepsia, stomach issues, amoebic ailments, and inflammatory disorders(Chahal et al., 2017). One of the most powerful and potent elements of medicinal plants that may be isolated from various sections is essential oil. These mostly oil-soluble compounds comes from major components including leaves, seeds, and bark(Zarshenas et al., 2014).EOs are very complex mixtures that may include hundreds of distinct aromatic elements. Even though they are not technically oils, essential oils can have a poor water solubility comparable to oils (Mahian& Sani, 2016).Many biological activities, such as antimutagenic, antibacterial, antimicrobial and antioxidant are present in ajwain essential oils(Chahal et al., 2017).

2. Materials and methods

2.1. Materials source and preparation

Fresh Ajwain seeds (*Trachyspermumammi* L.) were collected from a local farm in Timbi village, Bhavnagar, Gujarat. For further examination, the seeds were air dried for 8 days before being placed in a plastic bag with an airtight seal.

2.2. Determination of Proximate Composition

The sample's Ash, fat, crude fiber, and Moisture content have been evaluated using (AOAC 2000). For the determination of ash content, we take a 1 gm sample and put it in a muffle furnace. This was done by ashing at 550^0 C for 3 hours. The crude protein content has been identified using the Kjeldahl technique. A standard (AOAC 978.10) approach was used to determine the amount of crude fiber. Model FES04E crude fiber analysis equipment was used to find crude fiber. The Soxhlet extraction technique was used to evaluate the crude fat content. The difference between the total of all the proximate components and 100% was used to calculate the amount of total soluble carbohydrates.

2.3.Determination of TPC, TFC and Antioxidant activity

The total phenolic content in the ajwain seed was determined by using the Folin-Ciocalteu reagent method, as described by (Wanyo et al., 2014).Ajwain powder was dissolved in methanol at a concentration of 1 mg/ml (w/v) by adding 0.010 g of the powder to 10 ml of methanol. Add 9.6 ml of distilled water to 0.4 ml of an ajwain extract solution in a test tube. 2.5 ml of Folin-Clocalteu's reagent (10%) was then added. 2 ml of Na2CO3 (7.5% w/v) was added after the reaction had been going for 5 minutes. Three duplicates of the test samples were created. After that, it was left to develop for 30 minutes without light. Finally, a UV-VIS spectrophotometer was used to assess the absorbance of test samples at 730 nm.

Using the aluminium chloride method, the total flavonoid content of the Ajwain seed extracts was determined. (Khatiwora et al., 2010). 6 ml of several methanolic extracts were poured into the various test tubes to determine the total flavonoid concentration. 0.2 mL of correctly mixed 10% AlCl3 should then be added. A further addition of 0.2 ml of 5% Na-K tartrate solution was made. The last addition was 5.6 mL of distilled water. After 30 minutes of incubation, the solution was well mixed, and the absorbance at 415 nm was measured using a UV-V spectrophotometer. Using the quercetin calibration curve, the quantities of quercetin in the test samples were determined and expressed as mg of quercetin equivalent per g of sample. The method provided was used to determine the antioxidant activities. (Saikia et al., 2016). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical's ability to scavenge free radicals was used to assess the antioxidant activity of ajwain extracts. First, 4 mg of DPPH were dissolved in 100 ml

of methanol to create a DPPH solution. Test tubes were filled with a 0.4-ml solution of the methanolic extract. The mixture included 5.6 ml of DPPH solution. 30 minutes were given for the mixture to remain at room temperature after it had been mixed and kept in the dark. Using a spectrophotometer, the solution's absorbance at 515 nm was determined.

2.4. Evaluation of Antimicrobial Activity

Antibacterial properties in the isolates are allowed to permeate into the medium and interact in a plate freshly spread with the test organisms. If the test organisms are sensitive, it will result in the production of circular zones of inhibition (Murugan et al., 2018). The (MTCC 7190), *Escherichia coli* (MTCC 443) and *staphylococcus aureus* (MTCC 7190) strains were then serially diluted to 10^{-2}, and 0.2ml *E. Coli*, *bacillus cereus* and *S. Aureus* were dispersed separately on nutrient agar (himedia) plates. Then, drill a well and pour ajwain essential oil and cookies methanolic extract into it and using paraffin, seal the agar plates and place them in the incubator for 24-48 hours at 37°C.

2.5. Essential oil extraction of Ajwain seed

For extracting essential oil from ajwain seed, we are using the Soxhlet extraction method. In this, we take an ajwain powered sample in a thimble and put it into a solvent chamber, and then we set the temperature according to the solvent boiling point for 5–6 hours. After extracting a sufficient amount of oil from the solvent, we can go for distillation to remove the solvent by using a rotary evaporator.

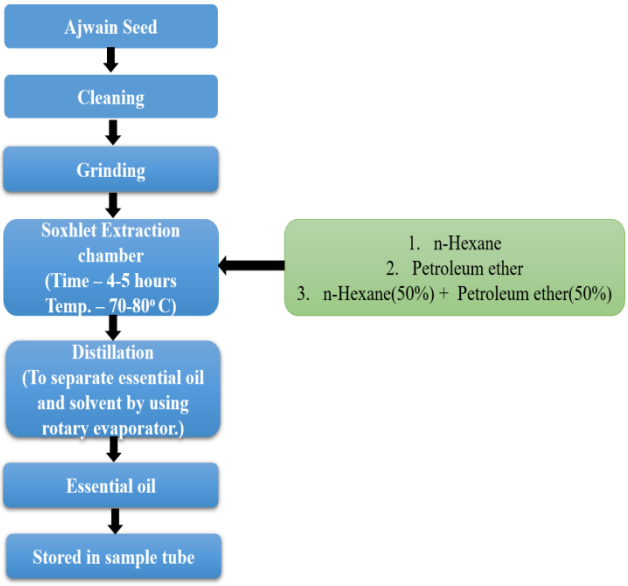

Figure 1. Essential extraction process.

2.6. Characterization of bioactive constitute of ajwain essential by using GC-MS

The identification of different bioactive constituents of Ajwain essential oil that were extracted by different solvents and their combinations, like n-hexane, petroleum ether, and mixtures of both solvents, was carried out using GC (model Clarus 680 GC), equipped with a HP-5 capillary column (60 meter long, 250 m diameter), and a mass spectrometer (model Clarus 600 MS). The analysis was conducted with a helium carrier gas flow rate of 2 ml/min and an injector temp. of 280°C. The column temperature was initially 60°C for 1 min, then continuously increase to 200°C at 7°C/min, and finally increased to 300°C at 10°C/min. For GC-MS detection, an electron ionization system was used with an ionization energy of 50 to 600 Da.

2.7. Development of ajwain essential oil fortified cookies.

By applying essential oils to cookies in different quantities, ajwain essential oil fortified cookies were developed.

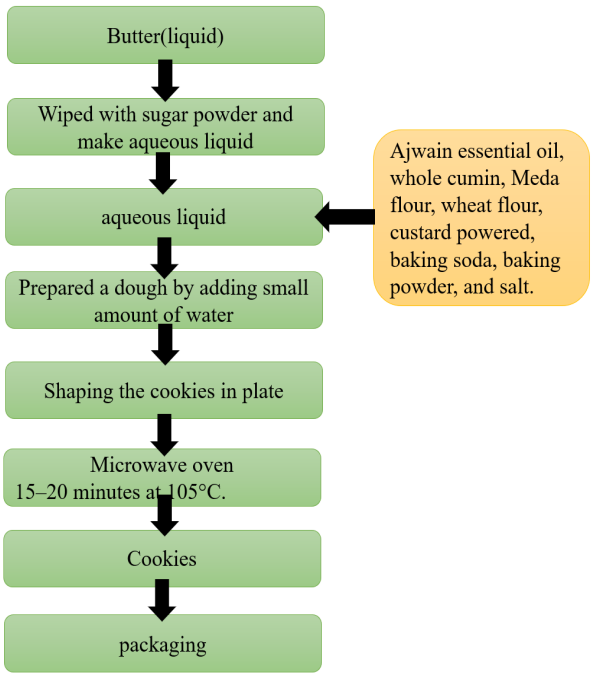

Figure 2. Preparation of development of ajwain essential oil fortified cookies.

2.7.1.Texture profile analysis and three-point bending test.

In order to determine the various textural attributes, such as hardness, chewiness, cohesiveness, gumminess, resilience, and springiness, a texture profile analysis (TPA) was used (Kek et al., 2013), and for fracturability measurement, we performed a three-point bending test (Hara et al., 2013).

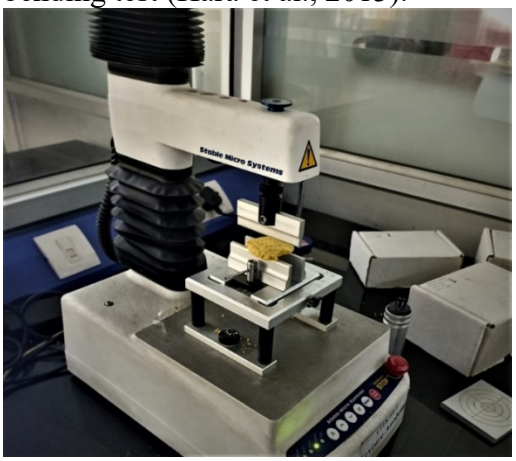

Figure 3. Model TA. XT plus texture analyser

2.8. Sensory Evaluation

According to (Singh et al., 2018), the nine-point hedonic rating test was used to evaluate the sensory attributes of ajwain essential oil fortified cookies.

3.Results and discussions

3.1. Proximate composition of ajwain seed

The results of the proximate composition of Ajwain seed are shown on Table 1. Result showed that Ajwain seed is rich in carbohydrate (55.38%), fat (10.08%) and protein (15.73%). Carbohydrates are listed first, followed by protein, and then lipids, with a range of nutritional values from 42.76 to 15.73%.We obtained a greater proportion of fat (10.23%) and a lesser percentage of fat (4.3%), as determined by (Javed et al., 2012). Because of High percentage of fat in Ajwain seed we can easily extract essential oil.

Table 1. Proximate Analysis of Ajwain seed

Ajwain seed constituents	Composition (%)
Carbohydrates	42.76±0.68
Protein	15.7325±0.87
Crude fibre	18.98±0.35
Fat	10.08±0.57
Moisture	5.44±0.40
Ash	7±0.27

All values are expressed as means ± SD. *Significant ($P \leq 0.05$).

3.2. Functional composition of ajwain seed.

Ajwain essential oil has a TPC, TFC, and DPPH radical-scavenging activity range at various concentrations of 14.29% to 56.17%, 6.02 to 16.52 mg GAE/g, and 0.04 to 3.89 mg QE/g, respectively, according to a research study (Chahal et al., 2017).The DPPH radical scavenging activity (total antioxidant activity) of ajwain seed was found as 36.36%. Occurrence of significant total phenolic content (TPC) and total flavonoid content (TFC) establishes its functional importance.

Table 2. Total phenolic, flavonoid and antioxidant content

Functional parameters	Result
TPC (Gallic acid equivalent µg/ml)	126.0216±0.46
TFC (mg Quercetin/ml)	2.0608±0.32
Antioxidant activity (mg/ml)	36.36±0.44

All values are expressed as means ± SD. *Significant (P ≤ 0.05).

3.3. Essential oil extraction yield with different solvent

As part of our investigation, we extract essential oils utilizing a variety of solvents and their mixtures. Our investigation guided us to the conclusion that solvents have an impact on the yield of bioactive compounds and extraction yield. The range of ajwain EO yields was within the ranges described in the literature(Chahal et al., 2017), ranging from 1.5 to 3.4% (v/w).In terms of extraction yield, we found that using a combination of solvents provides highest extraction yield 3.2%. Additionally, there is a little variation in the extraction yield between petroleum ether and n-hexane.

Table 3.Extraction yield of ajwain E.O.

Solvent	Yield % (v/w).
n-Hexane	2.1±0.64
Petroleum ether	2.5±0.76
N-Hexane (50%) + Petroleum ether (50%)	3.2±0.58

All values are expressed as means ± SD. *Significant (P ≤ 0.05).

3.4. GC-MS results

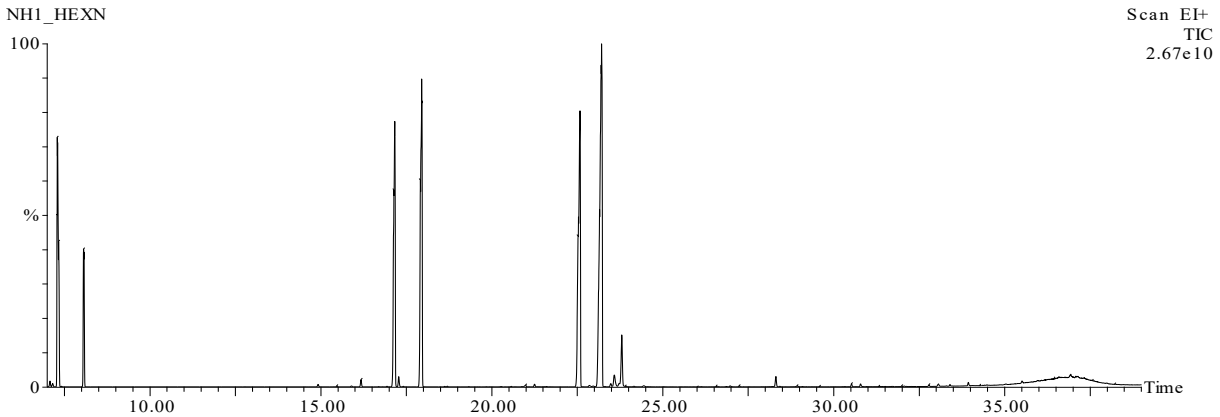

Figure 4. n-Hexane extracted essential oil GC-MS graph

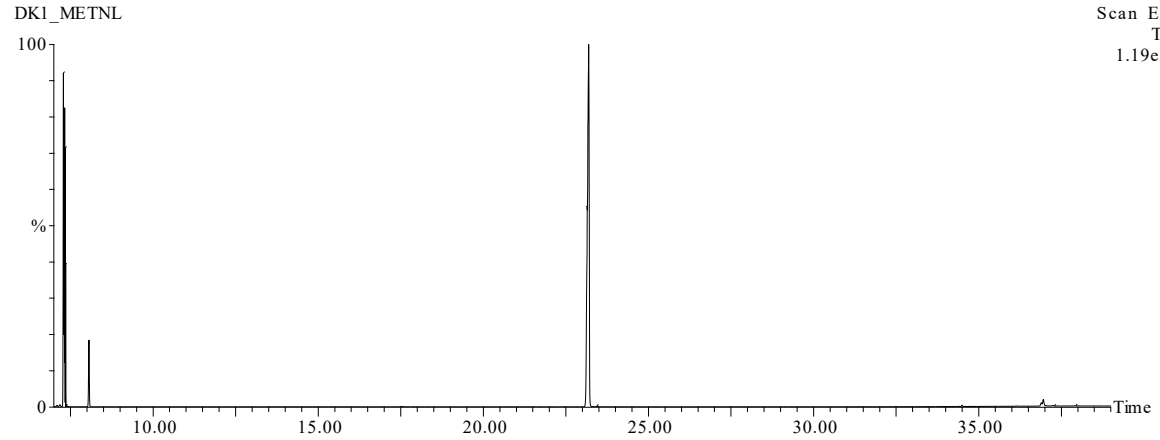

Figure 5. Petroleum ether extracted essential oil GC-MS graph.

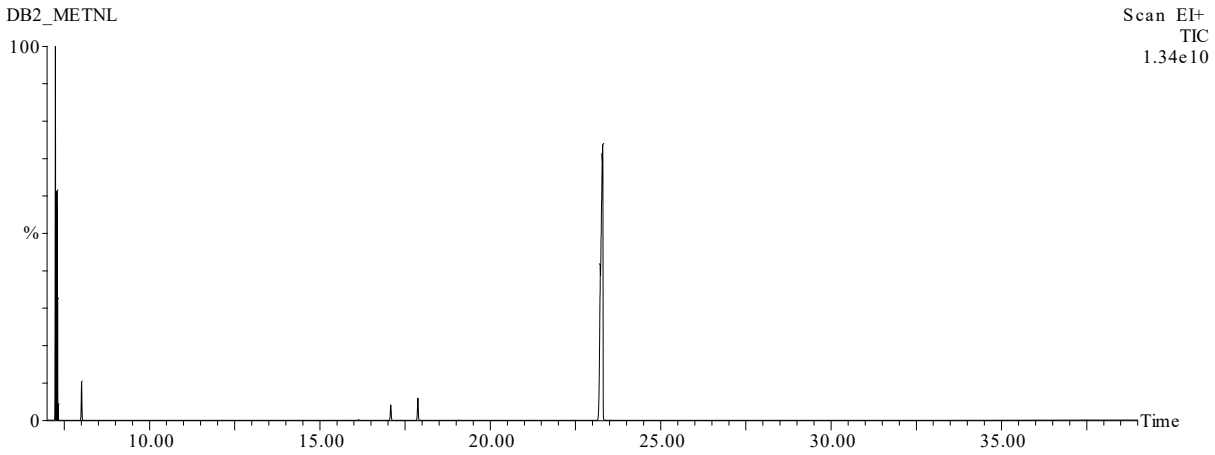

Figure 6. n-Hexane (50%) + Petroleum ether (50%) extracted essential oil GC-MS graph.

Table 4.GC-MS analysis of ajwain seed essential oil

Solvent	N-Hexane	Petroleum ether	N-Hexane (50%) + Petroleum ether (50%)
Terpinene-4-ol	-	10.426%	16.535%
P-cymene	12.029%	-	1.602%
Gama-terpene	14.793%	-	2.389%
Thymol	23.092%	75.831%	71.372%
Cyclopentanone, 2-(1-methylpropyl)-	4.360%	-	4.037%
3-heptene, 2-methyl-, (e)-	-	4.133%	-
Methyl 9-eicosenoate	-	0.955%	-
9,12-hexadecadienoic acid, methyl ester	-	0.420%	-
Methyl 8-methyl-nonanoate	-	0.138%	-
3,4-dimethylbenzyl alcohol	5.363%	-	-

About bioactive substances Petroleum ether is more effective in extracting the major bioactive components thymol than a mixture of solvents. More of the terpinene-4-ol compound is extracted using a mixture of solvents compared to petroleum ether. Additionally, petroleum ether is used to extract several minor chemicals that cannot be extracted using a mixture of solvents, such as 3-Heptene, 2-Methyl-, (E)-, Methyl 9-Eicosenoate, 9,12-Hexadecadienoic Acid, Methyl Ester, and Methyl 8-Methyl-Nonanoate. as well as several bioactive compounds that can only be extracted using a mixture of solvents, such P-Cymene, Gama-Terpene, and Cyclopentanonea 2-(1-Methylpropyl). The major bioactive elements identified by (Chahal et al., 2016) GC-MS study of ajwin essential are p-cymene, γ - terpinene, and thymol, with corresponding percentages of amount of 6.18, 12.31, and 31.4. Using innovative GC-MS techniques, (Gaba et al., 2018) investigated the properties of *Trachyspermumammi* L. essential oil and observed that thymol constituted an even higher percentage, with minor amounts of other bioactive compounds such as ρ-cymene (14.16%), β-phellandrene (0.27%), γ-terpinene (9.29%), and α-terpineol (0.17%). Based on this study, we have concluded that the phytochemicals found in solvent extracts of ajwain essential oil are different based on the solvent used. These phytochemicals include flavonoids, phenols, tannins, and alkaloids.

3.4.Antimicrobial activity in ajwain essential oil

Antimicrobial activity of essential oil and essential oil fortified cookie were tested against both gram-positive and gram-negative bacteria e.g. Bacillus cereus, staphylococcus aureus and Escherichia coli and represented in table 13. Maximum zone of inhibition in essential oil was found in bacillus cereus (34.2mm). However, maximum zone of inhibition in essential oil fortified cookie was found in staphylococcus aureus (33.8mm). Therefore, it was concluded that antimicrobial potential in both these two cases were significant. In our investigation, we observed a significantly larger inhibition zone when using ajwain seed extract as compared to the findings reported by Bhatt et al. in 2018. Specifically, the inhibition zones for *S. aureus* and *E. coli* were measured at 19.02 mm and 16.29 mm, respectively.

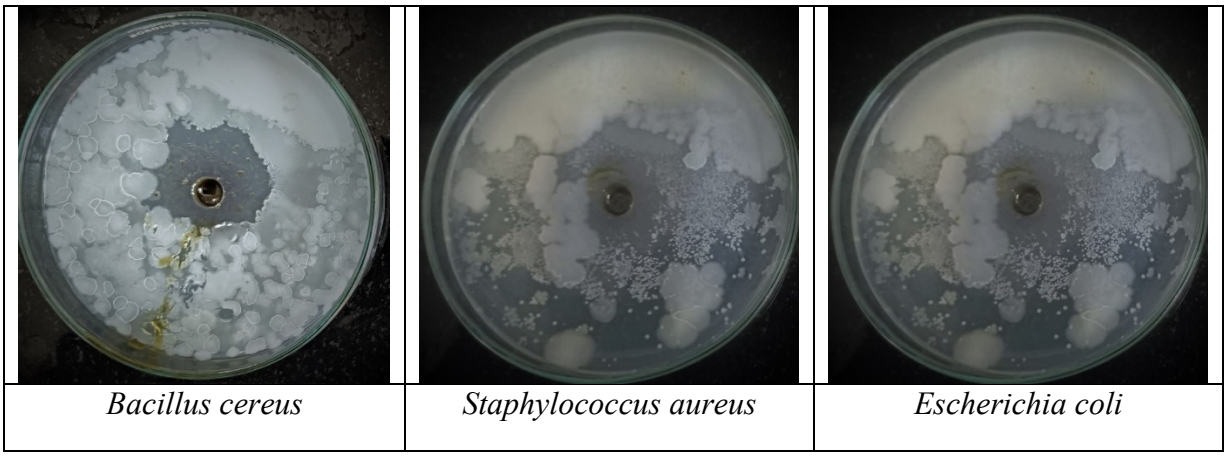

Figure 7. Antimicrobial zone of ajwain essential oil

Table 5. Zone of inhibition against ajwain essential oil

Bacteria name	zone of Inhibition (mm)
Bacillus cereus	34.2 ±0.26
staphylococcus aureus	21.7 ±0.36
Escherichia coli	18.9 ±0.24

All values are expressed as means ± SD. *Significant (P ≤ 0.05).

3.4.Sensory analysis of ajwain essential oil fortified cookies.

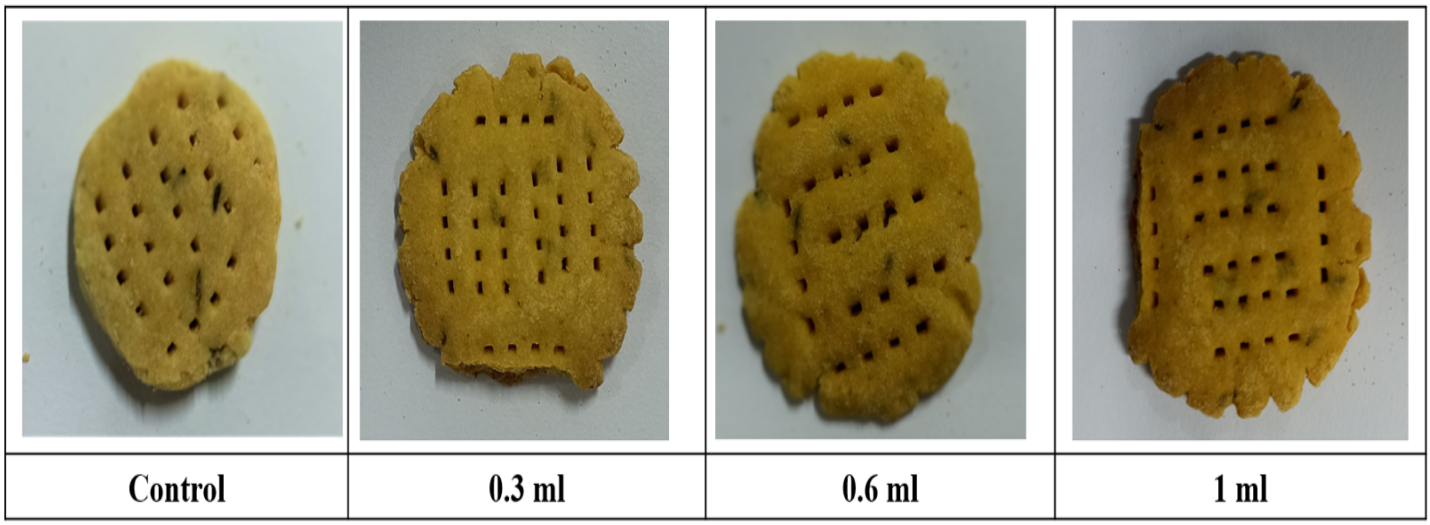

Figure 8. Pictorial representation of control and ajwain essential oil fortified cookies.

Here we developed 4 sample of ajwainjeera cookies. 10 eligible judges from the Department of Food Engineering and Technology at the Central Institute of Technology, Kokrajhar, participated in the sensory evaluation of ajwainjeera cookies.

The judges were asked to score for the sensory attributes viz. appearance, taste, flavour, mouthfeel and overall acceptability, on a 9-point Hedonic scale. It may be concluded that ajwain essential oil could be effectively added to cookies, as shown by the fact that overall acceptability of sample 4(1ml essential oil fortified cookies) was greater than that of other formulations.

The maximum mouth feel was produced by fortifying essential oil, according to a comparison of the control sample and 0.6 ml of fortified essential oil cookies.

Also, for 1 ml of essential oil fortification, we can find the highest flavor, texture, and overall acceptability. This is a novel food product; previously, there was no development on the basis of this concept, so there is not any sensory data for comparison.

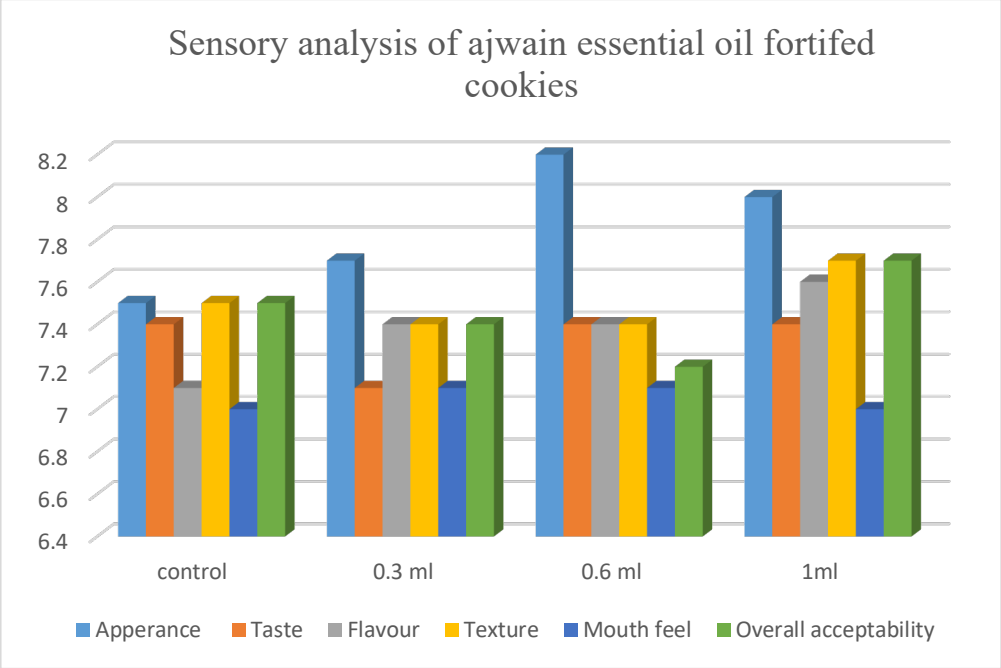

Figure 9. Sensory graph of a ajwain essential oil fortified cookies.
All values are expressed as means ± SD. *Significant ($P \leq 0.05$).
Scores are based on 9- point hedonic scale like extremely, 9; like very much, 8; like moderately, 7; like; slightly, 6; neither like or dislike,5; dislike slightly, 4; dislike moderately, 3; dislike very much, 2; dislike extremely, 1

3.5.Textural attributes of cookies

3.5.1. Texture profile analysis

Textural attribute like hardness, gumminess and chewiness are satisfactory for 1 ml ajwain essential oil fortifies cookies than that of other formulations**.**

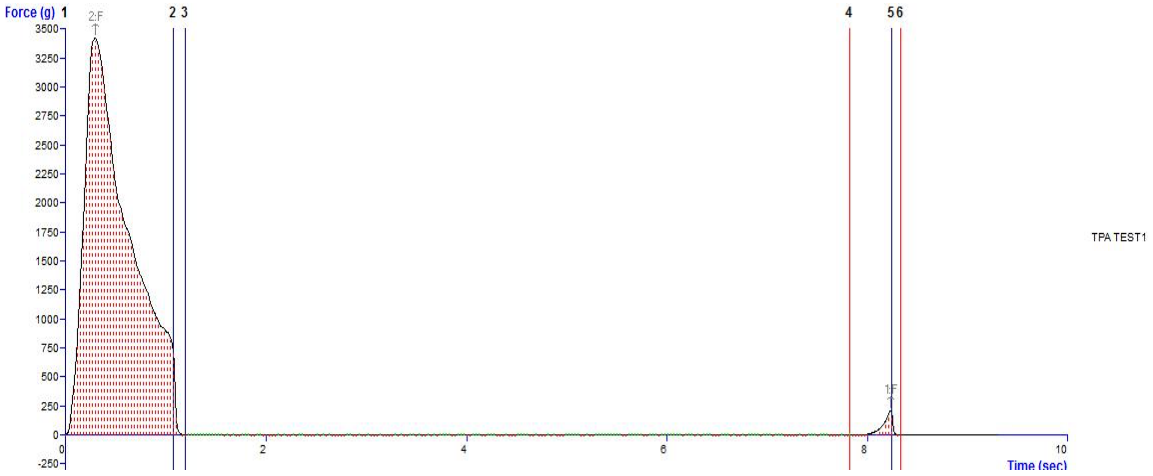

Figure 10. Graph of texture profile analysis of ajwain essential oil fortified cookie.

Table 6. Evaluation of textural attributes

Sample name	Hardness g	Adhesiveness g.sec	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
cookie	3104.698 ±0.36	-0.034±0.02	0.223±0.26	0.009±0.06	30.454±0.68	7.009±0.14	0.005±0.03

All values are expressed as means ± SD. *Significant ($P \leq 0.05$).

3.5.2. Three-point bending test

Generally three point bending test was performed for the measurement of facturability of product. Fracturability attribute of ajwain essential oil fortified cookie was found satisfactory and acceptable to consumers.

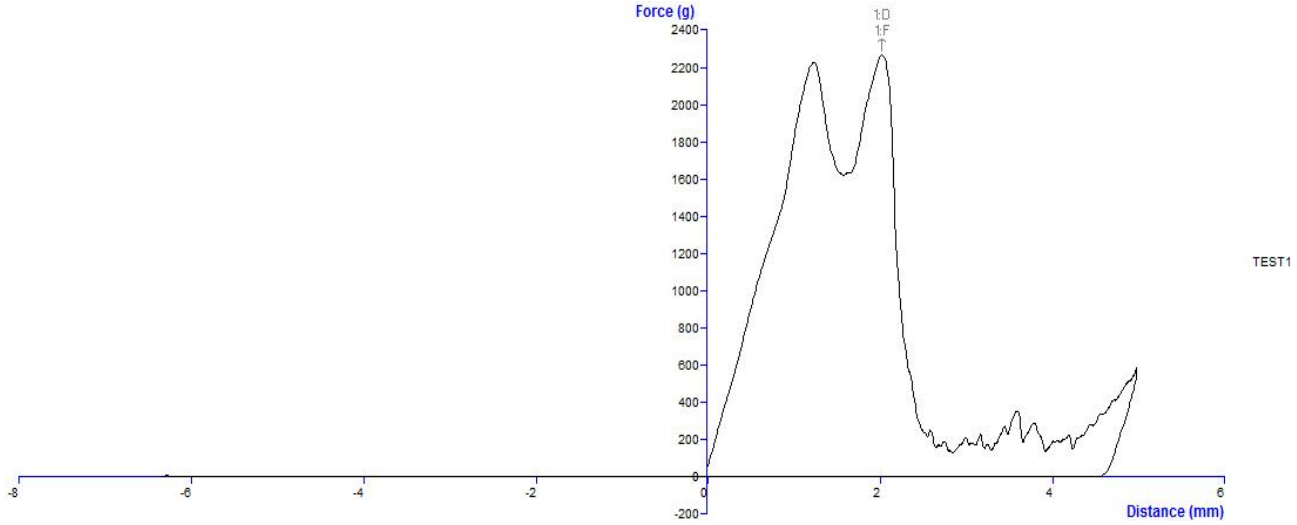

Figure 11. Graph of three-point bending test of ajwain essential oil fortified cookie.

Table 7. Value of Three-point bending properties of fortified cookies

Sample name	Hardness (g)	Fracturability (mm)
cookie	2267.94±0.84	2.04±0.27

All values are expressed as means ± SD. *Significant ($P \leq 0.05$).

While comparing our cookie's texture parameters, we found that the previous study (Bawa et al., 2020) on cookie texture parameters had established an accepted level within the specified range. The hardness, fracturability, and cohesiveness of the cookie texture profile analysis were given in Tables 6 and 7.

For mouthfeel, the Ajwain Jeera cookie sample's three textural qualities are important. The results of the study include cohesiveness, fracturability (mm), and hardness (g) of 0.009, 2.04, and 3104.698, respectively.

3.6.Nutritional quality of ajwain essential oil fortified cookies

Nutritional constituents of essential oil fortified cookies were evaluated and represented in table 8. Table 8 was shown the significant nutritional characteristics in terms of higher carbohydrate and fat content.

Table 8. Nutritional analysis of 1 ml essential fortified cookies

Cookies attributes	**(%) composition**
Ash	2.59±0.34
Carbohydrates	51.8926±0.76
Crude fiber	3.14±0.26
Fat	31.004±0.84
Moisture content	4.23±0.36
Protein	7.14±0.68

All values are expressed as means ± SD.
*Significant ($P \leq 0.05$).

4. Conclusions

The significant nutritional and functional potentials of Ajwain seed has been established as a suitable and effective nutraceutical. The high level of antioxidants principally polyphenols and flavonoids in Ajwain seed makes it as an effective source for creating nutraceutical. In view of the above facts, essential oils derived from Ajwain seed offer a broad range of biological activities. High level of antioxidant activity of Ajwain essential oil has help to preserve the moist food product and also responsible for the value-added bakery product development. Additionally, we are confirmed that the solvent plays a crucial role in solvent extraction techniques since it affects both the extraction yield and the bioactive substance's functionality. Experimental investigation revealed that a combination of solvents that is petroleum ether and n-hexane result satisfactory yields of essential oil. People are becoming more health conscious every day, which will lead to a growth in the popularity of herbal or nutraceutical products. Therefore, the developed ajwain essential oil fortified herbal cookies give significant health benefits due to availability of potential nutritional, functional and bioactive constituents. Moreover, the satisfactory sensory attributes and textural properties of the ajwain essential oil fortified cookie establish its significant importance in fortified cookie.

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Acknowledgments

We would like to thank the Lab Assistants of the Department of Food Engineering and Technology, Central Institute of Technology Kokrajhar, Kokrajhar, Assam, India for providing us all the basic requirements in order to conduct this study.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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BANANA PEEL POWDER AS THE POTENTIAL INGREDIENT TO SUBSTITUTE WHEAT FLOUR IN NOODLES PROCESSING

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https://doi.org/10.34302/crpjfst/2024.16.2.8

Article history:
Received: February 27th, 2023
Accepted: March 3rd, 2024

Keywords:
Banana peel;
Treatments;
Noodles;
Quality;
Sensory evaluation.

ABSTRACT

Banana production often produces abundant banana peels, which are considered agricultural waste. With the purpose of utilizing banana waste during the production process to create value-added products, the study was conducted to investigate the influence of pretreatment conditions ($NaHSO_3$ concentration 500-800 ppm) on color of banana peel powder and use it to replace part of wheat flour in noodle recipes (substitution ratio: 20-35% based on the total weight of banana peel powder and wheat flour). Research results have shown that when pretreating banana peels with $NaHSO_3$ at a concentration of 800 ppm for 30 minutes (28±2°C), banana peel powder with the brightest color was created. The recipe M3 with a ratio of 30:70% banana peel powder: wheat flour has created a noodle product with high quality, high sensory value and this product was the most acceptance by the consumers. This study is provided the added information for further development the the various product from agricultural waste as banana peel.

1. Introduction

In recent years, plant by-products accounted for a large part and were very worrisome because of its high density and moisture content. Peels/seeds from fruits and vegetables have an economic value below the cost of reuse and considered waste. By using appropriate treatment techniques, these wastes can be used to generate valuable by-products (Scheiber et al., 2001). Based on the 3R principle (reduce, reuse, and recycle), these materials cannot be considered as waste but become a valuable resource to be added to existing materials to increase the cost (Kabirifar et al., 2020). Therefore, the use of waste by-products to process valuable food both creates economic value and contributes to environmental protection. Banana (*Musa* spp.) is one of the most popular and important tropical fruits consumed by people all over the world, especially in Vietnam. Banana was favored by people for its nutritional properties and benefits to human health, ranking first in total fruit production due to its high consumption rate (Imam & Akter, 2011). Banana peel made up about 30% of the total weight of the fruit and contains about 20% of the dry matter, which also contained minerals, various amino acids and antioxidant compounds, but discarded as a waste product (Tai et al., 2021b; Vatanasuchart et al., 2012). In the banana peel contained antioxidant compounds and the fiber part has great potential in the production of functional foods. The flesh and skin of green bananas were high in resistant starch, ranging from 47% to 57%. The significant amount of carbohydrates, protein and fiber made banana peels an ideal source of raw materials to produce value-added products (Demir et al., 2004).

Pasta products are increasingly diversified through the addition of plant ingredients (such as potatoes, tapioca, pulse, sweet potatoes) (Kolarič et al., 2020; Melini et al., 2020; Thuy et

al., 2020). As mentioned, banana peel powder contains high levels of fiber, protein, and resistant starch, which can be used to partially replace wheat flour in recipes, creating pasta products with high levels of bioactive compounds that help reduce the oxidation process in body metabolism. Furthermore, the low energy and GI (glycemic index) in banana peels, when used for food processing, might create products with low energy value and help prevent obesity commonly found in developed societies (Kumar et al., 2023; Vatanasuchart et al., 2012). Thus, the aim of this study to determine the treatment condition for making banana peel powder and investigate their application on noodle processing. With this research might not only create the new and healthy recipes for noodle making but also reduce the impact of waste on environment to ensure sustainability.

2. Materials and methods

2.1. Materials

The banana peel (Figure 1) used for the study were obtained from banana production company at Can Tho city (Vietnam). Chicken eggs and salt were purchased at Mega Maket Hung Loi supermarket (Can Tho city). Global Wheat Flour (No. 13) produced by Interflour Vietnam Company. Potato starch is produced by Vinh Thuan Import-Export Production Trading Co., Ltd. Semolina durum flour imported from Singapore, xanthan gum manufactured by Hodgson Mill Company (US).

Figure 1. Banana peel for this study

2.2. Experiment design

Banana peels were pre-sorted to remove damaged parts, washed and drained. Sample was soaked in 0.5% citric acid solution, the banana peel must be submerged in water to avoid contact with air, producing browning pigment. Then, sample were treated in $NaHSO_3$ solution with different concentrations (500-800 ppm) for 30 minutes. Banana peels were dried at 70°C until the moisture content is 6÷8% (Tai et al., 2021a). Dried banana peel was ground into powder (80 mesh) and packaged into dark bag. Bana peel powder (BPP) was analysis color, total phenolic content, total flavonoid content to choosing pretreatment condition.

After choosing appropriate pre-treatment condition, BPP was applied into yellow alkaline noodle. Wheat flour was replaced with a portion of BPP with the weight of 40, 50, 60 and 70 g, respectively, for the formulas M1 (20% replacement), M2 (25% replacement), M3 (30% replacement) and M4 (35% replacement) [M0: the control sample (using 100% wheat flour)] (Table 1).

Table 1. Formulation for noodle production

Ingredient (g)	M0	M1	M2	M3	M4
BPP	0	40	50	60	70
Wheat flour	200	160	150	140	130
Semolina	50	50	50	50	50
Potato starch	50	50	50	50	50
Salt	2	2	2	2	2
Egg	65	65	65	65	65
Water	63	63	63	63	63
Xanthan gum	5.8	5.8	5.8	5.8	5.8
Total	425.8	425.8	425.8	425.8	425.8

BPP was mixed with wheat flour, semolina, and xanthan gum. The mixture was then sifted and put into the PHILIPS noodle maker. Stirring well for the first time, after the mixture was stirred for 1.5 minutes, start adding the egg, salt and water into mixture. Continue to stir for 5 minutes and let the dough rested for 30 minutes. After incubation, the mixture was stirred for the second time and extruded (Figure 2). After pressing, the noodles were boiled in boiling water at 100°C for 2.5 minutes and dried at 60°C for 4.5 hours (Thuy et al., 2020).

Figure 2. Pasta with banana peel powder and control noodles after extruded

2.3. Quality analysis

The color of dried noodles enriched with BPP and control sample was measured on the Hunter scale for L, a, and b using a Minolta chroma meter (CR-400, Konica Minolta, Tokyo, Japan) (Thuy, Chi, et al., 2020). Protein, carbohydrate and moisture content were analyzed as Thiex (2009). The Folin-Ciocalteu assay was used for determination of total phenolic content (TPC), based on the coloration reaction between the hydroxyl groups of phenol with the reagent Folin-Ciocalteu (Fu et al., 2011). TPC was calculated as mg gallic acid equivalent per gram sample (mgGAE/g) through standard curve (Tai et al., 2021b). The Aluminum Chloride Colormetric method was applied to determine the flavonoid content (TFC) in the product (Mandal & Madan, 2013; Tai et al., 2021b). Texture attributes were analyzed with a Brookfield CT3 Texture Analyzer equipped with a 1,500 g load cell and software version 1.8 (Brookfield Engineering Laboratories, Middleboro, MA, USA) (Thuy et al., 2020). Scanning election microscopy was used as method of Thuy et al. (2020). Briefly, samples were examined at 15 kV, the sample distance to the 7 cm ejection glass, using a JEOL model J550 scanning electron microscope (Japan). Cooking quality and sensory evaluation also was followed as performance of Thuy et al. (2020).

2.4. Data analysis

The experiments were performed with three replicates. One-way analysis of variance was performed to test the differences using Stagraphic Centurions XV.I. Experimental results were expressed as the mean ± standard deviation (SD). Sensory data was analyzed by XLSTAT 2014 (USA).

3.Results and discussions

3.1 Effect of pretreatment condition on color and antioxidant compounds of BPP

Evaluation of the color change of BPP samples presented through L, a and b values. The color change of banana peel powder after treatment with $NaHSO_3$ solution at different concentrations is shown in Table 2 and Figure 3.

Table 2. Effect of concentration of $NaHSO_3$ on color of BPP

Concentration of $NaHSO_3$ (ppm)	Colorimeter		
	L	a	b
Control (without $NaHSO_3$)	41.44^{a}	3.43^{d}	11.50^{a}
500	50.62^{b}	0.74^{c}	26.49^{b}
600	51.55^{c}	0.66^{c}	27.55^{c}
700	57.35^{d}	-0.36^{b}	29.52^{d}
800	60.52^{e}	-0.54^{a}	30.59^{e}

Values with different superscripts are significantly different (P<0.05).

Figure 3. Color of BPP treated at different concentrations of $NaHSO_3$

The L value [brightness value: black (0) to white (100)] of BPP increased with increasing concentration of NaHSO3 solution from 500 to 800 ppm. The b (-b to +b: showing the color from blue to yellow) value also increased with increasing NaHSO3 solution concentration from 500 ppm to 800 ppm. This may be due to the retardation of polyphenol oxidase (PPO) (Iqbal et al., 2019). Sulfites inhibit PPO through the reaction between sulfite and quinine ions, inhibiting PPO activity and depleting oxygen (Thamsenanupap & Prommi, 2020). All samples of BPP that were treated with $NaHSO_3$ solution at different concentrations had more attractive colors than untreated samples, the highest L value was obtained when soaking the banana peels in the $NaHSO_3$ 800 ppm solution. Pretreatment with $NaHSO_3$ solution can prevent the development of brown color of banana peels during the drying process. Research results of Vatanasuchart et al. (2012) showed that sulfite pretreatment prevented significant color loss, while water and steam blanching also suppressed enzymatic browning in convection drying, resulting in relative stability for the color parameter. The quality of raw materials is one of the important factors determining the quality of the final product. The results of content analysis of some components in BPP were observed. The moisture content of the BPP fluctuated in the range of 7.21±0.08%, the protein content was 1.54±0.08%. While the largest content in BPP was carbohydrate 73.57±0.35%. Vitamin C content in BPP accounted for 41.62±0.73 mg%, this value is only half of the study of Mosa and Khalil (2015). In addition, BPP contained a large amount of biologically active compounds. TPC and TFC in BPP were 54.84±0.56 mgGAE/g and 15.33±0.29 mgQE/g, respectively, as consist with our pervious result for extraction (Tai et al., 2021b). The difference of the above values is due to the difference in banana varieties, soil, growing conditions.

3.2. Effect of mixing ratio of BPP:wheat flour (%) on the quality of noodle

3.2.1. Physicochemical properties

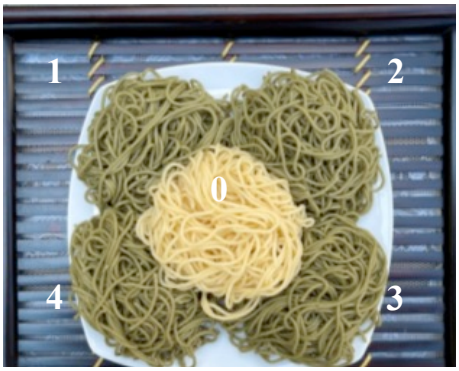
Color is one of the important factors in assessing the quality of the final product. Table 4 showed the color values L of noodle products supplemented with BPP in different proportions. The results show that the L and b values in sample M3 were 67.04 and 31.90, respectively, higher than that of another sample. When partially replacing the BPP, the noodles was more yellow, and the brightness also increased. The measured a value of sample M3 is -3.07, so the noodle product processed from formula M3 is greener than the control sample (Figure 4), mainly due to the color of the BPP used. The hardness of noodle could be measured through impact force (Wang et al., 2008). Hardness of noodles supplemented with BPP was affected when changing the different mixing ratios of BPP:wheat flour. The hardness of sample tended to increase when gradually increasing the mixing ratio of BPP:wheat flour. Specifically, samples at the mixing ratio of BPP:wheat flour (20:80%) showed the lowest value of hardness (88.04 g force), while samples at the ratio 35:65% shows the highest hardness value (96.39 g force). One of the causes affecting the hardness of noodle was probably the high amylose content and this is considered an important factor affecting the hardness of cooked noodle (Guo et al., 2003). Research by Ravi and Mustaffa (2013) showed that starch content in the peel of different banana varieties

was recorded in the range of 80.35 to 86.76%. The content of amylose in banana peel has been reported to range from 24.41 to 36.87%. The amylose content in the starch of the banana peel was significantly higher than that in the pulp of the banana (Li et al., 2011). Therefore, when increasing the mixing ratio of BPP:wheat flour (%) into the noodle recipe, the hardness of the noodles increased gradually. After extrusion, samples of noodles with different compositions were dried at a fixed temperature of 70°C until the moisture content reached 6-8%. The content of total polyphenols and flavonoids in the products was analyzed (Table 3). When increasing the mixing ratio of BPP:wheat flour (%), TPC and TFC in the samples of boiled noodles also increased. Specifically, at the mixing ratio of BPP:wheat flour of 20:80%, TPC and TFC reached the smallest value of 0.97 mgGAE/g and 0.43 mgQE/g, respectively. When increasing the mixing ratio of BPP:wheat flour (%) to 35:65%, TPC and TFC reached the highest value of 2.45 mgGAE/g and 1.98 mgQE/g, respectively. Banana peel was a source rich in antioxidant compound (Tai et al., 2021b), as increase the supplementation proportion of BPP also led to increase antioxidant compound in noodle.

Table 3. Physicochemical of noodle from different formulations

Sample	L	a	b	Hardness (g-force)	TPC (mgGAE/g)	TFC (mgQE/g)
M1	68.50^{c}	-0.77^{d}	29.71^{a}	88.04^{a}	0.97^{a}	0.43^{a}
M2	67.54^{b}	-1.38^{c}	31.14^{b}	92.84^{b}	1.43^{b}	1.07^{b}
M3	67.04^{b}	-3.07^{a}	31.90^{b}	95.68^{c}	2.17^{c}	1.77^{c}
M4	62.29^{a}	-2.19^{b}	33.39^{c}	96.39^{d}	2.45^{d}	1.98^{d}

Note: L, a, b and hardness of the control sample was 65, -2.7, 12 and 53.47 g-force, respectively. Values with different superscripts are significantly different (P<0.05)



0 is M0 sample
1 is M1 sample
2 is M2 sample
3 is M3 sample
4 is M4 sample

Figure 4. Noodle sample with different recipes and control noodle sample

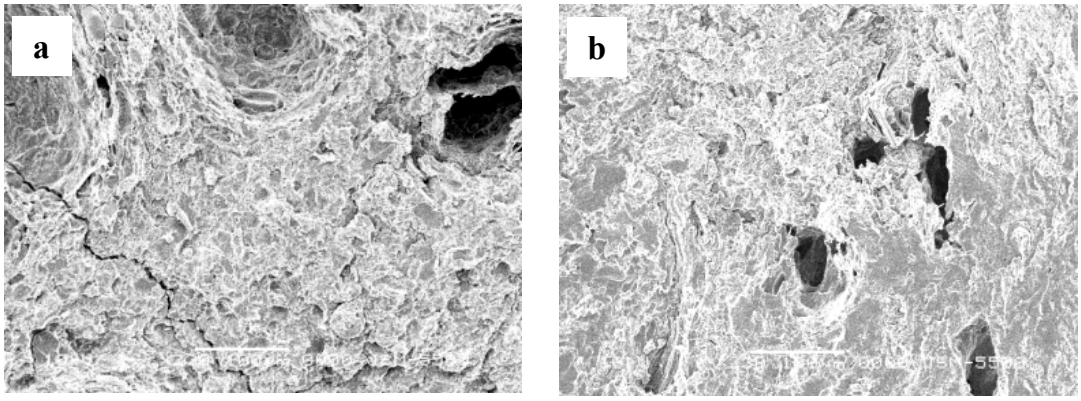


Figure 5. SEM of noodle samples: a. M0 sample; b. M3 sample

3.2.2. Morphological of noodle

Scanning electron microscopy (SEM) helps to provide information about the size, shape, and arrangement of particles in the pasta matrix (Tudorica et al., 2002). Scanning electron microscopy images can help analyze the appearance, texture, or integrity of food products (Gorinstein et al., 2004). Scanning electron microscopy cross-section of the pasta sample with partial replacement of flour by banana peel powder and the control sample (100% flour) shows the binding force between starch particles and proteins of both samples were very tight (Figure 5).

The grain size and starch grain distribution in the gluten network of the two samples are quite similar. However, the structure of the noodles with a portion of banana peel powder has a higher hardness than the control sample, leading to the noodles breaking more easily.

3.2.3. Cooking quality

Three important factors related to the quality of noodles during cooking are cooking time, percent mass gain and volume increase after heating and percent dry matter loss (Ho & Noomhorm, 2011). The mixing ratio made a statistically significant difference for cooking quality. Specifically, when increasing the mixing ratio of peel powder, the percentage of pasta volume after cooking also increased as the smallest was in sample M1 (65.70%) and the highest was in sample M4 (67.33%) (Table 4). Besides, the percentage of cooking loss was one of the parameters to evaluate the amount of dry matter lost to the cooking water (Sozer et al., 2007). The maximum cooking loss was obtained in sample M1 (5.13%). While there was no statistically significant difference between sample M2, M3, and M4. The expansion rate of pasta was calculated from the volume increase during cooking (Kaur et al., 2012; Rani et al., 2019). The obtained results showed that volume of noodles tended to increase when increasing the substitution rate of BPP into the recipe, the lowest was 77.72% corresponding to the proportion of BPP : wheat flour was 20:80% (M1) and the highest was 79.14% at the mixing ratio of 35:65% (M4).

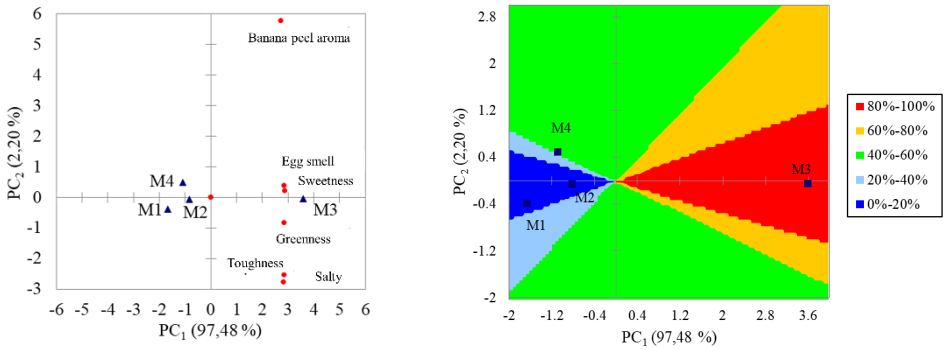
Table 4. Cooking quality of noodle from different recipes

Sample	Weight increase (%)	Cooking loss (%)	Volume increase (%)
M1	65.70^{a}	5.13^{c}	77.72^{a}
M2	66.32^{b}	4.94^{b}	78.39^{b}
M3	66.77^{c}	4.88^{ab}	78.80^{c}
M4	67.33^{d}	4.81^{a}	79.14^{d}

Values with different superscripts are significantly different (P<0.05)

3.2.4. Sensory characteristic

The method of Quantitative Descriptive Analysis (QDA), Principle Component Analysis (PCA) and Preference mapping have been applied and shown to be effective in describing sensory properties of many products such as milk, soup, pickled vegetables and fruits (Ghosh & Chattopadhyay, 2012; Nazir et al., 2018). The sensory properties of the noodles were analyzed using the PCA method and preference mapping is presented in Figure 6. To describe the organoleptic properties of prepared noodle with the addition of BPP, the relationship between the attributes and sample is shown Figure 6a. When showing noodle samples and sensory attributes on the PCA graph, noodles samples located close to each other have similar sensory properties. The dispersion of samples on the graph shows that changing the proportion of BPP in the noodle recipe greatly affected the organoleptic properties. Sample M3 was evaluated as having egg smell, sweet taste, and green color because it is located near the first major component axis. Sample M2 and M4 was located close to each other, so they have similar properties. Preference mapping to confirm PCA questions and results, sample M3 accounted for 80-100% of the highest, samples M1 and M2 belonged to the position with the lowest percentage (0-20%) (Figure 6b). Therefore, the sensory results have shown that the best rated was in sample M3, which determined as the most acceptability of consumers in noodles supplemented with BPP.



a b

Figure 6. Sensory properties of noodles as analysis of PCA method (a) and Preference mapping (b)

4. Conclusions

Sodium bisulfite ($NaHSO_3$) has been shown to have a great influence on the color of banana peel powder. An increase in the brightness of banana peel powder was observed as the concentration of $NaHSO_3$ in the treatment solution increased. The selected condition is to treat the sample (banana peel) in 800 ppm $NaHSO_3$ solution for 30 minutes. Furthermore, this type of flour is also used effectively in noodle making recipes. Based on the results of physicochemical analysis and sensory testing, 30% of wheat flour was replaced by banana peel powder in the recipe chosen to produce noodles. This study provides further information for further utilization of banana wastes, which can be considered more in the future to ensure the sustainability of agriculture.

Acknowledgement

The authors acknowledge the financial support to Can Tho University (Vietnam) from the Research fund project code number: T2020-67.

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CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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DEVELOPMENT AND QUALITY ANALYSIS OF WAFER PREMIXES USING DIFFERENT TYPES OF MILLETS

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https://doi.org/10.34302/crpjfst/2024.16.2.9

Article history:
Received: September 7th, 2023
Accepted: April 22nd, 2024

Keywords:
Wafers;
Finger millet;
Pearl millet;
Sorghum;
Premix;
Gluten free.

ABSTRACT

In the current study, four different varieties of wafer premixes incorporated with malted finger millet flour, pearl millet flour, sorghum flour and mixed millet flour (finger millet, pearl millet and sorghum) were developed by replacing refined wheat flour as millets are rich in dietary fiber, vitamins, and minerals including other nutrients and have several beneficial effects to our health. Malting helps to increase digestibility by breaking down complex substances into simple one and helps in increasing the bioavailability of some nutrients. The variety of developed premixes has four distinct flavours namely, chocolate flavour for ragi wafer premix, cinnamon flavour for bajra wafer premix, vanilla flavour for jowar wafer premix and strawberry flavour for the mixed millet wafer premix. Raw materials were procured from local grocery shops and e-commerce platform. Trials were taken by developing ice cream cones using malted millet flour and other raw materials. The amount of ingredients to be used to develop the premixes was decided through the organoleptic evaluation performed using a 5-point hedonic scale. Among these four premixes, wafers made with ragi (finger millet) wafer premix got the most overall acceptability score followed by jowar (sorghum) wafer premix, mixed millet wafer premix and bajra (pearl millet) wafer premix. Standby pouches made of LDPE were used as packaging material to perform the primary functions of packaging such as protection, preservation, and presentation of the product inside the packet. Developed premixes were subjected to physical, chemical, and microbial analysis to evaluate the quality and storage behaviour. The main motive of this study was to develop healthier version of wafers without compromising their taste as well as texture and to know the effect of using different kind of millets in the processing of wafers. Cereals can be replaced by millets to add more nutrition in daily diet. So, if millets are used in commercial snack products, then it can contribute to the upliftment of total health of a community. Wafers in the form of cones, bowls etc. can contribute to edible cutlery which is sustainable and can reduce the load of waste from the food industry.

1.Introduction

In food technology generally, wafer means light, thin and crispy food items those are baked from batters. In the United States wafers are also known as crisp cookies or biscuits. The word Wafel is originated in German. Some studies found that wafel comes from the Old High German words *waba* and *wabo* (honeycomb). Later the word around 1377 was conjoined into Middle English as wafer. Wafers are available in different shapes namely, flat wafers, communion wafers, fan wafers, wafer sticks or flute wafers, hollow wafers, sandwich wafer bars, noncreamed wafers, wafer breads, wafer cones etc. In industry, wafers are baked in hot

metal molds. There are two basic types of wafers-1. No or low sugar wafers 2. Sugar wafers. As suggested by the name no or low sugar wafers contain zero to a few percentages of free sugar on a flour base, while sugar wafers are composed of more than 10% of sugars on a flour base. No sugar wafers are baked in closed molds under pressure and heat. The sugar wafers are given different shapes by rolling, pressing, or deep forming but this is possible while the product is hot, as the sugar resolidifies during the process of cooling down and makes the product crispy (Tiefenbacher, 2017).

Generally refined wheat flour or corn flour is used as a flour base in wafers available in market. But intake of refined-grain foods is linked to a higher risk of weight gain or higher BMIs, according to certain large cohort studies, if intake of refined-grain foods is higher than advised. In areas like India with high carbohydrate and/or rice intakes (>200 g/d), refined-grain foods were linked to an elevated risk of type 2 diabetes (Jones et al., 2020).

With the changes in lifestyle and increment of non-communicable diseases, the need to develop not only tasty but also healthy and nutritious food products has become important. That is why alternating refined flour with a better ingredient without changing the physical characteristics of traditional wafers is also in trend to upgrade the nutritional value of the product.

Zanariah, M. et al. (2019) utilized Saba Banana (Musa bablisiana) peel flour as fiber ingredient in the waffle cones. The high-water holding capacity of banana peel powder caused lowering the tensile strength of wafer cone but low oil holding capacity helps in reducing oil absorption during baking.

Two graduate students' team from department of Animal Sciences and Industry at Kansas State University developed, 'Gluten-Free Fun Flavored Waffle Cones', using brown rice flour and secured first position at the 2009 AACC International Student Division Product Development Competition (Daniel et al., 2010)

"Premix is a combination of two or more fortificants in a specific proportion with or without additives packed and meant for use in formulating a product falling under any category" (FSSAI, 2018).

In this study 4 different variety of premixes of sugar wafers incorporated with malted finger millet flour, pearl millet flour, sorghum flour and mixed millet flour (finger millet, pearl millet and sorghum) were developed by replacing refined wheat flour as millets are rich in dietary fibre, vitamins and minerals including other nutrients and have several beneficial effects to our health. Germination enhances the bioavailability of some micronutrients like calcium and iron while reducing some antinutritional elements like phytates and tannins. By reducing complicated compounds to simple ones, malting enhances the amount of nutrients in food and facilitates digestion. Iron and calcium are two minerals whose bioavailability is improved by malting (Vijay et al., 2021). According to a study germination of finger millet seeds results in elevated protein content of the seeds (Swami et al., 2013). Rice flour was also used to develop the crispy texture of wafer and for binding purpose.

Millet belongs to the cereal of grass family, *Poaceae* and is mainly cultivated for its seeds. Finger Millet's (*Eleusine coracana*) grains vary in colour from white to brown, are consumed in different form including chapati, porridge, cakes by using milled flour (Traditional Crops, FAO). Finger millet is one of the oldest crops in India. Unlike other millets like sorghum, pearl millet, proso millet, and foxtail millet, finger millet has a multilayered (five layered) testa, which may be one of the potential explanations for the increased dietary fiber content of finger millet (Shobana et al., 2013). In native Indian languages, finger millet is also known as ragi (Kannada), nachni (Marathi), mandua (Bengali), ragulu (Telugu), kelvaragu (Tamil), etc. (Dayakar Rao et al., 2017).

Pearl millet (*Pennisetum glaucum)* is a tropical cereal grass with small grains that is also known as *P. typhoides*, *P. americannum*, or *P. spicatum*. India and northern Africa are the primary regions for pearl millet cultivation (Taylor et al., 2006). The pearl millet plant can grow anywhere between 0.5 and 4 meters tall, and the grain can be almost all white, pale

yellow, brown, grey, slate blue, or purple. The ovoid grains are substantially larger than those of other millets, measuring between 3 and 4 mm in length, and the average weight of a thousand seeds is 8g (FAO, 1995). In India, pearl millet is also known as bajra (Hindi, Bengali, and Punjabi, sajjai (Kannada), kamboo (Tamil, Malayalam), and bajri (Marathi, Gujrati), among other names (Dayakar Rao et al., 2017).

Sorghum (*Sorghum bicolor (L.) Moench)* is a member of the grass family *Poaceae*'s Andropogonae tribe. Sorghum seems to have come from Ethiopia to eastern Africa around 200 AD or earlier. During the first millennium BC, sorghum was probably carried from eastern Africa to India. The sorghum kernel can be white, reddish-brown, pale yellow, or deep purple-brown, among other colours. Although their size and form might vary, most kernels are spherical (FAO, 1995). Sorghum is primarily a warm-season, daylength-sensitive plant with a C4 metabolism (Blum, 2004). The grain is made up of naked caryopsis, which includes a pericarp, endosperm, and germ. In India, sorghum millet is also known as jowar (Hindi), jola (Kannada), cholam (Tamil, Malayalam), jowari (Marathi), juar (Bengali, Gujrati), among other names (Dayakar Rao et al., 2017).

Millets are a great source of several nutrients; they are superior in nutritive value as compared to major cereals like wheat and rice. Complex carbohydrates are more in millet seeds than the simpler ones. Millets contain a good amount of dietary fibre, for example, finger millet has 18.6% dietary fibre and 3.6% crude fibre (Dayakar Rao et al., 2017). Millets do not contain gluten as a source of protein. Essential amino acids except lysine and threonine are present in millet grains with relatively high amounts of methionine (Abah et al., 2020). The chemical score (a measure of protein quality determined as the ratio of the amount of an amino acid in a test protein over a reference protein expressed as a percentage) of finger millet protein is 52 as opposed to 37 for sorghum and 63 for pearl millet (FAO, 1995). Millets have a good amount of vitamins (vitamin B complex except vitamin B12, vitamins A, D, E and K) and minerals such as calcium, magnesium, phosphorus, iron etc. In comparison to other grains and millets, finger millet has eight times more calcium (344 mg%) (Shobana et al., 2013). Pearl millet has more niacin as compared to other cereal grains and sorghum is high in beta-carotene which the body can convert into vitamin A, leutin, and zeaxanthin (Dayakar Rao et al., 2017). Various functional components such as tannins, flavonoids, polyphenols, phytate and phytic acid are present in millets. Due to this nutritional composition, millets can help to prevent several non-communicable diseases like elevated blood pressure and sugar, obesity, cardiovascular diseases, constipation etc. including different types of cancers.

Trials were taken to standardize the amounts of ingredients to be used and the procedures of making wafer from the premixes. After developing the premixes 100gm LDPE zip lock standy pouches were used for packaging and stored at room temperature away from direct sunlight and moisture. Sensory evaluation was performed while conducting trials as well as in different stages of storage period to know the acceptability of the final products prepared using the premixes. The combination of various modalities of perception that are used in the selection and consumption of food is referred to as sensory quality. The acceptance of the food is determined by appearance, flavour, and texture. In the final analysis, this response, which is heavily influenced by a range of psychological and social elements, is crucial to the acceptance and choice of foods (Srilakshmi, 2018). Proximate analysis, microbial analysis, and chemical analysis were accomplished to evaluate the quality of the premixes and to study their storage behaviour. The motive of this study was to develop a healthier version of wafers from premixes and to study their physical, chemical, and microbial properties.

2.Material and methods

For the preparation of millet wafer premixes materials such as finger millet seeds, finger millet flour, sorghum seeds, pearl millet seeds, rice flour, milk powder, sugar, cocoa powder, cinnamon powder, baking powder and oil were

procured from local grocery store in Pune. Vanilla powder and strawberry powder were purchased from an e-commerce platform. For control, All-purpose flour was used along with other ingredients.

Equipment and utensils required for the study were used from the Nutrition and Food Processing laboratory of the Food Science and Nutrition department in S. N. D. T. College of Home Science, Pune.

A standby pouch made of low-density polyethylene (LDPE) was used as a primary packaging material.

Chemicals required for quality analysis were obtained from the Department of Food Science and Nutrition.

2.1.Processing of raw materials

After the procurement of millets (finger millet, sorghum, and pearl millet), they were subjected to sorting, washing, soaking, germinating, drying, roasting, grinding, and sieving. Various steps of processing are given below (Figure 1).

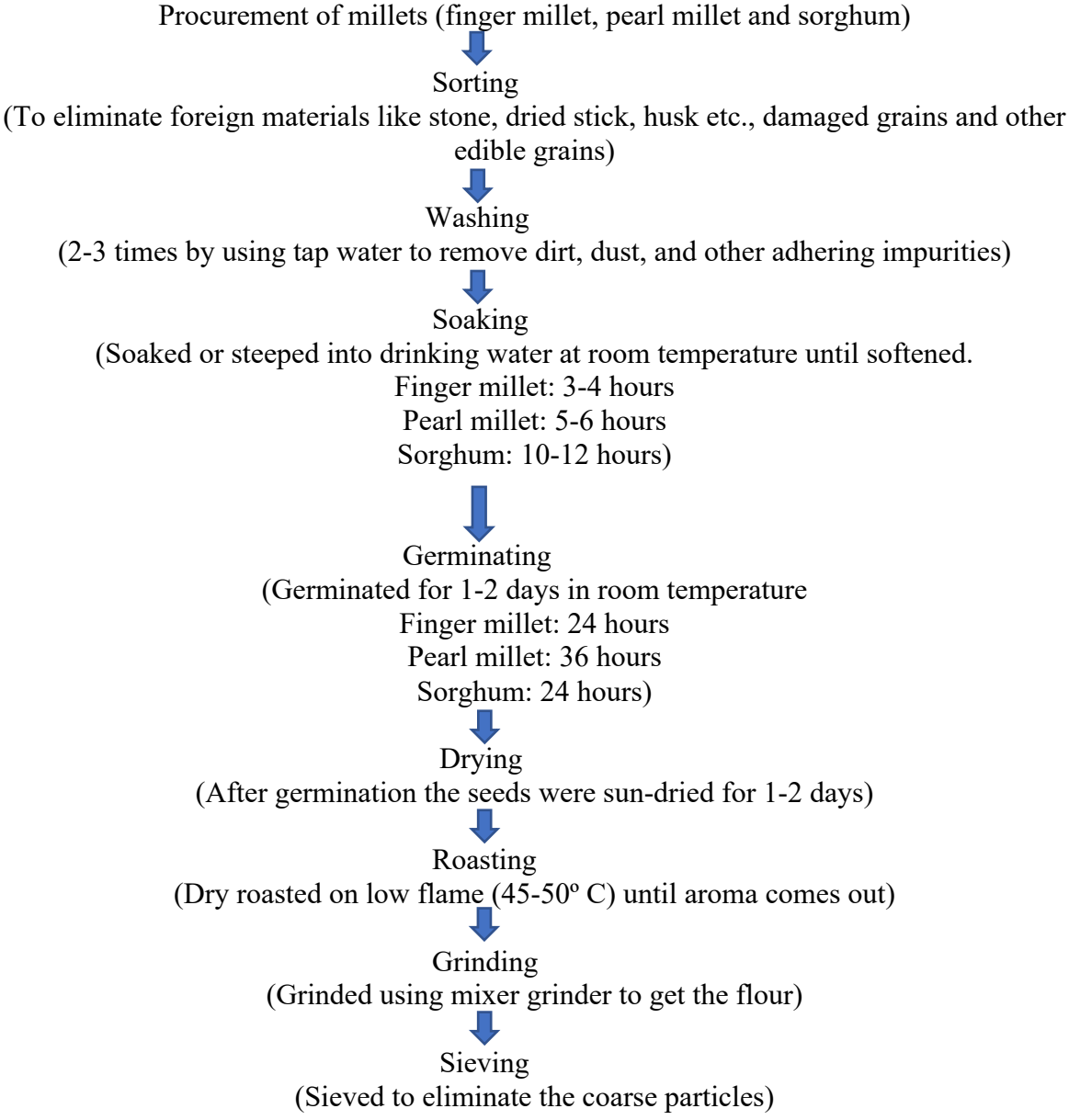


Figure 1. Processing of raw millets

2.2. Trials

The trials for the product were conducted in the Nutrition and Food Processing Laboratory of S.N.D.T College of Home Science, Pune. Following trials have been made to prepare millet-based wafer premix in the laboratory.

Table 1. Ingredients used to prepare wafers in trials

Ingredients	**Trial 1 for Finger millet wafer premix (T1) (gm)**	**Trial 2 for Finger millet wafer premix (T2) (gm)**	**Trial 3 for Finger millet wafer premix (T3) (gm)**	**Trial 1 for Pearl millet wafer premix (T4) (gm)**	**Trial 1 for Sorghum wafer premix (T5) (gm)**	**Trial 1 for Mixed millet wafer premix (T6) (gm)**	**Trial 2 for Mixed millet wafer premix (T7) (gm)**
Finger Millet Flour	25	25	-	-	-	-	-
Malted Finger Millet Flour	-	-	30	-	-	10	10
Malted Pearl Millet Flour	-	-	-	30	-	10	10
Malted Sorghum Flour	-	-	-	-	30	10	10
Rice Flour	10	15	20	20	20	20	20
Sugar (Powdered)	30	30	30	30	30	30	25
Milk powder	17	12	14	14	14	14	12
Cocoa powder	5	5	5	-	-	-	-
Cinnamon powder	-	-	-	5	-	-	-
Vanilla powder	-	-	-	-	5	-	-
Strawberry Powder	-	-	-	-	-	5	12
Baking Powder	1	1	1	1	1	1	1
Oil	12 ml	12 ml	18 ml	18 ml	18 ml	18 ml	18 ml
Luke warm water	60 ml	68 ml	70 ml	70 ml	70 ml	70 ml	70 ml

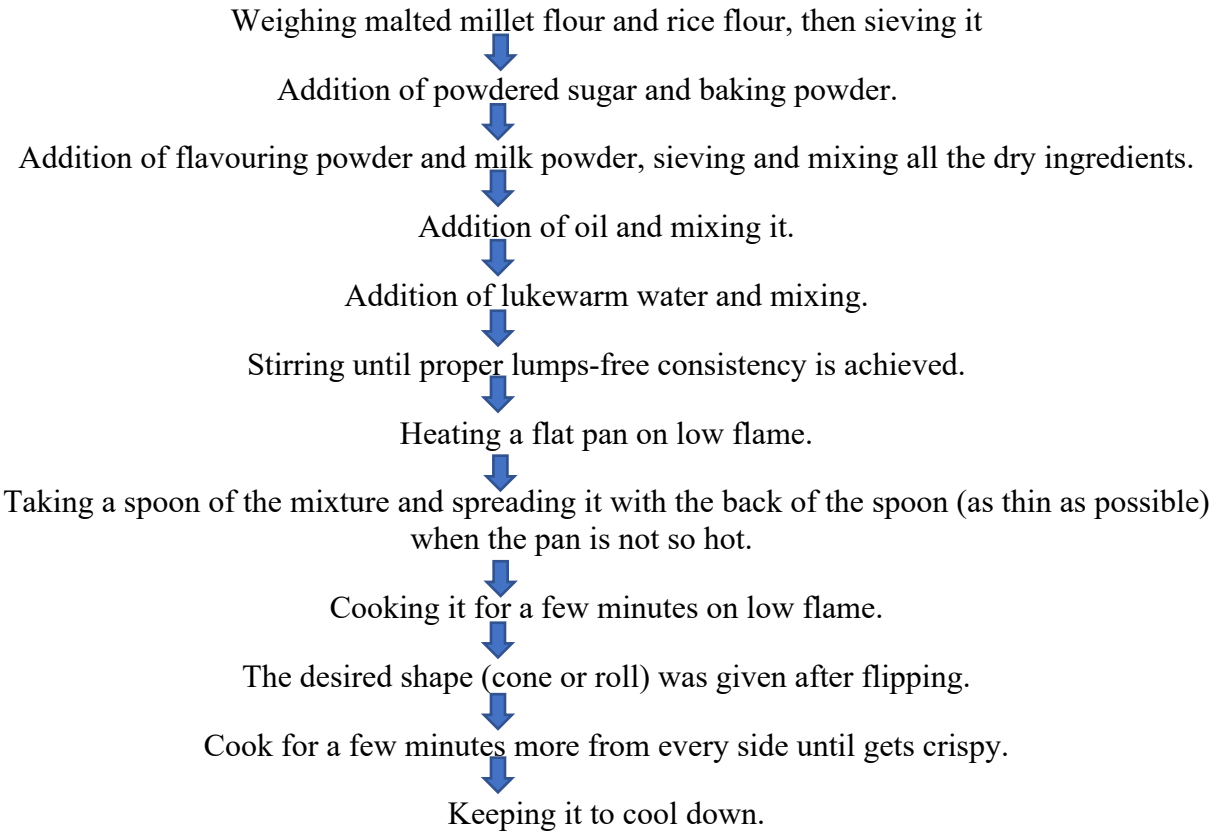


Figure 2. Procedure of making wafers

Table 2. Ingredients used to prepare premixes (in 100 gm): -

Ingredients	**Control (all-purpose flour) (gm)**	**Finger millet wafer premix (gm)**	**Pearl millet wafer premix (gm)**	**Sorghum wafer premix (gm)**	**Mixed millet wafer premix (gm)**
All-purpose flour	35	-	-	-	-
Malted Finger Millet Flour	-	30	-	-	10
Malted Pearl Millet Flour	-	-	30	-	10
Malted Sorghum Flour	-	-	-	30	10
Rice Flour	20	20	20	20	20
Sugar (Powdered)	30	30	30	30	25
Milk powder	14	14	14	14	12
Cocoa powder	-	5	-	-	-
Cinnamon powder	-	-	5	-	-
Vanilla powder	-	-	-	5	-
Strawberry Powder	-	-	-	-	12
Baking Powder	1	1	1	1	1

A B C

D

Figure 3. Processing of wafers

(A) Developed premix, (B) Prepared mixture by addition of oil and water, (C) Cooking of wafer, and (D) Prepared ice-cream cones from different premixes (1. Finger millet wafer, 2. Mixed millet wafer, 3. Pearl millet wafer and 4. Sorghum wafer)

2.3. Sensory evaluation

Sensory evaluation of the test samples was done using the 5-point Hedonic Rating Scale for parameters: Taste, Appearance, Colour, Flavour, Texture and Overall Acceptability. Organoleptic analysis was performed for the sample size (n) of 30 people in SNDT College of Home Science to know the acceptability of the product.

2.4. Packaging

Packaging of the premixes was done as per FSSAI standard [Food safety and standards (packaging and labelling) regulations, 2011]. Standy pouches made of LDPE were used as packaging material to perform the primary functions of packaging such as protection, preservation, and presentation of the product inside the packet.

2.5. Quality evaluation:

After developing the premix, quality was checked by physical analysis, physicochemical analysis, microbial analysis, and sensory evaluation. Finished goods were subjected to the following tests-Parameters for testing millet wafer premixes:

A. Physicochemical Analysis:
   - Moisture content (FSSAI, 2015)
   - Estimation of total sugar (FSSAI, 2015)
   - Estimation of alcoholic acidity (IS: 1155-1968)
   - Estimation of carbohydrate content (calculated by using the food composition table given by ICMR, 2017)
   - Estimation of protein content (calculated by using the food composition table given by ICMR, 2017)
   - Estimation of fat content (calculated by using the food composition table given by ICMR, 2017)

B. Microbial Analysis:
   - TPC (Total Plate Count) (FSSAI, 2012)

A. Physicochemical Analysis:

2.5.1. Moisture estimation of developed premix:

The standard method of oven drying as mentioned in the FSSAI manual was followed to calculate the moisture content of the sample (FSSAI, 2015).

$$\text{Moisture (\%)} = \frac{(W1 - W2)}{W1 - W} X\ 100 \quad (1)$$

Where,

W1 = Weight in gm of the dish with the material before drying

W2 = Weight in gm of the dish with the material after drying

W = Weight in gm of the empty dish

1. Estimation of total sugar: -

To estimate the reducing sugar, total reducing sugar, and total sugar Lean and Eynon method was referred from FSSAI manual (FSSAI, 2015).

$$\text{Fehling Factor (for Invert Sugar)} = \frac{Titre\ x\ Weight\ of\ sucrose\ in\ gm}{500} \quad (2)$$

$$\text{Reducing sugar \% (as invert sugar)} = \frac{Dilution\ x\ Factor\ of\ Fehling\ (in\ gm) x\ 100}{Weight\ of\ sample\ x\ titre} \quad (3)$$

$$\text{Total reducing sugars \% (as invert sugar)} = \frac{Dilutions\ x\ Fehling\ factor\ x\ 100}{Weight\ of\ sample\ x\ titre} \quad (4)$$

2.5.2. Estimation of alcoholic acidity in developed premix:

Standard procedure of alcoholic acidity with 90 % alcohol as given by BIS was followed and calculated as $H_2\ SO_4$ on dry basis (IS: 1155-1968, 2006). Due to less time the sample solution was allowed to stand for 3 hours with manual shaking.

$$\text{Alcoholic acidity (as } H_2SO_4) = 24.52\ X\ \frac{AN}{W} \quad (5)$$

Where,

A = volume in ml of standard NaOH used in titration

N = normality of standard NaOH solution used in titration (i.e., 0.05 N)

W= weight in gram of the sample taken for analysis

B. Microbial analysis:

To estimate the microbial load of the product standard procedure given by FSSAI for total plate count was followed with three dilutions ($10^{-1,}$ 10^{-2} and 10^{-3}) and spread plate technique (FSSAI, 2012).

$$\text{Colony Forming Unit / ml} = \frac{no.of\ colonies}{volume\ used}\ x\ dilution\ factor \quad (6)$$

2.5.3. Storage studies for quality evaluation

Changes in quality during storage of the product were examined by determination of physicochemical constituents, microbial examination, and organoleptic evaluation for about 30 days of storage period by conducting random sampling at an interval of 15 days for physicochemical analysis and 30 days for microbial examination and organoleptic evaluation.

2.5.4. Statistical analysis

Mean and standard deviations of the data were calculated using Microsoft Excel.

3.Result and Discussion:

3.1. Sensory evaluation of wafers prepared using developed premixes:

For sensory evaluation the first two trials (T1 and T2) were conducted with finger millet flour and two different variations were prepared. To increase the digestibility and palatability malted flour has been added to prepare the product in third trial. As mentioned in Table 3 The third trial (T3) obtained maximum, acceptability; 4.48 $\pm$0.50, 4.32 $\pm$0.56, 4.46 $\pm$0.44, 4.40 $\pm$0.57, 4.43 $\pm$0.45 and 4.40 $\pm$0.42 for texture, colour, appearance, taste, flavour, and overall acceptance. The amount of dry ingredients used in this trial was the final amount to develop the chocolate flavour finger millet wafer premix. We have observed that the malting process helped to decrease the cooking time as compared to that of the flour without malting, also the acceptability (Figure 4) was comparably better.

To develop the pearl millet wafers (T4) equal quantity of ingredients was used as finger millet wafer premix as well as the time consumed for cooking and other proportions was the same. In organoleptic evaluation pearl millet wafers obtained 4.13 $\pm$0.71, 4.05 $\pm$0.58, 4.08 $\pm$0.68, 4.03 $\pm$0.74, 3.85 $\pm$0.73 and 4.05 $\pm$0.65 for texture, colour, appearance, taste, flavour, and overall acceptability (Table 4). This indicates that the developed premix based experimental wafer was found to be fallen under the category of "very good to excellent" according to overall acceptability. An equivalent amount of dry ingredients was taken to prepare the cinnamon flavour pearl millet wafer premix.

Another trial (T5) was taken with malted sorghum flour to develop a vanilla-flavoured sorghum wafer premix. An equal amount of ingredients was taken to get the desired characteristics of the wafers. The time consumption for the sorghum wafer was observed to be more than that of the ragi wafer. As mentioned in Table 4, the obtained score of sorghum wafer cones indicates that the experimental wafer was to fall under the category of "very good to excellent." Premix was developed with the same amount of ingredients.

Lastly, to develop strawberry flavoured mixed millet wafer premix same amount (T6) of ingredients was taken as the above-mentioned wafer cones. In the prepared cones though, the proper texture was achieved but, the flavour of the strawberry was not noticeable. That is why the quantity of ingredients was modified to develop proper taste and flavour with the same cooking time as finger millet and pearl millet wafers. In sensory evaluation mixed millet wafer cones obtained 4.12 $\pm$0.73, 4.18 $\pm$0.58, 3.95 $\pm$0.71, 4.02 $\pm$0.74, 3.85 $\pm$0.79and 4.08 $\pm$0.67 for texture, colour, appearance, taste, flavour, and overall acceptability (Table 4) respectively. So, the amount of dry ingredients used in the last trial (T7) was the final amount to develop a mixed millet wafer premix.

Among these four different varieties of wafers, finger millet wafer cones scored highest

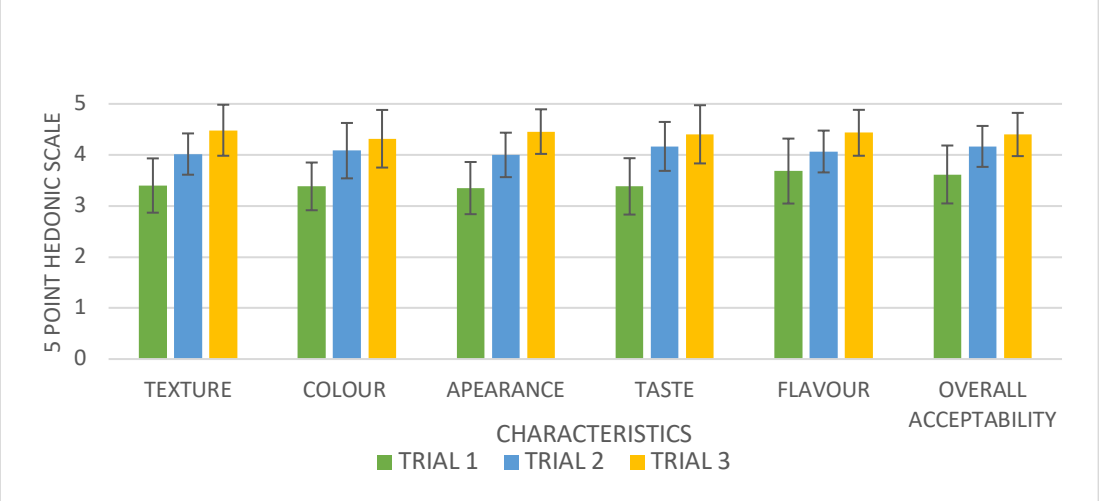
based on texture, appearance, taste, flavour, and overall acceptability, while sorghum millet cones obtained the highest score in colour. Mixed millet wafer cones obtained the lowest score for texture, appearance, and taste while pearl millet wafer cones obtained the lowest for colour and overall acceptability. For flavours, both pearl millet and mixed millet wafer cones got 3.85 which is the lowest. The graph of sensory evaluation (Figure 5) shows that all the millet-based wafer cones come under the category of "very good to excellent" according to their overall acceptability.

After 30 days no significant change in sensory parameters was observed. Finger millet wafer cones secured the highest score based on appearance, taste, and flavour (4.28± 0.52, 4.35± 0.51, 4.46± 0.46) while cones made of sorghum millet obtained the highest score in texture and colour (4.37± 0.49, 4.17± 0.48). Both the finger millet wafer cone and sorghum millet wafer cone got maximum overall acceptancy (4.35). This time, pearl millet wafers obtained lowest score based on texture and taste (4.13 ±0.71 and 4.03 ±0.74) whereas in the case of colour, appearance, flavour, and overall acceptance both pearl millet wafer cones and sorghum wafer cones scored almost same marks as shown in Table 5. The graph of sensory evaluation after 30 days (Figure 6) shows that all the millet-based wafer cones still come under the category of "very good to excellent" according to their overall acceptability.

Table 3. Mean values of sensory evaluation of wafers prepared from finger millet

TRIAL NO.	TEXTURE	COLOUR	APPEARANCE	TASTE	FLAVOUR	OVERALL ACCEPTANCY
TRIAL 1 (T1)	3.40 ±0.53	3.38±0.47	3.35 ±0.51	3.38 ±0.55	3.68 ±0.64	3.62 ±0.57
TRIAL 2 (T2)	4.02 ±0.40	4.08 ±0.54	4.00 ±0.43	4.17 ±0.48	4.07 ±0.41	4.17 ±0.40
TRIAL 3 (T3)	4.48 ±0.50	4.32 ±0.56	4.46 ±0.44	4.40 ±0.57	4.43 ±0.45	4.40 ±0.42

*Data represents mean ±standard deviation for (n= 30)



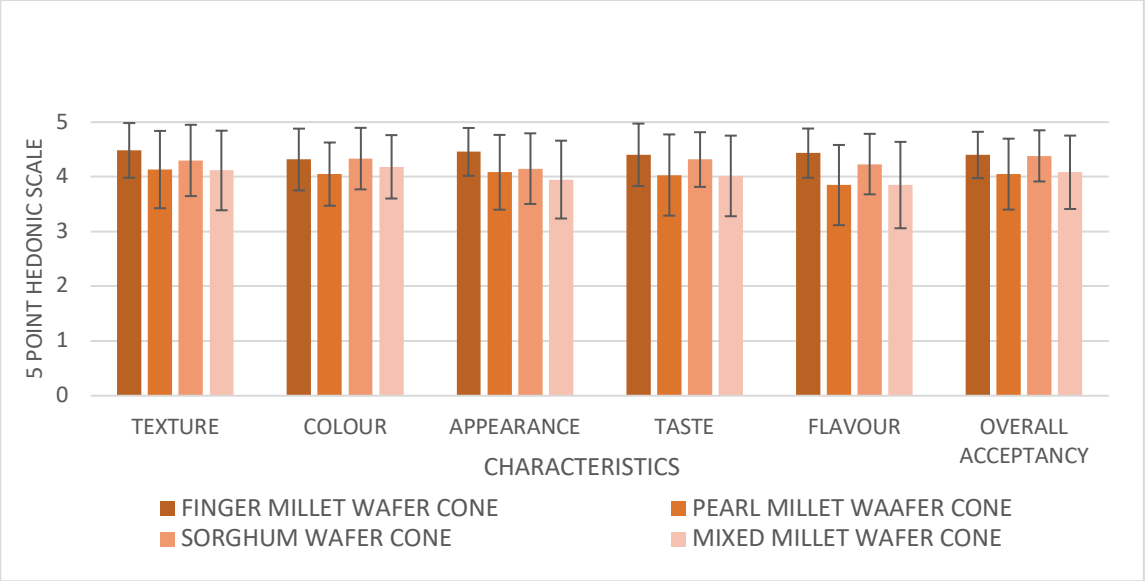
*Data represents mean ±standard deviation for (n= 30)

Figure 4. Comparison between sensory evaluation of wafers prepared from finger millet

Table 4. Mean values of sensory evaluation of wafers prepared from different millets

NAME OF THE WAFER	TEXTURE	COLOUR	APPEARANCE	TASTE	FLAVOUR	OVERALL ACCEPTANCY
Finger millet wafer cone	4.48 ±0.50	4.32 ±0.56	4.46 ±0.44	4.40 ±0.57	4.43 ±0.45	4.40 ±0.42
Pearl millet wafer cone	4.13 ±0.71	4.05 ±0.58	4.08 ±0.68	4.03 ±0.74	3.85 ±0.73	4.05 ±0.65
Sorghum wafer cone	4.30 ±0.65	4.33 ±0.56	4.15 ±0.64	4.32 ±0.50	4.23 ±0.55	4.38 ±0.47
Mixed millet wafer cone	4.12 ±0.73	4.18 ±0.58	3.95 ±0.71	4.02 ±0.74	3.85 ±0.79	4.08 ±0.67

*Data represents mean ±standard deviation for (n= 30)



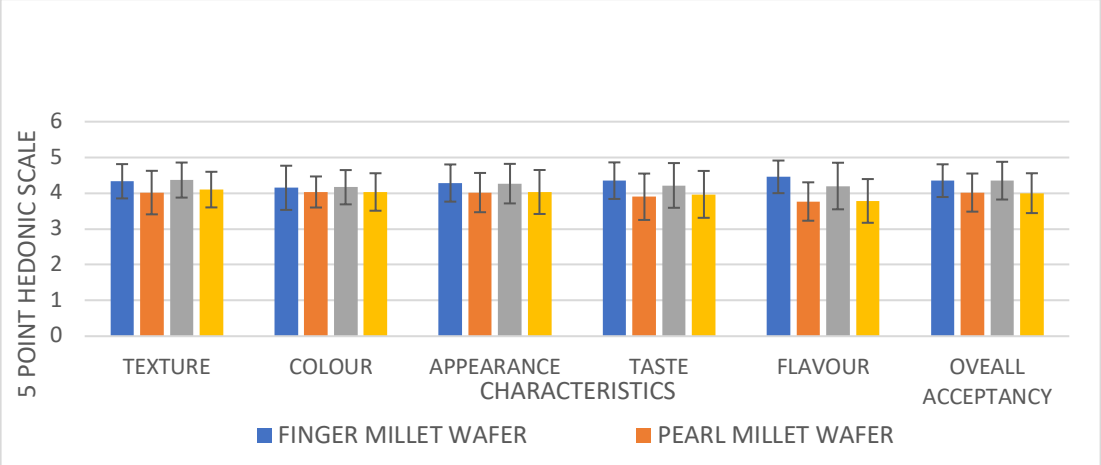
*Data represents mean ±standard deviation for (n= 30)

Figure 5. Comparison between sensory evaluation of wafers prepared from different millets

Table 5. Mean values of sensory evaluation of wafers prepared from different millets after 30 days:

NAME OF THE WAFER	TEXTURE	COLOUR	APPEARANCE	TASTE	FLAVOUR	OVERALL ACCEPTANCY
Finger millet wafer cone	4.33± 0.48	4.15± 0.62	4.28± 0.52	4.35± 0.51	4.46± 0.46	4.35± 0.46
Pearl millet wafer cone	4.01± 0.61	4.03± 0.43	4.01 ± 0 .52	3.90 ± 0.65	3.77 ± 0.54	4.02± 0.53
Sorghum wafer cone	4.37± 0.49	4.17± 0.48	4.27± 0.55	4.22± 0.63	4.20± 0.65	4.35± 0.53
Mixed millet wafer cone	4.10± 0.50	4.03± 0.52	4.03± 0.61	3.97± 0.65	3.78± 0.61	4.00± 0.56

*Data represents mean ±standard deviation for (n= 30)



*Data represents mean ±standard deviation for (n= 30)

Figure 6. Comparison between sensory graphs of wafers made of different millets after 30 days

3.2. Quality evaluation

3.2.1. Physicochemical analysis:

3.2.1.1. Moisture estimation of developed premix:

The developed premixes were subjected to moisture determination test by oven drying method. The change in moisture content is shown in Table 6. The initial moisture content of the finger millet wafer premix, pearl millet wafer premix, sorghum wafer premix and mixed millet wafer premix were 5.92%, 5.76%, 4.89% and 4.68%. After 15 days there were little moisture gain has been observed specifically, in the sorghum wafer premix and mixed millet

wafer premix while finger millet wafer premix and pearl millet wafer premix had no such changes in moisture content. After 30 days increased moisture content was observed in the pearl millet wafer premix, in the sorghum wafer premix and in mixed millet wafer premix whereas moisture content of the finger millet wafer was approximately same as before. According to FSSAI moisture content of millets should not be more than 16% by weight [Food safety and standards (food products standards and food additives) regulations, 2011]. As per the specification these premixes matched the standard.

Table 6. Moisture content of developed premixes

DAYS	CONTROL MOISTURE %	MOISTURE % (IN FINGER MILLET WAFER PREMIX)	MOISTURE % (IN PEARL MILLET WAFER PREMIX)	MOISTURE % (IN SORGHUM WAFERPREMIX)	MOISTURE % (IN MIXED MILLET WAFER PREMIX)
0th day	3.52	5.92	5.76	4.89	4.68
15th day	3.22	5.98	5.71	5.24	4.89
30th day	2.98	5.94	5.88	5.86	4.92
SE±	0.271	0.031	0.087	0.491	0.131

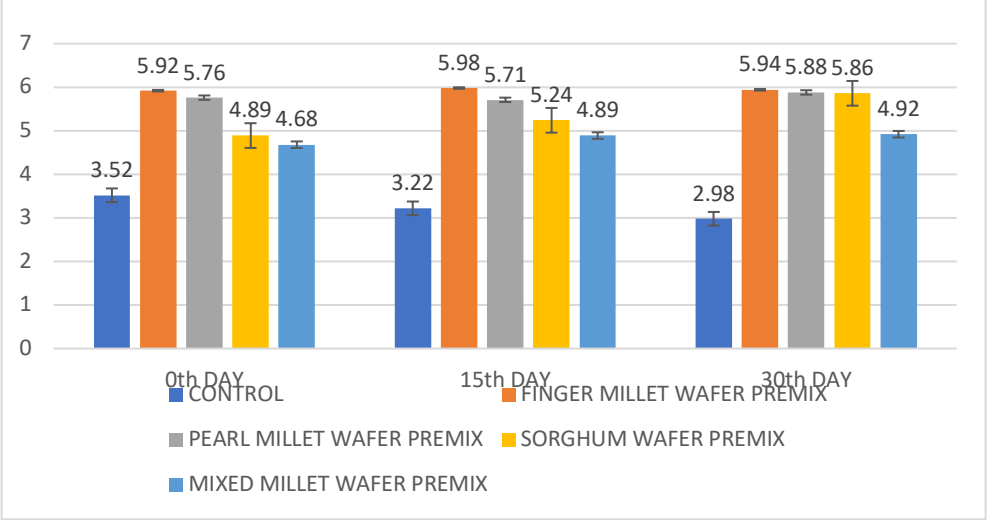


Figure 7. Comparison between moisture content of developed premixes

3.2.1.2. Estimation of total sugar:

Total sugar content of the developed premixes was estimated by Lean and Eynon method. Initially total sugar content of finger millet wafer premix, pearl millet wafer premix, sorghum wafer premix and mixed millet wafer premix were 19.33 gm, 18.60 gm, 20.86 gm, and 27.83 gm per 100 gm respectively. After 15 days and 30 days the amount of total sugar decreased gradually as shown in Table 7. This decrease in sugar content might be due to microbial activity in the premixes during storage.

Table 7. Total sugar content of developed premixes:

DAYS	CONTROL (% OF TOTAL SUGAR)	% OF TOTAL SUGAR (IN FINGER MILLET WAFER PREMIX)	% OF TOTAL SUGAR (IN PEARL MILLET WAFER PREMIX)	% OF TOTAL SUGAR (IN SORGHUM WAFER PREMIX)	% OF TOTAL SUGAR (IN MIXED MILLET WAFER PREMIX)
0th day	Reducing sugar =3.88 Non reducing sugar=17.88 Total sugar = 21.76	Reducing sugar = 3.22 Non reducing sugar=16.11 Total sugar = 19.33	Reducing sugar = 3.26 Non reducing sugar = 15.34 Total sugar = 18.60	Reducing sugar = 2.70 Non reducing sugar = 18.16 Total sugar = 20.86	Reducing sugar = 4.86 Non reducing sugar = 22.97 Total sugar = 27.83

15th day	Reducing sugar = 3.78 Non reducing sugar=17.76 Total sugar = 21.54	Reducing sugar = 2.84 Non reducing sugar=16.16 Total sugar = 19.00	Reducing sugar = 3.22 Non reducing sugar = 15.11 Total sugar = 18.33	Reducing sugar = 2.36 Non reducing sugar = 18.30 Total sugar = 20.66	Reducing sugar = 4.59 Non reducing sugar = 22.70 Total sugar = 27.29
30th day	Reducing sugar = 3.72 Non reducing sugar=17.56 Total sugar = 19.33	Reducing sugar = 2.48 Non reducing sugar=15.89 Total sugar = 18.37	Reducing sugar = 3.00 Non reducing sugar = 14.79 Total sugar = 17.79	Reducing sugar = 2.08 Non reducing sugar = 17.65 Total sugar = 19.73	Reducing sugar = 4.39 Non reducing sugar = 21.78 Total sugar = 26.17
SE+	1.344	0.488	0.412	0.603	0.847

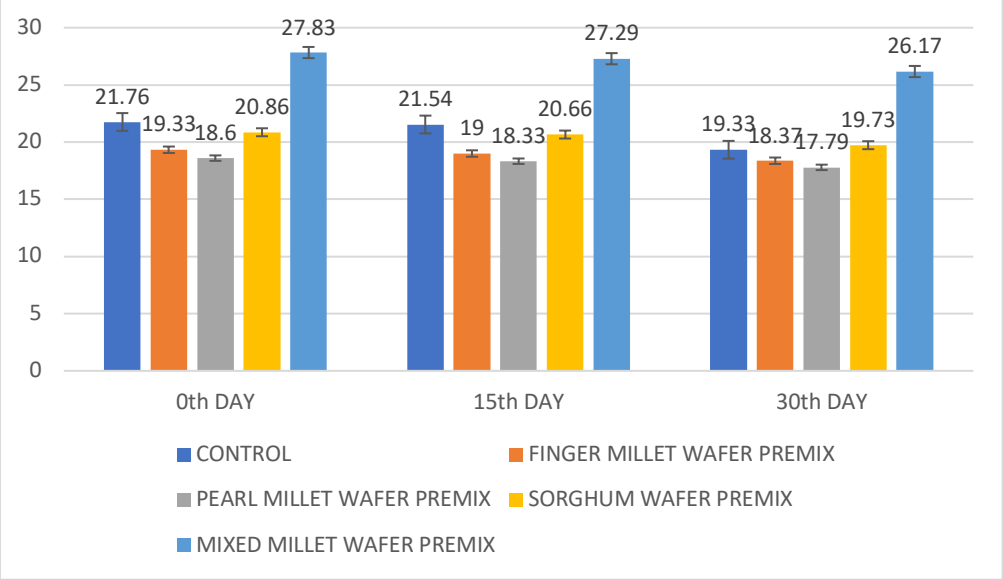


Figure 8. Comparison between total sugar content of developed premixes

3.2.1.3. Estimation of alcoholic acidity in developed premix

Alcoholic acidity (The amount of H_2SO_4 (mg) needed in 100 g of the sample to produce the equivalent amount of alcohol-soluble acids is known as the alcoholic acidity) is determined to check the quality of flour (egyankosh). Lower the alcoholic acidity means fresher the flour. Initial alcoholic acidity of finger millet wafer premix, pearl millet wafer premix, sorghum wafer premix and mixed millet wafer premix were 0.133%, 0.135%, 0.101% and 0.135% respectively. After 15 days and 30 days the percentage of alcoholic acidity increased. It can be due to the enzymatic hydrolysis of phytin, protein and/or fat.

Table 8. Alcoholic acidity of developed premixes: -

DAYS	CONTROL	% OF ALCOHOLIC ACIDITY (IN FINGER MILLET WAFER PREMIX)	% OF ALCOHOLIC ACIDITY (IN PEARL MILLET WAFER PREMIX)	% OF ALCOHOLIC ACIDITY (IN SORGHUM WAFER PREMIX)	% OF ALCOHOLIC ACIDITY (IN MIXED MILLET WAFER PREMIX)
0th day	0.150	0.133	0.135	0.101	0.135
15th days	0.145	0.175	0.133	0.180	0.168
30 days	0.166	0.223	0.166	0.224	0.223
SE+	0.011	0.045	0.019	0.062	0.044

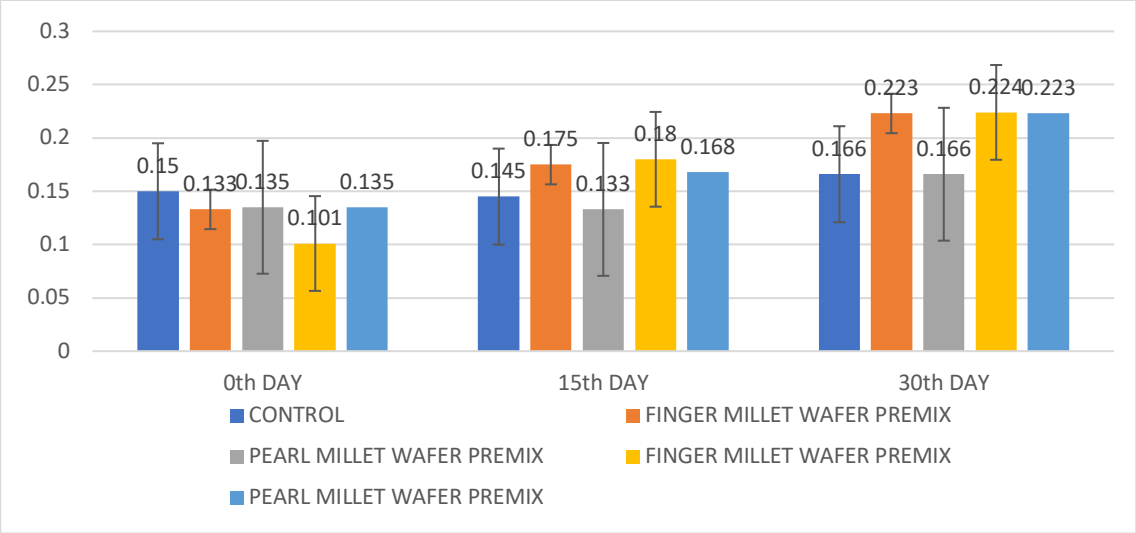


Figure 9. Comparison between alcoholic acidity of developed premixes

3.2.1.4. Estimation of carbohydrate, protein, and fat in developed premixes:

The nutritive values of developed premixes were calculated with the help of 'Food Composition Table' by ICMR (Longvah et al., 2017). Table 9 shows the total carbohydrate (CHO), protein and fat content of the developed premixes.

Table 9. Carbohydrate, protein, and fat content of developed premixes: -

NUTRIENT	CONTROL	FINGER MILLET WAFER PREMIX	PEARL MILLET WAFER PREMIX	SORGHUM WAFER PREMIX	MIXED MILLET WAFER PREMIX
CHO (gm)	72.100	68.295	66.783	68.553	62.613
Protein (gm)	8.210	9.611	10.751	10.454	9.419
Fat (gm)	2.181	0.742	1.795	0.685	1.064

3.2.1.5. Microbial analysis: -

Microbial load of the developed premixes was estimated by spread plate technique. According to a study by K. Geetha, Geetha M. Yankanchi and Netravati Hiremath (2019) the range of total bacterial count in millet based high fibre food mix was 3550 CFU/ ml to 5120 CFU/ ml from initial to 30th day of storage. In this study initially microbial growth was found on the plates with 10^{-1} and 10^{-2} dilution after 48 hours of incubation. On 30th day microbial growth was increased with the storage period as mentioned in Table 10.

Table 10. Total Plate Count of developed premixes

DAYS	CFU/ml (IN FINGER MILLET WAFER PREMIX)	CFU/ml (IN PEARL MILLET WAFER PREMIX)	CFU/ml (IN SORGHUM WAFER PREMIX)	CFU/ml (IN MIXED MILLET WAFER PREMIX)
0th day	2050.00	1900.00	2000.00	2600.00
30th day	3600.00	2650.00	3400.00	3850.00

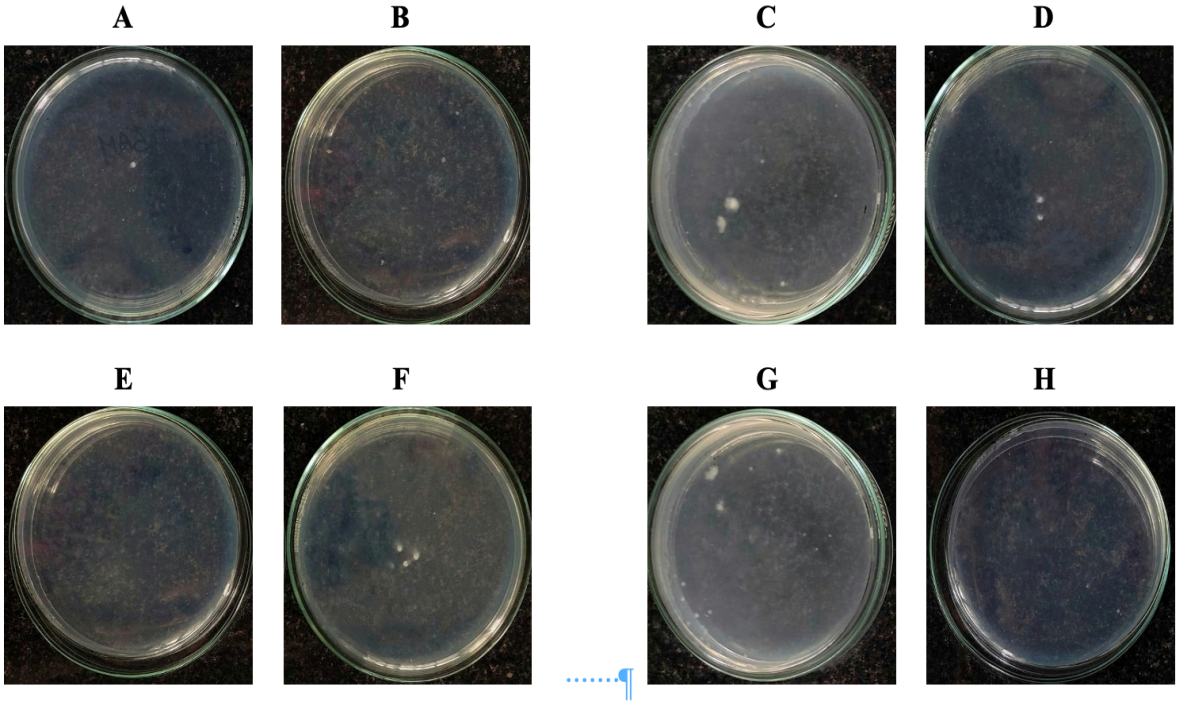


Figure 10. Microbial growth on agar plates with 10^{-1} & 10^{-2} dilution respectively on 0^{th} day (A & B. Finger millet wafer premix, C & D. Pearl millet wafer premix, E & F. Sorghum wafer premix, G & H. Mixed millet wafer premix)

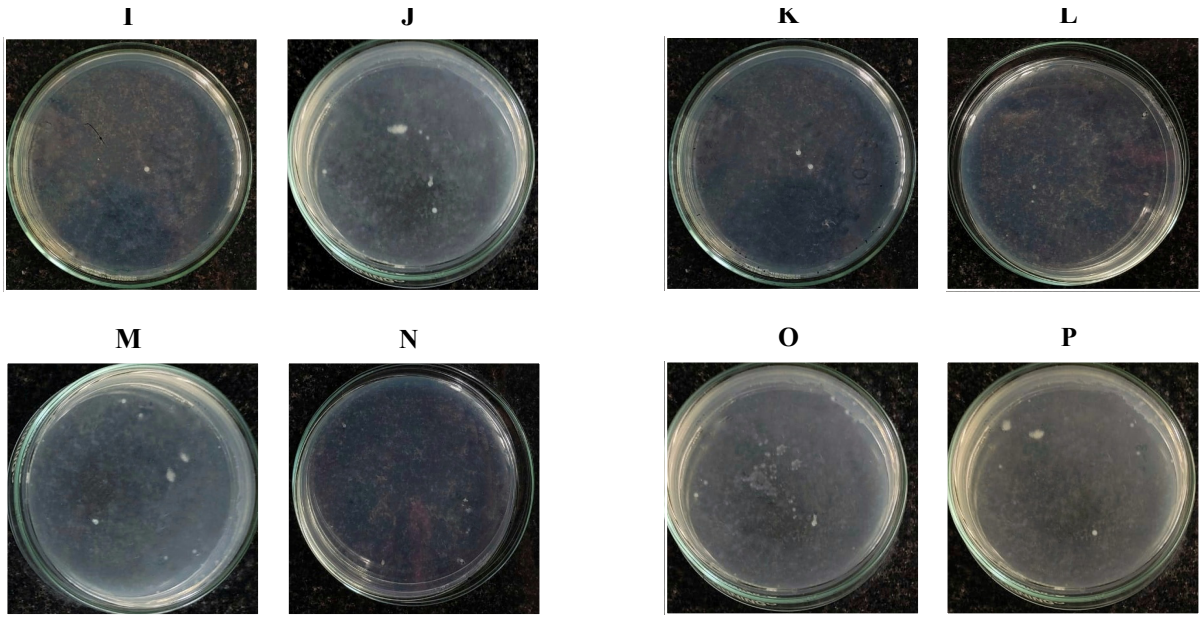


Figure 11. Microbial growth on agar plates with 10^{-1} & 10^{-2} dilution respectively on 30^{th} day (I & J. Finger millet wafer premix, K & L. Pearl millet wafer premix, M & N. Sorghum wafer premix, O & P. Mixed millet wafer premix)

4.Conclusions

In this study 4 different varieties of millet-based wafer premixes: finger millet wafer premix with chocolate flavour, pearl millet wafer premix with cinnamon flavour, sorghum wafer premix with vanilla flavour and mixed millet wafer premix with strawberry flavour were developed which were of acceptable quality according to the chemical, microbial and sensory evaluation. The final products were accepted by the sensory panel members. It has been observed that malting helped to reduce the preparation time of the wafers. Due to the germination of the seeds, the bioavailability of nutrients should be increased. Rice flour was added to improve the textural quality. According to the sensory panel wafers made of finger millet wafer premix and sorghum millet wafer premix with chocolate and vanilla flavour respectively got more acceptability.

Developed premixes matched the standard moisture content given by FSSAI, which is below 16% by weight. The sugar content of the premixes gradually decreased with storage, while alcoholic acidity increased. This may cause due to physical changes and/or microbial activity. There were no significant changes both in quality and sensory aspects during the study period. After 30 days also all the premixes were acceptable.

As the products are millet based, they contain high amounts of fibre and minerals such as iron, calcium, phosphorus etc. Millets contain more amount of complex carbohydrates rather than simpler ones and due to this property millets can help to reduce the risk of elevated blood pressure, blood sugar and other non-communicable diseases. Various essential amino acids and unsaturated fatty acids are present in millet. Millets do not contain gluten so; it can be consumed by celiac patients. Millets are called 'superfoods' due to their functional properties. They are proven antioxidants and show antimicrobial and anticarcinogenic activity. By using millets in commercial snack products, the upliftment of the total health of a community is possible. More over wafers in the form of cones, bowls etc. can contribute to edible cutlery which is sustainable and can reduce the load of waste from the food industry.

The accurate shelf life of the products can be studied in future as there were no significant changes observed in the product's quality during this study. Different packaging technology such as vacuum seal packaging can be introduced, moreover, we can think about better options for the packaging material rather than LDPE to make it eco-friendly as well as to protect the quality of the product inside for a longer time. Further study can be conducted to understand the effect on the products after malting and to compare the changes in the nutrient content of developed premixes with and without malting of millets. Also, by using minor millets like proso millet, foxtail millet etc. a new product can be developed. Future study can be conducted to develop new flavours. Last but not the least by modifying the amount of water and baking powder or by modifying the composition of the ingredients premix for muffins or cupcakes can be developed by following the same process.

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INHIBITORY EFFECT OF SELECTED SPICES ON POLYPHENOL OXIDASE FROM ICEBERG LETTUCE (*LACTUCA SATIVA* L.)

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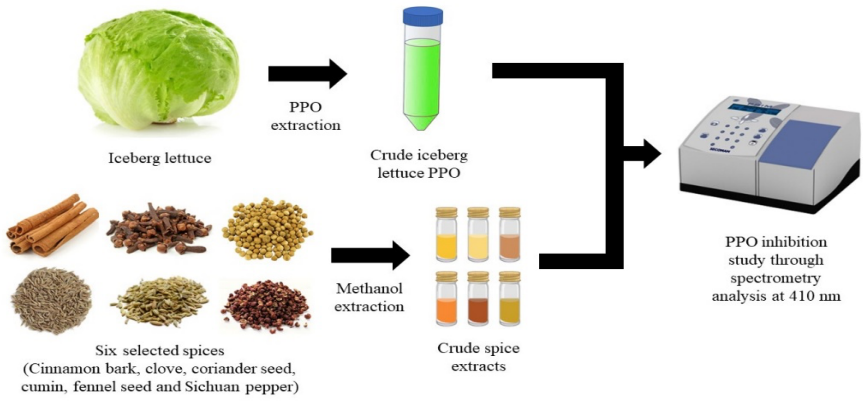
https://doi.org/10.34302/crpjfst/2024.16.2.10

Article history:
Received: May 20th, 2024
Accepted: June 25th, 2024

Keywords:
Polyphenol oxidase
Enzymatic browning
Iceberg lettuce
Spices
Inhibitor

ABSTRACT

Food browning is an undesirable phenomenon that alters sensory properties and nutritional value of fresh-cut produces, which had a significant economic impact owing to decreased customer acceptability. In this study, the effect of selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper on iceberg lettuce polyphenol oxidase (PPO) inhibition was investigated to replace synthetic browning inhibitor that often used by food manufacturers. Iceberg lettuce PPO has an enzyme activity of 13677.04 ± 21.00 EU/mL, using pyrocatechol as substrate. Ascorbic acid was used as the synthetic inhibitor of Iceberg lettuce PPO and it acted as a mixed inhibitor with the IC_{50} of 4.20 ± 0.19 mM. Among all the browning inhibition effect of the selected spices on Iceberg lettuce PPO, cinnamon bark was the best inhibitor among the six selected spice extracts, with the inhibition percentage of 32.39 ± 1.47 % inhibition was determined, followed by fennel seed (16.93 ± 1.47 %), Sichuan pepper (14.24 ± 0.83 %), cumin (13.72 ± 1.35 %), coriander seed (9.85 ± 0.75 %), and clove (9.64 ± 0.47 %) at 2.0 mg/mL. The present findings suggested the potential to expand the application of the spices to be used as food-based anti-browning inhibitors directly on the surface of iceberg lettuce as well as other fresh-cut produces or used as active ingredient of enzymatic browning-based active packaging of fresh-cut produces.



Graphical Abstract

1.Introduction

Enzymatic browning (EB) is an oxidation reaction that is caused by the catalytic reaction of the enzyme polyphenol oxidase (PPO) with the presence of phenolic compounds and oxygen (Arnold and Gramza-Michałowska, 2022). It is an undesirable reaction on fresh-cut produces as it causes darkening on their surfaces which make them unappealing to the consumers. Physical and mechanical injury such as cutting, peeling, dicing as well as extreme temperature fluctuations can destroy the tissue of the fruits and vegetables during processing or handling (Moon et al., 2020), allowing the interaction of PPO and its phenolic substrates under the presence of oxygen.

PPO (EC: 1.14.18.1) is a copper-containing enzyme from the oxidoreductase family. It can be furthered classified based on cresolase and catecholase activity, where the former catalysed monophenolase activity and the latter catalysed diphenolase activity (Biundo et al., 2020). For instance, cresolase responsible for the hydroxylation of monophenols into *o*-diphenols and catecholase responsible for the oxidase of *o*-diphenols to *o*-quinones. The formation of *o*-quinones will further undergo a nonenzymatic polymerization process, which results in the formation of high molecular-weight pigments, complexed and insoluble brown pigment known as melanin (Jiang et al., 2016).

EB is estimated to result in more than 50 % loss of global fruits and vegetables production (Moon et al., 2020). Therefore, there are various methods used by food industry to prevent the EB such as physical treatments including freezing, blanching, high-temperature short-time (HTST) method, high-pressure process and the application of synthetic anti-browning agents such as reducing agents, antioxidants, acidulants and chelating agents (Ioannou, 2013). However, physical treatments may alter the sensory properties and nutritional qualities of food, while the use of synthetic inhibitor may cause irritation and asthma that could affect the health status of the consumers (Se Hoo *et al.* 2022).

In recent times, the utilization of food and plant-based inhibitors has emerged as a viable option, as they are both safe for consumption and promote food sustainability. Spices are rich in bioactive compounds and can be utilised as food-based preservatives since they improve the nutritional value of food while also increasing its shelf life (Siew et al., 2022). Hence, it may enable food industries to use spices as food-based browning inhibitors to preserve and extend the quality and shelf life of fresh-cut fruits and vegetables, respectively (Siew et al., 2022, Sikora et al., 2021).

Iceberg lettuce (*Lactuca sativa* L.) is one of the famous *Asteraceae* family leafy vegetable. It contains numerous health benefit nutritional contents including carotenoids, polyphenols, vitamin B9, vitamin C and vitamin E that can protect against cancer, cardiovascular diseases, hyperlipidemia, metabolic syndrome, neurodegenerative diseases and osteoporosis (Lafarga *et al.* 2020). However, it is vulnerable to EB that significantly alter the organoleptic and biochemical properties of lettuce, thereby affecting its commercial value (Mai and Glomb, 2013). In this study, six selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper were examined with their respective PPO inhibition ability on iceberg lettuce.

2. Materials and methods

2.1. Plant and spices materials

Iceberg lettuce (*Lactuca sativa* L.) originated form Cameron Highlands, Malaysia was used as the source of PPO in this study. It was purchased from TF Value-Mart, Cheras, Malaysia. The selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper were purchased from a local market in Kuala Lumpur, Malaysia. The purchased iceberg lettuce and spices were in good condition and free from damage and spoilage. Iceberg lettuce and spices were stored in 4ºC and room temperature before usage, respectively. All the chemicals and reagents used in this study were analytical grade.

2.2. PPO extraction

The extraction of crude iceberg lettuce PPO was conducted according to Lim and Wong (2018) with slight modifications. Fresh iceberg lettuce (100 g) was washed and cut into fine strips, followed by homogenisation with 200 mL of pre-chilled (4 °C) phosphate buffer (0.1 M, pH 6.8) mixed with 4 g of polyvinylpyrrolidone (PVP) using a pre-chilled (4 °C) blender (Philips, HR2021/75, Malaysia) at maximum speed (3600 rpm) for 1 minute. The homogenate was then centrifuged (Universal 320 R, Hettich, Tuttlingen, Germany) at 7000 rpm at 4 °C for 20 minutes. The enzyme-containing supernatant obtained was proceeded with filtration with the aids of Whatman No. 1 filter paper. The resultant filtrate was the crude PPO extract of iceberg lettuce and store in small aliquots at -20 °C prior to analysis.

2.3. PPO assay

The PPO assay was adapted from the method of Lim and Wong (2018) with slight modifications. The reaction mixture consisted of 1.9 mL of 0.1 M phosphate buffer (pH 6.8) and 1.0 mL of 0.1 M pyrocatechol. The crude PPO (0.1 mL) was added into the reaction mixture and immediately transferred into a cuvette after mixing. The absorbance readings were taken at 15 second intervals over 5 minutes at 410 nm using a spectrophotometer (PRIM, Secoaman, France). The blank solution was consisted of 2.0 mL of phosphate buffer (0.1 M, pH 6.8) and 1.0 mL of pyrocatechol. The initial velocity was calculated from the initial slope of the absorbance against time curve. One unit (EU) of PPO activity is defined as the amount of the enzyme that increased the absorbance by 0.001 per min (Siew *et al.* 2022).

2.4. Effects of ascorbic acid on PPO activity

The effect of ascorbic acid on the iceberg lettuce PPO activity was determined according to the method by Lim et al. (2018) with slight modifications. Ascorbic acid was used as a synthetic inhibitor at different concentrations ranging from 0.05 mM to 0.2 mM. The reaction mixture consisted of 1.0 mL of ascorbic acid, l.0 mL of pyrocatechol at different concentrations (0.025 M, 0.05 M, 0.075 M, and 0.1 M) and 0.9 mL of phosphate buffer (0.1 M, pH 6.8). The crude PPO (0.1 mL) was then added into the reaction mixture and immediately transferred into a cuvette after mixing. The absorbance readings were taken at 15 second intervals over 5 minutes at 410 nm by using a spectrophotometer (PRIM, Secoaman, France). The blank solution was consisted of 1.0 mL ascorbic acid, l.0 mL pyrocatechol and 1.0 mL phosphate buffer (0.1 M, pH 6.8). The type of inhibition, inhibition percentage, Michaelis constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i) of the synthetic inhibitor were determined. The inhibition percentage of ascorbic acid was also expressed as IC_{50}, which is the concentration of the ascorbic acid required to inhibit 50 % of the enzyme activity (Se Hoo *et al.* 2022).

2.5. Preparation of selected spices extracts

The spices extracts were prepared according to Siew et al. (2022) with slight modifications. Each spice (50 g) was dried overnight in an oven (UNB 100, Memmert, Germany) at 50°C, before grinding (Philips, HR2021/75, Malaysia) them into fine powder at maximum speed (3600 rpm) for 1 minute. Each of the fine grinded spices (10 g) were extracted with 100 mL methanol for 1 hour via maceration. The spices extracts were then filtered by using Whatman No. 1 filter paper to remove the fine grinded spices. The extracts were recovered by using a rotary evaporator (R-200, Buchi, Switzerland) at 50 °C, followed by oven-dried (UNB 100, Memmert, Germany) at 50 ± 1 °C until constant weights were obtained. The weight of the dried extracts was recorded before storage at 4 °C prior to analysis.

2.6. Effect of the selected spices extracts on PPO activities

The effect of selected spices extracts on iceberg lettuce PPO activities was conducted according to the methods reported by Lim et al. (2018) with slight modification. The reaction mixture including 0.9 mL of phosphate buffer

(0.1 M, pH 6.8), 1.0 mL of pyrocatechol (0.025 M, 0.05 M, 0.075 M and 0.1 M), and 1.0 mL of 2 mg/mL spice extract dissolved in 5 % v/v DMSO. The crude PPO solution was then added quickly into the reaction mixture at 0.1 mL and then immediately transferred into a cuvette. The absorbance readings were taken at 15 second intervals over 5 minutes at 410 nm by using a spectrophotometer (PRIM, Secoaman, France). The blank solution consisted of 1.0 mL of phosphate buffer (0.1 M, pH 6.8), 1.0 mL of substrate and 1.0 mL of 2 mg/mL spice extract dissolved in 5 % v/v DMSO. The type of inhibition, inhibition percentage, Michaelis constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i) for each spice extract was determined.

2.7. Statistical Analysis

All the experiments in this study were performed with triplicate (n=3) and statistical analysis were done by using Microsoft Office Excel 2016 and IBM SPSS statistics 26. All the data collected were expressed in means ± standard deviation (SD). Analysis of variance (ANOVA) was analysed by using the Tukey's Post Hoc test with significant difference at $p < 0.05$.

3.Results and discussions

3.1. PPO extraction

PPO is an intracellular enzyme that only can be obtained by disrupting the cellular structure of plants. The iceberg lettuce tissues were homogenised using a blender to rupture cell walls and facilitate the release of PPO into the buffer (Sabarre & Yagonia-Lobarbio, 2021).

The pre-chilled phosphate buffer was employed to solubilize the released PPO while retaining its stability (Salis et al., 2007; Sabarre and Yagonia-Lobarbio, 2021). The pH of phosphate buffer was kept at 6.8 to ensure the stability of the enzyme. It coincided with the study of Taranto et al. (2017) that the optimal pH of lettuce PPO reported were between pH 5.0 to 8.0. Apart of the buffer pH, the concentration of the buffer will also affect PPO stability, where the ability to stabilise the pH of the buffer increases with the buffer concentration. Nevertheless, most enzymes preferred the moderate ionic strength between 0.05 to 0.2 M (Papaneophytou, 2021).

The phenolic compounds naturally present in iceberg lettuce can transform into polymeric pigments by the catalytic action of PPO (Sabarre & Yagonia-Lobarbio, 2021). Inhibit phenolic oxidation and polymerization during homogenization can be done by adding polyvinylpyrrolidone (PVP) to PPO extract in order to adsorb the phenolic substrates as well as inhibit oxidation (Lim and Wong, 2018). Centrifugation was carried out to separate PPO from the suspended particle in the homogenate, where the PPO remains in the supernatant. The PPO extract was then stored at -20 °C to retain enzyme activity (Tian et al., 2014).

3.2. PPO activity

The iceberg lettuce PPO had an enzyme activity of 13677.04 ± 21.00 EU/mL at pH 6.8 and room temperature, when pyrocatechol was used as substrate. It had superior PPO activity when compared to ginger PPO (9040 EU/mL), pearl brinjal PPO (11200 EU/ml) and '*Mas*' banana peel PPO (9000 ± 43.2 EU/mL), respectively when pyrocatechol was used as the substrate (Lim & Wong 2018; Se Hoo et al., 2022; Siew et al., 2022).

Iceberg lettuce was usually stored at refrigerated temperature to maintain the freshness and its quality to a greater extent of period (Meena et al. 2022). However, storage of iceberg lettuce at low temperature will only reduce the PPO activity but not deactivate the PPO activity completely. Therefore, PPO inhibitor can be incorporated to further delay the browning process of the iceberg lettuce where they are ready to be sold on the refrigerated shelf spaces. The application of browning inhibitor may also reduce the needs of refrigerated shelf spaces to store iceberg lettuce and other fresh-cut produces.

3.3. Effect of ascorbic acid on PPO activity

Ascorbic acid is an antioxidant and acidifying-based synthetic browning inhibitor. It

suppresses EB by reducing oxidised substrates and lowering the pH of the environment (Moon et al., 2020). However, browning will still occur when ascorbic acid is completely oxidized into dehydroascorbic acid in the later stage. The inhibition percentage of the ascorbic acid increased significantly ($p < 0.05$) from 4.51 ± 0.26 to 26.40 ± 0.91 % with its concentration from 0.05 to 0.2 mM (Table 1). Similar findings were reported by Sikora et al. (2019) that increasing the concentration of ascorbic acid increased the inhibition percentage. The IC_{50} of ascorbic acid was 4.20 ± 0.19 mM, which is the concentration of ascorbic acid required to inhibit 50 % of iceberg lettuce PPO activity.

It was observed that the V_{max} decreased when increasing the concentration of ascorbic acid (Table 1). This implies that ascorbic acid lowered the maximal reaction rate by reducing PPO catalytic reaction. The reduction of PPO catalytic reaction was supported by the increase of K_m, (Table 1), which indicates the binding of ascorbic acid to the PPO reduces the binding affinity between PPO and pyrocatechol.

The decreased of V_{max} and increased of K_m also suggested that ascorbic acid acts as a mixed inhibitor on iceberg lettuce PPO. The mixed inhibition of ascorbic acid was aligned to the previous study conducted by Doğan & Salman (2007) on iceberg lettuce PPO. However, ascorbic acid was also previously reported as competitive inhibitor and non-competitive inhibitor on blackberry, lotus root and lettuce PPOs (Doğan & Salman, 2007; Wong et al., 2019; Azzouzi et al., 2022). The diverse inhibition types observed in different studies underscore the influence of different PPO origin and substrates on inhibition characteristics (Gouzi et al., 2010).

K_i is the dissociation constant of the enzyme–inhibitor complex, which represents the binding affinity of inhibitor towards PPO (Doğan & Salman, 2007). The K_i values were present in Table 1, where the K_i' values were higher than K_i. The K_i'/K_i ratio was expected to be greater than one, indicating that the inhibitor's affinity for free PPO is greater than PPO-pyrocatechol complex (Gouzi et al., 2010). This finding indicates that the inhibitor can bind to both free PPO and PPO-pyrocatechol complex with different equilibrium constants for each interaction (Gouzi et al., 2010).

Table 1. Effects of ascorbic acid on iceberg lettuce PPO.

Inhibitor	[I] (mM)	Inhibition (%)	IC_{50} (mM)	V_{max} (EU/mL)	K_m (mM)	K_i ' (mM)	K_i (mM)	Type of inhibition
Control	-	-	-	16030.96	19.04	-	-	-
Ascorbic acid	0.05	4.51 ± 0.26[a]	4.20 ± 0.19	15715.09	23.00	2.51	0.22	Mixed
	0.10	14.26 ± 0.54[b]		14734.97	26.87	1.14	0.19	
	0.20	26.40 ± 0.91[c]		13323.54	32.96	0.99	0.20	

[a-c]Means ± standard deviations followed by different superscript letters within the same column are significant different at $p < 0.05$ according to Tukey's test.

3.4. Extraction yields of selected spices

Methanol was used as an extraction solvent in the preparation of spice extract. This is because methanol was an effective solvent to extracting potential anti-browning polyphenol and flavonoid compounds with high yields (Norsyamimi et al., 2014; Rezaei & Ghasemi Pirbalouti, 2019). The studies of Dong et al. (2004), El-Ghorab et al. (2010) and Shahwar et al. (2012) showed that methanol can obtain greater spices extracts' yield as compared to ether and hexane.

The extraction yield of the six selected spices was tabulated in Figure 1. Clove shows the highest extraction yields (21.94 ± 1.50 %) among the six selected spices, followed by Sichuan pepper (13.25 ± 0.99 %) and cinnamon bark (12.00 ± 1.31 %). There was no significant ($p > 0.05$) different between the extraction yields of coriander seed, cumin and fennel seed. These

spices show the lowest extraction yields, ranging from 4.89 ± 0.17 to 6.03 ± 0.67 %.

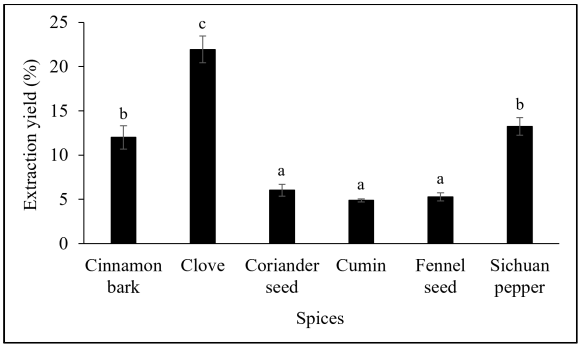


Figure 1. Extraction yield of the six selected spices.

[a-c]Different superscript letters within the same column are significant different at $p < 0.05$ according to Tukey's test.

3.5. Effects of selected spices extracts on PPO activities

The inhibition effect of the selected spices extracts on iceberg lettuce PPO were showed in Table 2. The inhibition percentage of the selected spices extracts were ranging from 9.64 ± 0.47 to 32.39 ± 1.47 %. Cinnamon bark extract (32.39 ± 1.47 %) had the highest inhibition percentage among all the selected spices extracts. It acts as a mixed inhibitor on iceberg lettuce PPO based on the Lineweaver-Burk plot plotted as shown in Figure 2.

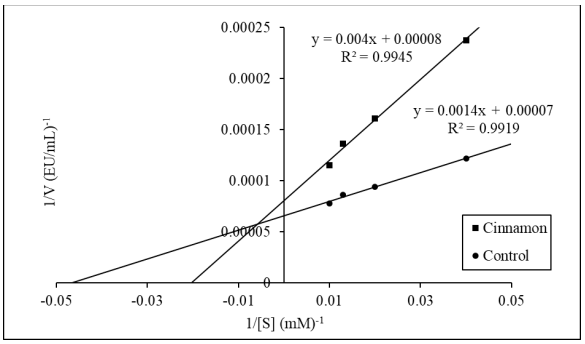


Figure 2. Lineweaver-Burk plot of the effect of cinnamon bark extract on iceberg lettuce PPO.

The mixed inhibition of cinnamon bark extract was also supported by the decrease of V_{max} and increase of K_m. Clove and Sichuan pepper extracts were also identified as mixed inhibitors based on the decreasing and increasing of the V_{max} and K_m, respectively (Table 2). Meanwhile, coriander seed, cumin and fennel seed extracts were acted as competitive inhibitors, where the V_{max} remained unchanged and the K_m increased (Table 2). Mixed inhibitor exhibits inhibition activity by binding to both free PPO and PPO-pyrocatechol complex, altering catalytic capacity, whereas competitive inhibitor exhibits inhibition activity by competing the PPO binding site with pyrocatechol (Roberts & Gibb, 2013; Ramsay & Tipton, 2017).

Cinnamon bark appeared to be the best and most effective inhibitor among the selected spices used in this study. The anti-browning activity of cinnamon bark were contributed by various bioactive compounds such as cinnamaldehyde, eugenol and cis-2-methoxycinnamic acid (Siew et al., 2022). The browning inhibition activity of cinnamon bark had also been demonstrated on banana, mushroom and soursop PPO, with the inhibition percentage ranging from 40 to 51.97 % (Marongiu et al., 2007; Weerawardana et al., 2020). It was also identified as the most effective browning inhibiting spices by Rossi et al. (2019) and Weerawardana et al. (2020).

Eugenol is also the most prevalent bioactive compound found in clove. It is responsible for its fragrant as well as the anti-browning properties. The browning inhibition of clove essential oil and eugenol emulsion had been reported by Chen et al. (2017) and Teng et al. (2020), which they were effective to inhibit the browning of fresh-cut lettuce and Chinese water chestnut.

The browning inhibition activity of cumin could be related to its major bioactive compound known as cuminaldehyde (Sunohara et al., 2021). Peng et al. (2023) reported cuminaldehyde is a strong tyrosinase inhibitor that can be found in cumin. Siew et al. (2022) had demonstrated the browning inhibitory effect of cumin and suggested that cumin could exhibited greater browning inhibition activity than the medicinal plant parts such as *Swietenia macrophylla* seeds and *Eurycoma longifolia* roots reported by Hassan et al. (2015).

Sichuan pepper contained a wide range of bioactive compounds including volatile oils, phenols and alkaloids, which phenols and alkaloids were the major antioxidant of Sichuan pepper (Sun et al., 2020). Phenolic compounds such as quercetin, hyperoside, kaempferol-3-O-rhamnoside, myricetin-3-O-β-D-galactopyranoside, rutin and dicoumarol and alkaloids such as alkylamides, tetrahydroberberine, α-sanshool, bungeanool, isobungeanool and dihydrobungeanool present in Sichuan pepper could also possessed anti-browning activity (Sun et al., 2020). According to Nirmal et al. (2015), phenolic compounds from plant extract can function as antimelanotics, owning to their metal chelating activity and structural similarity with PPO substrate (Nirmal et al., 2015).

The browning inhibition of both coriander seed and fennel seed had been reported previously. Lee & Kim (2020) observed a significant ($p < 0.05$) decrease in potato PPO activity with increasing fennel concentration. Meanwhile, coriander seed was found to have inhibitory effect on tyrosinase enzyme with IC_{50} of 13.94 ± 1.86 mg/mL (Siew et al., 2022). Their inhibition activity may be attributed to their flavonoid compounds (Mandal & Mandal 2015; Barakat et al., 2022). The flavonoid compounds might have synergistic browning inhibition effect with the presence of phenolic hydroxyl group in the fresh-cut produce, where increasing the concentration of phenolic hydroxyl group in the flavonoid compound showed positive impact on suppressing potato PPO (Jun et al., 2019). Nevertheless, the particular mechanism of driving flavonoid synergy is yet unknown and has to be researched further.

Table 2 Effects of selected spices extracts on iceberg lettuce PPO at 2 mg/mL

Spices extracts	Inhibition (%)	V_{max} (EU/mL)	K_m (mM)	K_i ' (mM)	K_i (mM)	Type of inhibition
Control (5 % v/v DMSO)	-	15215.84	21.41	-	-	-
Cinnamon bark	32.39 ± 1.47^{d}	12431.67	49.08	9.00	1.11	Mixed
Clove	9.64 ± 0.47^{a}	14098.05	24.15	25.31	9.20	Mixed
Coriander seed	9.85 ± 0.75^{a}	15221.34	35.11	-	3.13	Competitive
Cumin	13.72 ± 1.35^{b}	15210.58	39.44	-	2.38	Competitive
Fennel seed	16.93 ± 1.47^{c}	15219.01	45.70	-	1.76	Competitive
Sichuan pepper	14.24 ± 0.83^{bc}	13889.12	28.57	20.98	4.33	Mixed

$^{a-d}$Means ± standard deviations followed by different superscript letters within the same column are significant different at $p < 0.05$ according to Tukey's test.

4. Conclusion

The potential of spices of being food-based browning inhibitor had been studied on the inhibition of iceberg lettuce PPO. The browning inhibition percentage of the selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper on iceberg lettuce PPO were ranging from 3.80 ± 0.67 to 32.39 ± 1.47 % at 2 mg/mL. Cinnamon bark extract exhibited the highest significant ($p < 0.05$) inhibition percentage (32.39 ± 1.47 %) on iceberg lettuce PPO via mixed inhibition. Although the synthetic ascorbic acid was more effective in inhibiting RB than cinnamon bark extract at lower concentration, the preference for spices being used as food-based browning inhibitor aligns with contemporary consumer demands for health, organic and sustainable options. Besides extending the shelf life of fresh-cut fruits and vegetables, the application of spices also addresses health concerns by eliminating synthetic additives. Therefore,

spices are the potential food-based browning inhibitor to replace synthetic inhibitor to inhibit EB of fresh-cut produces.

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Acknowledgment

The authors would like to thank UCSI University on their financial support through Research Excellence & Innovation Grant (REIG-FAS-2022/008).

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journalhomepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

OPTIMIZING THE AQUEOUS EXTRACTION OF CROCIN FROM SAFFRON AND MODELING THE KINETICS OF ITS DEGRADATION DURING STORAGE AND HEAT TREATMENT

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https://doi.org/10.34302/crpjfst/2024.16.2.11

Article history:
Received: September 1st, 2023
Accepted: April 25th, 2024

Keywords:
Crocin;
Extraction method;
Degradation kinetic;
Storage;
Heat treatment.

ABSTRACT

This study aims to optimize the extraction of crocin from saffron through various methods and energy levels, and to investigate its stability during storage and heat treatment. Three extraction techniques−maceration, microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE)−were evaluated at different energy levels to determine the most efficient method. The resulting extracts were then subjected to stability tests under varying storage temperatures (-12 to 35 °C) and heat treatment conditions (100 to 200 °C). Our findings indicate that MAE and UAE, particularly at higher energy levels for 5 minutes, yielded the most efficient extraction, with an average coloring strength of 265. During storage, crocin degradation followed a zero-order kinetic model, with the degradation rate increasing with higher storage temperatures. The shortest half-life was observed at freezing temperature (100 hours), while the shortest half-life at 35 °C was less than 10 hours. Similarly, during heat treatment, crocin degradation followed a zero-order kinetic model at 100 and 150 °C, with half-lives of 260 and 74 minutes respectively. At 200 °C, the degradation kinetics shifted to first order, with a half-life of 20 minutes. Our results suggest that MAE and UAE at high energy levels are optimal for crocin extraction, and highlight the impact of temperature on crocin stability during storage and heat treatment.

1.Introduction

Crocus sativus L. is a highly prized species within the Iridaceae family, renowned for producing saffron, the world's most expensive spice. Saffron is derived from the carefully harvested stigmas of the flowers, which are meticulously dried to yield the coveted red filaments. With a history dating back to ancient times, saffron has been used not only as a spice but also as a medicinal plant and a natural coloring and flavoring agent in culinary applications. Extensive research has highlighted the myriad health benefits of saffron, including its anti-inflammatory, antioxidant, anti-carcinogenic, anti-

proliferative, and cardioprotective properties. Moreover, saffron exhibits neuroprotective qualities and has been associated with alleviating depression and stress (Dhiman and Kharkwal, 2020).

The therapeutic attributes of saffron stem from three primary compounds: crocin, picrocrocin, and safranal, which impart its distinctive coloring, bitter taste, and aromatic qualities, respectively. Beyond its traditional role as a coloring and flavoring agent in home cooking, saffron finds applications in various industries, including food, pharmaceuticals, cosmetics, and perfumery (Karbasi and Zandi Dareh Gharibi, 2022; Mzabri *et al.*, 2019). Due to its high value, saffron is incorporated into a wide range of dietary formulations to enhance the nutritional profile and sensory appeal of various functional food products. These include dairy items such as milk, cheese, and yogurt, as well as pasta, jams, baked goods, cookies, chocolates, and beverages like herbal teas and bitter drinks (Lage and Cantrell, 2009).

Crocin, also known as crocins, comprises a group of water-soluble compounds formed through the esterification of a fat-soluble dicarboxylic acid carotenoid known as crocetin with one or two molecules of glucose and/or gentiobiose (El Khoudri *et al.*, 2021; Poma *et al.*, 2012). This yellow coloring compound is highly concentrated in saffron, comprising up to 37% of its total weight (Lage and Cantrell, 2009).

The extraction of crocin, along with other bioactive compounds present in saffron, employs various methods and extraction durations. Originally, a standardized 24-hour extraction period was employed, utilizing the maceration technique as prescribed by (ISO.3632, 1980). This method has been used in previous studies for the extraction of secondary compounds from saffron (Esfanjani *et al.*, 2015; Oukhrib *et al.*, 2015). Other extended extraction durations, such as overnight (Escribano *et al.*, 1996) or 5 hours (Najafi *et al.*, 2021), have been investigated. However, these prolonged periods were subsequently reduced to one hour after observing the degradation of saffron's bioactive compounds (Orfanou and Tsimidou, 1996). The one-hour extraction method has been widely adopted by several authors (Masoumi *et al.*, 2021; Moradi *et al.*, 2022). Additionally, sonication at various durations (1, 3, 5, and 10 minutes) has been explored for extraction purposes (Kadkhodaee and Hemmati-Kakhki, 2006). Hence, these variations in extraction methods and conditions may contribute to differences in results.

Once crocin is extracted from its original stigma tissues, this highly water-soluble compound becomes remarkably susceptible to degradation, with various factors accelerating its decomposition. Studies on crocin stability have shown that variations in medium pH, whether acidic or basic, can induce its decomposition. Temperature is another crucial parameter, as it can facilitate oxidation processes. Furthermore, the presence of oxygen and exposure to light also contribute to crocin degradation (Karasu *et al.*, 2019; Tsimidou and Tsatsaroni, 1993).

This investigation seeks to establish the optimal extraction conditions using three methods set at varying energy levels: maceration (at 0, 200, 600, and 1000 rpm), microwave-assisted extraction (at powers of 100, 300, and 500 W), and ultrasound-assisted extraction (using amplitudes of 20, 60, and 100%). Subsequently, the stability of the extract was evaluated through a kinetics modeling study conducted during storage at different temperatures (−12, 4, 22, and 35 °C) and heat treatment at 100, 150, and 200 °C.

2. Materials and methods

2.1. Saffron sampling

Saffron flowers (*Crocus sativus*) were procured from the Safran Tariki Association after their harvest at the Constantine research farm in Algeria. The stigmas were manually extracted from the flowers and subsequently air-dried in the shade at an average temperature of 23 °C for 10 days. Upon drying, the samples were transported to the Laboratory of Applied Biochemistry (LBA) at the University of

Bajaia. The stigmas were then ground using a mortar and sieved, with particles smaller than 250 nm retained for subsequent analyses.

2.2. Optimization of crocin extraction with different methods

To investigate the optimization of crocin extraction, three distinct methods were employed: one conventional method involving maceration, and two non-conventional methods, namely microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). The extraction process entailed mixing 10 mg of saffron powder with 50 ml of distilled water. Extractions were carried out at various time intervals (0, 0.5, 1.5, 3, 5, 10, 15, and 20 minutes). The resultant extracts were recovered via centrifugation at 5000 rpm for 5 minutes using a Növe NF 200 centrifuge. Crocin content was determined by measuring the absorbance at 438 nm using a spectrophotometer (UvLine 940, Secomam, France). Crocin results were expressed as the coloring strength of the solutions using a 1-cm quartz cell, as per the following equation (Eq. 1).

$$E_{1cm}^{1\%}(\lambda_{440nm}) = \frac{Abs \times 200}{\mathrm{W}(100 - \mathrm{Wmv})} \qquad (1)$$

Where, $E_{1cm}^{1\%}(\lambda_{440nm})$ is the specific extinction, Abs is the absorbance at 438 nm, W is the used sample weight (g), Wmv is the weight of moisture and volatiles in the sample (8%), 200 was the dilution factor.

For each method, different levels were tested. Maceration was conducted using a magnetic stirrer (Multistirrer Digital 15, VELP Scientifica, Italy) at three rotation speeds: 200, 600, and 1000 rpm. Additionally, extraction without agitation (0 rpm) was evaluated. The microwave (Maxipower, China) was operated at three power levels: 100, 300, and 500 W. The sonicator (Sonics Vibra Cell VCX 130, Sonics, USA) was equipped with a probe, and three amplitudes were tested: 20, 50, and 100%.

2.3. Effect of storage temperature on the stability of crocin

To assess the stability of the aqueous crocin extract during storage, various temperatures were examined. Crocin extract obtained under optimal conditions was divided into 20 ml aliquots and sealed in test tubes to prevent water evaporation. These test tubes were then placed in different environments: a freezer at −12 °C, a refrigerator at 4 °C, and ovens set to 20 °C and 35 °C. Crocin levels were measured at different time intervals throughout the storage period (0, 2, 5, 10, 15, and 20 days). The results were expressed as percentages relative to the initial crocin levels.

2.4. Effect of heating on the stability of crocin

To evaluate the thermal stability of the crocin aqueous extract, various temperatures were applied during heat treatment. The optimized crocin extract was subjected to different temperatures for varying durations. Specifically, 20 ml of the aqueous extract was placed in test tubes and exposed to temperatures of 100, 150, and 200 °C for durations ranging from 1 to 90 minutes. Crocin levels were quantified as percentages relative to the absorbance measured at the initial time point (t = 0).

2.5. Degradation kinetics

The evaluation of three reaction models (orders 0, 1, and 2) for predicting crocin degradation indicated that only the zero and first-order models effectively represented the degradation rate (Eq. 2 and Eq. 3).

$$[\mathrm{C}]_t = -kt + [\mathrm{C}]_0 \qquad (2)$$

$$\mathrm{Ln}\left(\frac{[\mathrm{C}]_t}{[\mathrm{C}]_0}\right) = -\mathrm{kt}. \qquad (3)$$

Where, $[C]_t$ is the concentration of crocin at the time *t*, $[C]_0$ is the initial concentration of crocin, t is time, and k is constant rate of degradation.

The half-life time ($t½$) for the zero-order reaction is calculated as $[C]_0/2k$, while for the first-order reaction, it is calculated as $Ln(2)/k$.

2.6. Statistical analysis

The data underwent processing using Microsoft Office Excel 2013 for calculating the means and standard deviations of the triplicates, graph plotting, and kinetic modeling. The analysis of variance (ANOVA) along with the HSD test was conducted using Statistica software (StatSoft, Inc., version 7.0.61.0).

3. Results and discussion

3.1. Effect of extraction method and time on crocin yield

The extraction kinetics of crocin from saffron powder varied according to the extraction method, as illustrated in Figure 1. The initial extraction, occurring immediately upon contact of the solvent with the powder (t = 0 min), yielded an average value of 74.64 across all the three methods. This initial phase represents the rapid dissolution of crocin molecules present on or near the surface of the powder particles, requiring minimal extraction time. In the case of maceration, crocin concentration increased quickly at all the three rotation speeds (200, 600, and 1000 rpm) during the initial minutes of extraction, followed by a gradual decrease in extraction rate until stabilization. Statistical analysis revealed that maximum crocin extraction values were achieved after 10 minutes at all rotation levels, reaching a value of 205.80. However, extraction without agitation showed a continuous increase in crocin concentration throughout the extraction period, albeit resulting in a lower value of 178.06 at the end of extraction compared to agitation-assisted methods. Stirring facilitated the diffusion of crocin particles from the plant matrix into the solvent, explaining the slower extraction rate observed in the absence of agitation. While the speed of agitation did not significantly affect the crocin extraction rate, minimal agitation proved necessary for effective extraction.

In a study investigating the optimization of bioactive compound extraction from apricot kernel shells, it was observed that the extraction rate increased with the stirring speed from 300–700 rpm to 1100 rpm, after which it stabilized at 1500 rpm (Teffane *et al.*, 2022). Similarly, research on the extraction of polyphenols from mango seed kernels demonstrated that higher agitation speeds enhanced extraction, although speeds of 400–450 rpm resulted in a detrimental effect (Anta *et al.*, 2020). It's noteworthy that elevated stirring speeds do not invariably translate to improved yields; in fact, they can sometimes lead to reduced yields because of compound degradation caused by oxygen dissolution, which triggers the oxidation of already extracted molecules, particularly during prolonged extractions and at high temperatures. Moreover, exceeding a certain speed threshold represents unnecessary energy consumption.

The kinetics of Microwave-Assisted Extraction (MAE), depicted in Figure 1, illustrated an escalating crocin recovery rate over time during the initial extraction phase across all the tested power levels. Crocin concentration stabilized after 5 minutes at 500 W, reaching 257.61. However, extraction stability was achieved after 10 minutes for 300 W and 100 W. While 300 W attained a similar extraction level as 500 W, it necessitated twice the duration. Statistical analysis encompassing the entire extraction period identified 500 W as the optimal power for crocin extraction, followed by 300 W and then 100 W. Nonetheless, during the latter phase of extraction (post-10 min), no discernible difference in crocin yield was observed between 300 W and 500 W. Consequently, for energy efficiency considerations, a microwave power of 300 W was deemed preferable for saffron crocin extraction, notwithstanding the longer extraction duration.

Numerous studies have highlighted the substantial impact of microwave power on the extraction rate of target molecules. For instance, in the recovery of carotenoid pigments from *Citrus clementina* peels, the extraction rate surged with increasing power until reaching 560 W; beyond this threshold, the yield notably decreased (Kadi *et al.*, 2022).

Similarly, the extraction efficiency of antioxidant compounds from *Opuntia ficus-indica* seeds exhibited an increase in yield between 100 and 500 W, with no observed effect beyond this range (Boudjouan *et al.*, 2021). Conversely, the extraction of phenolic compounds from *Bellis perennis* flowers revealed that escalating microwave power led to a significant reduction in yield and degradation of the compounds (Bouallag *et al.*, 2022).

The use of reasonable microwave levels improved extraction yields. Indeed, during microwave extraction, the waves generated penetrate the particles and interact with polar molecules having a positive dipole moment, in particular water, to induce magnetic interactions and rotational movements, which cause molecular friction and an increase in heat at the center of the particle. The difference in heat between the variable levels of the granule creates a pressure gradient, which causes a draining force of the compounds from the inside of the particle to the outside (Khaled Khodja *et al.*, 2020). However, the choice of microwave power for the extraction of the desired compounds must be made with care, as compounds can be decomposed by the effects of irradiation and increased temperature, which accelerate chemical reactions, especially with the use of extended extraction times and in the presence of oxygen (Bachir-bey *et al.*, 2013; Ismail-Suhaimy *et al.*, 2021).

The ultrasound-assisted extraction results for crocin are depicted in Figure 1. The extraction process exhibited two distinct phases. Initially, there was a rapid acceleration phase lasting from 0 to 5 minutes for 100% amplitude and from 0 to 10 minutes for 60% and 20% amplitudes. Subsequently, a stationary phase ensued until crocin extraction reached stability. Statistical analysis encompassing the entire extraction period revealed significantly different efficiencies among the three amplitudes, with 100% exhibiting the highest efficiency, followed by 60%, and then 20%. However, during the stationary phase, the last two amplitudes exhibited comparable crocin yields, averaging around 243.57. Consequently, the 100% amplitude, with a 10% higher efficiency compared to the other two, is recommended for optimal crocin extraction from saffron.

The findings from the ultrasound-assisted extraction (UAE) align with previous research by Kadkhodaee and Hemmati-Kakhki (2006), demonstrating that increasing the sonication amplitude enhances the extraction rate while significantly reducing the required time. The authors concluded that an amplitude of 100% and a sonication duration of 10 minutes provided optimal conditions for crocin extraction, yielding an estimated 239.3.

The efficacy of ultrasound-assisted extraction (UAE) in extracting target compounds has been highlighted in various studies. For instance, in the extraction of phenolic compounds from carob and date pulps, it was observed that yields increased with the amplitude up to 85%; however, concentrations decreased beyond that threshold (Benkerrou *et al.*, 2018a; Saci *et al.*, 2018). Similarly, optimal amplitudes of 74% and 65% were identified during the extraction of bioactive compounds from other plant resources using ultrasound extractors (Benkerrou *et al.*, 2018; Zemouri-Alioui *et al.*, 2018).

The efficiency of extraction is significantly enhanced with the amplification of mechanical action resulting from increased ultrasonic amplitude. This phenomenon triggers cavitation bubbles, which collapse on the particle surface, releasing substantial energy that ruptures cell tissue and facilitates compound release. However, at certain amplitudes, extraction efficiency may decline due to elevated temperatures and the production of free radicals through sonochemical reactions, leading to compound oxidation. Additionally, cavitation efficiency may decrease as bubbles are destroyed upon formation (Benkerrou *et al.*, 2018; Tiwari *et al.*, 2010; Zemouri-Alioui *et al.*, 2018).

Statistical analysis revealed that crocin extraction stabilized between 5 and 10 minutes

for all methods except for maceration without agitation (Table 1). Initially, extraction commenced with crocin molecules diffusing from the matrix, driven by concentration gradients between particles and solvent. However, this diffusion slowed over time until equilibrium was reached between the interior and exterior of the powder particles. Molecules nearer the surface were more accessible to the solvent compared to those in deeper layers, resulting in a gradual slowdown of extraction until stability was attained. This equilibrium could be influenced by the extraction method and energy level employed.

Notably, the most efficient crocin extraction was achieved with 100% ultrasound amplitude, as well as microwave powers of 300 W and 500 W. Conversely, maceration proved to be the least efficient method, regardless of agitation level. Maceration without agitation and microwave extraction at 100 W exhibited a significant loss of approximately one-third of the expected crocin amount and should thus be avoided.

The selection of an appropriate extraction duration is pivotal for achieving optimal results. Traditionally, a prolonged extraction period of 24 hours was advocated in the ISO 3632 standard ISO.3632 (1980). However, this extended timeframe often resulted in a notable loss of color intensity due to the rapid degradation of saffron pigments. Recognizing this, subsequent revisions in ISO 3632-2010 (ISO.3632-2, 2010) reduced the recommended extraction time to just 1 hour. Supporting this adjustment, a study by (Orfanou and Tsimidou, 1996) demonstrated a 15% increase in crocin yield when utilizing a 1-hour extraction compared to the conventional 24-hour period. In line with these findings, our investigation determined that a duration of 5 to 10 minutes proved optimal for achieving maximum crocin extraction efficiency.

This study underscores the significant influence of extraction method, energy level (such as agitation, power, or amplitude), and extraction duration on the efficiency of crocin extraction from saffron.

3.2. Effect of storage temperature on crocin stability

Saffron is not only valued for its therapeutic compounds but also cherished for its vibrant yellow hue attributed to crocin. Given its widespread use in functional foods, products containing saffron are often subjected to various storage conditions and temperatures, which may accelerate the degradation of bioactive compounds. Thus, this section delves into the kinetics of crocin degradation during storage at different temperatures.

To investigate, a crocin extract obtained under optimal extraction conditions (UAE at 100% for 5 minutes) was divided into four aliquots and stored at varying temperatures (-20, 4, 22, and 35 °C) to simulate freezing, refrigeration, room temperature, and warm conditions, respectively. Crocin levels were measured at different intervals (0, 2, 5, 10, and 20 days), and the results were expressed as percentages (Figure 2).

Analysis of the extract over 20 days of storage revealed significant crocin degradation across different temperatures. Notably, degradation intensified with higher temperatures. At freezing temperatures (-12 °C), crocin degradation was minimal, with only a 10% loss observed at the end of the storage period. Preservation of crocin was satisfactory at 4 °C, albeit with gradual degradation over time. Conversely, room temperature (22 °C) led to rapid crocin degradation, with an 80% reduction by the end of the storage period. Lastly, storage at 35 °C resulted in swift crocin degradation, nearly depleting its levels entirely within 20 days.

Similar findings were noted in studies investigating the stability of saffron aqueous extract, wherein crocin degradation escalated with rising temperatures (Tsimidou and Tsatsaroni, 1993). Notably, crocin degradation was observed even during the storage of saffron stigma and powder (Atyane *et al.*, 2017; Chaouqi *et al.*, 2018).

To enhance comprehension of crocin degradation kinetics during storage, modeling of the degradation reaction rate was

undertaken. Statistical analysis revealed that this rate conforms to a zero-order reaction, as described by the following model:

$$V = -k[C]^0 \text{ or } V = -k \qquad (4)$$

Where V is the reaction rate, $-k$ is the reaction constant, the minus sign indicates the decrease in crocin, and the number in superscript indicates the order of the degradation rate.

The concentration of crocinat a time "t" ($[C]_t$) is given by the following equation:

$$[C]_t = -kt + [C]_0 \qquad (5)$$

Where $[C]_0$ is the initial concentration of crocin.

The parameters used for modeling crocin degradation kinetics are presented in Table 2. The negative sign signifies crocin depletion during storage, with degradation becoming more pronounced as the value of k escalates. A minimal degradation rate was observed at −12 °C, whereas degradation at 4 °C was fourfold that of freezing. Elevating the temperature from 4 to 22 °C and from 22 to 35 °C increased the degradation rate by 150%. Moreover, the high coefficients of determination (R^2), ranging from 0.976 to 0.996, indicate a strong agreement between the experimental results and the calculated values by the models. The low probability values (P-values) suggest the significant fit of the four models, indicating that crocin degradation conforms to the zero-order reaction rate.

To determine the shelf life of crocin at various temperatures, the half-lives were computed for the zero-order reaction rate using the formula: $t_{1/2} = [C]_0/2k$. As per the results depicted in Table 2, the longest half-life (100 days) was observed at freezing temperature, followed by refrigeration (4 °C), room temperature, and finally 35 °C, where the estimated half-life was less than 10 days.

In a study examining the stability of microencapsulated crocin in aqueous environments, crocin degradation followed first-order kinetics, with increasing k values observed at higher storage temperatures (Karasu *et al.*, 2019). The researchers reported a half-life of 52 hours for crocin microencapsulated at pH 6 when stored at 60 °C. However, this duration decreased notably at higher temperatures of 70, 80, and 90 °C, with corresponding half-lives of 27, 17, and 11 hours, respectively. Another investigation showed that the aqueous extract of crocin at pH 7 experienced degradation during storage that was 20 times more pronounced than at 40 °C (Tsimidou and Tsatsaroni, 1993).

The degradation of bioactive compounds can fellow different pathways. The degradation kinetics of ascorbic acid were investigated in an intermediate moisture model food system, varying with water activity (0.69-0.90) and temperature (61-105°C). The degradation of ascorbic acid in each scenario adhered to a zero-order kinetic model (Laing *et al.*, 1978). Whereas, the study on the stability of anthocyanin extracts from mangos teen peel under varying storage temperatures revealed that the extracts exhibited also first-order kinetics, with the highest half-life observed at 5°C (4006 hours), followed by 28°C (370 hours), 40°C (125 hours), and 50°C (93 hours) (Chisté *et al.*, 2010).

The findings from the storage experiments underscore the importance of storing food products containing saffron or saffron extracts at lower temperatures to maintain the coloring power of crocin over extended periods.

3.3. Heating effect on crocin stability

During food processing or culinary preparation, heat treatments are frequently employed to eliminate microorganisms, inhibit enzymes, and develop desired organoleptic characteristics. Consequently, this section addresses the effect of heating on aqueous crocin extract to study its impact on crocin degradation. Three temperatures were tested: 100, 150, and 200 °C, to simulate cooking in an aqueous medium at atmospheric pressure, under pressure, and in the oven, respectively. The remaining crocin content was measured as a percentage at various time intervals over a

total duration of 90 minutes, corresponding to the maximum cooking time.

The results of the study on crocin thermal degradation kinetics in aqueous extracts are depicted in Figure 3. Minimal degradation was observed during the heating treatment at 100 °C, with only one-fifth degraded during the 90-minute heating period. At 150 °C, crocin degradation occurred gradually, while at 200 °C, an accelerated degradation was observed initially, followed by a slowdown over time.

To comprehend crocin degradation behavior during heating, a kinetic study was conducted. Modeling of the three kinetics revealed that crocin degradation at temperatures of 100 and 150 °C followed zero-order reaction kinetics, indicating a linear decrease in concentration over time, similar to storage conditions as described previously.

However, treatment at 200 °C fitted the first-order degradation kinetics according to the equation: $V = -k\,[C]^1$. The half-life time ($t_{1/2}$) is equal to 0.693/k. The kinetic parameters for the three temperatures are summarized in Table 3. Based on the R^2 and P-values, the models of crocin degradation kinetics showed a strong fit with the established kinetic orders. Moreover, at 100 °C, the longest half-life was observed, followed by 150 °C, while 200 °C revealed very rapid crocin degradation.

It has also been observed that the degradation of aqueous crocin extracts increases with rising temperatures, from 5 to 70 °C (Sánchez *et al.*, 2008). Since crocin is a carotenoid derivative, heat treatment induces its isomerization from *trans* to *cis* form, oxidation, and degradation (Atencio *et al.*, 2022; Meléndez-Martínez *et al.*, 2023). Therefore, the high temperatures applied during various food preparations lead to crocin degradation; consequently, incorporating saffron filaments or infusions in food preparations should occur towards the end of heat treatments, approximately five to ten minutes before processing is completed.

The kinetics of color degradation in pineapple puree were examined during heat treatment within the range of 70-110 °C by Chutintrasri and Noomhorm (2007). The alterations in L and b values conformed closely to the first-order kinetic model, whereas a value and Browning index adhered to the zero-order kinetic model. The order of reaction can even vary for the same compounds, depending on the medium. For instance, the decolorization reaction of carotenoids, including β-carotene, di-esterified capsanthin, and capsanthin, was examined. It was observed that reactions occurring in an anhydrous medium follow zero-order kinetics, whereas those occurring in an aqueous medium adhere to first-order kinetics (Minguez-Mosquera and Jaren-Galan, 1995).

Table 1. Crocin amounts at optimal times according to the extraction method and energy level

Extraction method	Energy level	Crocin	Required time for extraction (min)
Maceration	0 rpm	178.06 ± 5.12 ^{e}	20
	200 rpm	208.70 ± 4.09 ^{d}	10
	600 rpm	204.57 ± 7.16 ^{d}	10
	1000 rpm	204.13 ± 7.16 ^{d}	10
MAE	100 W	168.04 ± 8.18 ^{e}	10
	300 W	264.78 ± 9.21^{a}	10
	500 W	257.61 ± 4.09 ^{ab}	5
UAE	20%	238.04 ± 7.16 ^{c}	10
	60%	242.09 ± 6.14 ^{bc}	10
	100%	270.65 ± 5.74 ^{a}	5

The results of crocin with different letters are statistically different (ANOVA, HSD test, $p < 0.05$, a>b>c>d>e).

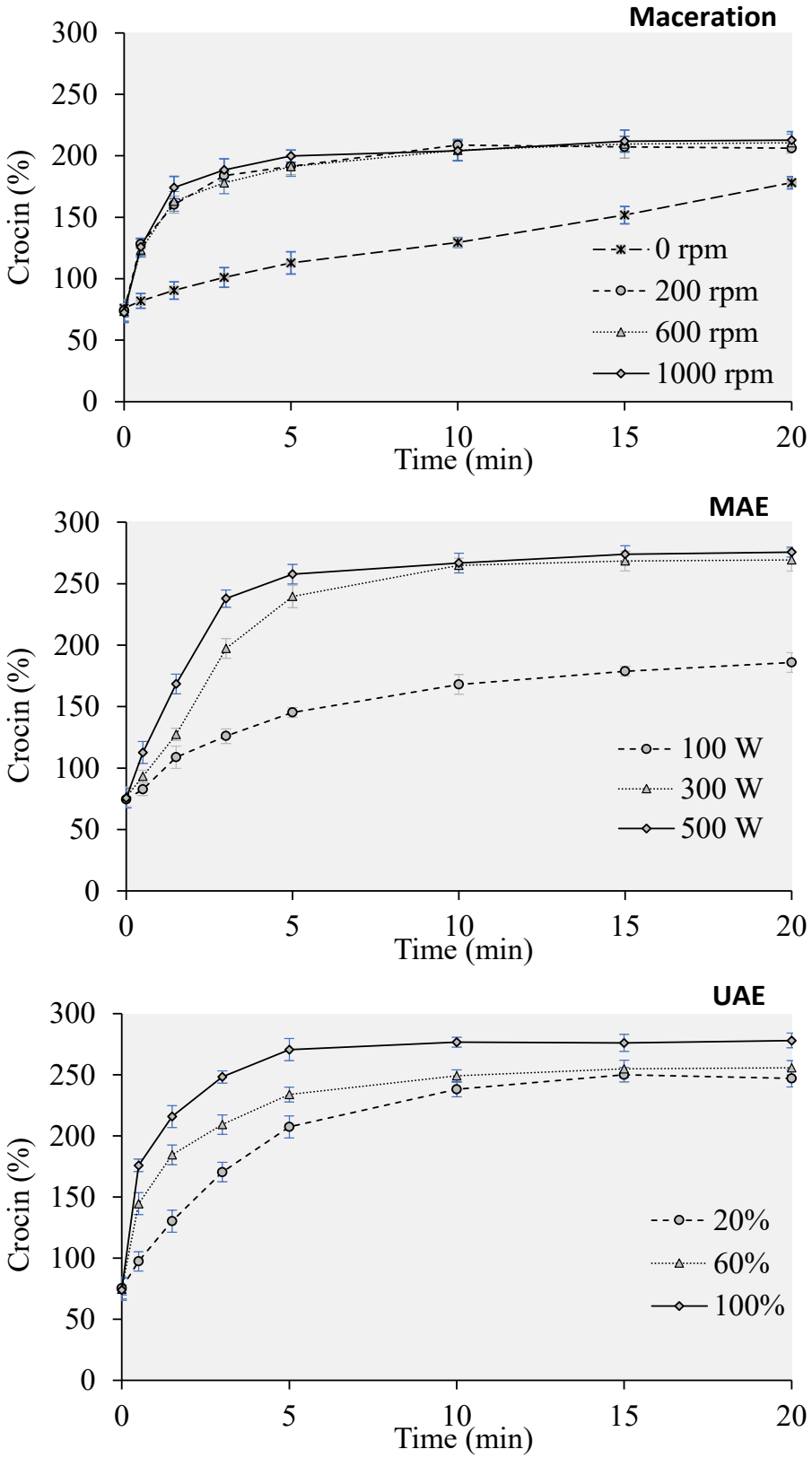

Figure 1. Evolution of crocin extraction using maceration

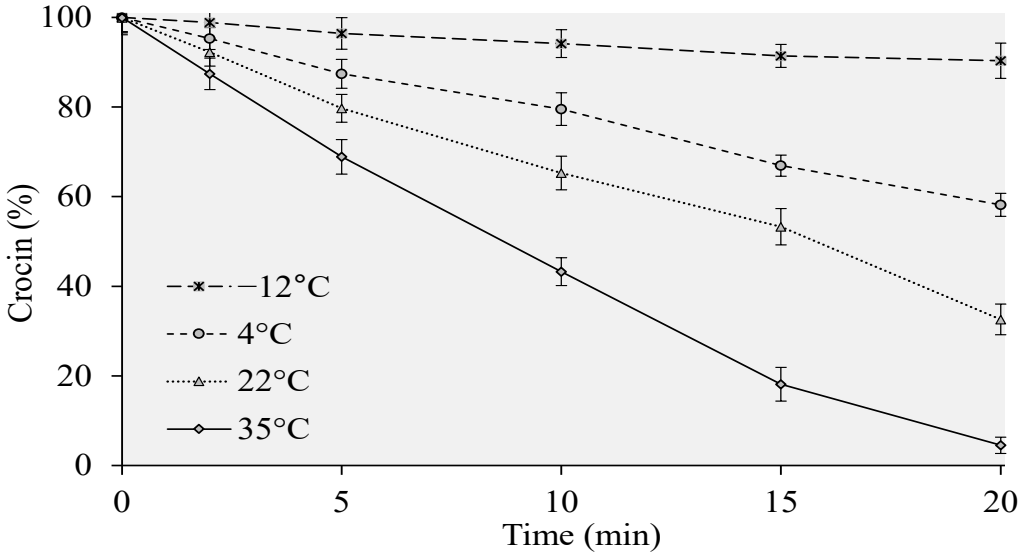

Figure 2. Evolution of crocin percentage during storage of aqueous extract at different temperatures

Table 2. Kinetic parameters of crocin degradation at different storage temperatures

Parameter Temperature	Crocin concentration	R^2	P-value	$t_{1/2}$ (days)
−12 °C	$[C]_t = -0.497\ t + 99.505$	0.976	0.0002	100.050
4 °C	$[C]_t = -2.087\ t + 99.315$	0.996	< 0.0001	23.791
22 °C	$[C]_t = -3.232\ t + 98.543$	0.993	< 0.0001	15.244
35 °C	$[C]_t = -4.874\ t + 95.939$	0.986	< 0.0001	9.842

$[C]_t$, amount of crocin; t, any time in the studied interval period; R^2, coefficients of determination; P-value, the level of significance of the model; $t_{1/2}$, the half-life of crocin degradation expressed in days.

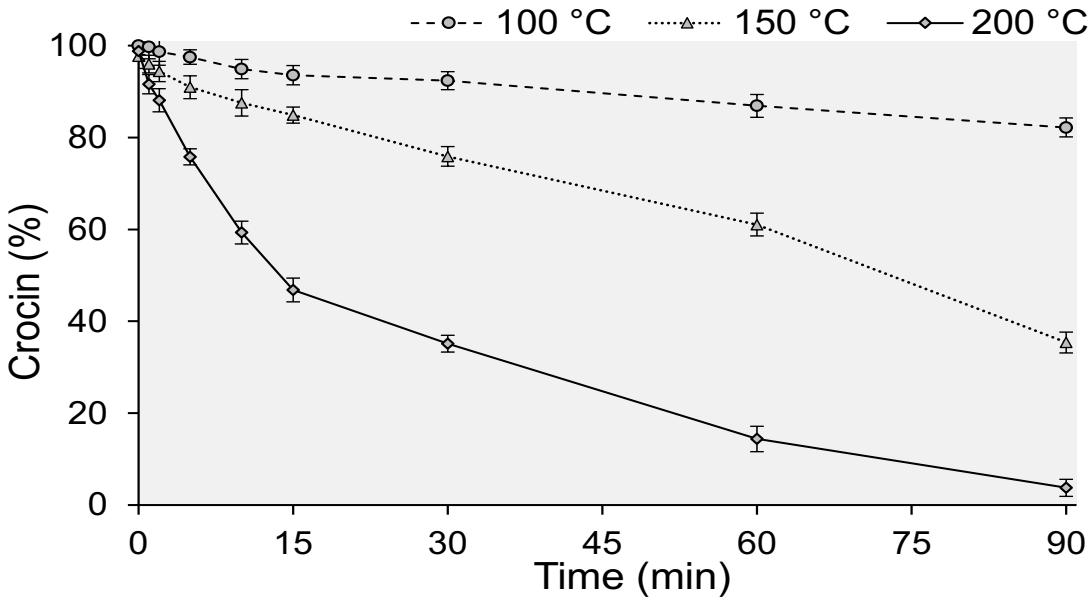

Figure 3. Evolution of crocin percentage during heat treatment

Table 3. Kinetic parameters of crocin degradation at different treatment temperatures

Parameter Temperature	Crocin concentration	Reaction Order	R^2	P-value	$t_{1/2}$ (min)
100 °C	$[C]_t = -0.189\ t + 99.505$	0	0.958	< 0.0001	260.051
150 °C	$[C]_t = -0.647\ t + 99.315$	0	0.990	< 0.0001	74.018
200 °C	$Ln[C]_t = -0.034\ t + 1.509$	1	0.989	< 0.0001	20.211

$[C]_t$, amount of crocin; t, any time in the studied interval period; R^2, coefficients of determination; P, the level of significance of the model; $t_{1/2}$, the half-life of crocin degradation expressed in minutes.

4. Conclusion

Saffron contains numerous bioactive compounds, particularly crocin, responsible for sensory characteristics and medicinal properties but which are sensitive to different handling during extraction, storage, and heat treatment. The experiments regarding the method and conditions of crocin extraction revealed considerable variations. Microwave (500 W) and ultrasound (100%) extractions for 5 minutes were the best procedures for crocin recovery. The storage of the aqueous extract of crocin demonstrated its sensitivity as the temperature rose and adopted the zero-order degradation rate. The heat treatment revealed good resistance of crocin at 100 °C, but the degradation was high at 150 °C and more pronounced at 200 °C. The degradation rate follows a zero-order kinetics at 100°C and 150°C, while at 200°C, it exhibits first-order kinetics. Consequently, the temperature during storage and heat treatment of the aqueous saffron extract must be taken into account, and the use of low temperatures is recommended to reduce crocin degradation.

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Acknowledgment

We are very grateful to the Algerian Ministry of Higher Education and Scientific Research for the financial support, particularly the DGRSDT.

Authors contribution

Methodology: BACHIR-BEY M., BENCHIKH Y.; Data collection: CHOUALI M., DJEBELLANNOUAR S.; Data processing: KHALED KHODJA Y., BENCHIKH Y.; Writing – original draft preparation: BACHIR-BEY M.; Writing – review and editing: BENBOURICHE A., BELLIK Y.; Supervision: KATI D. E.

Conflicting interests

The authors have no competing interests to declare that are relevant to the content of this article.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

DETERMINATION OF ANTIBACTERIAL EFFECTS OF PEEL POWDERS OBTAINED FROM ZIVZIK POMEGRANATE GROWN IN SOUTHEAST TÜRKİYE AGAINST SOME PATHOGENIC BACTERIA

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https://doi.org/10.34302/crpjfst/2024.16.2.12

Article history:
Received: February 20th, 2024
Accepted: June 28th, 2024

Keywords:
Antibacterial effect;
Pathogen;
Pomegranate peel;
Zivzik pomegranate.

ABSTRACT

In this study, the antibacterial effects of peels obtained from Zivzik pomegranate against Staphylococcus aureus, Escherichia coli, Bacillus cereus, Enterococcus faecalis, and Pseudomonas aeruginosa were investigated. The pomegranate peel was ground into powder form and their control (0%), 2%, 4% and 8% solutions were prepared, and firstly pH, Oxidation/Reduction (O/R) and electrical conductivity (EC) values were measured. Then, these bacteria were inoculated with selected standard antibiotics according to the disc diffusion method and with pomegranate peel powder according to the well/hole method and the zone diameters formed because of incubation were determined. In addition, the counts of bacteria formed after incubation was determined by inoculating these bacteria and each peel solution on special media. In analyses, the pH value of the 0% concentration solution was determined as 5.96, O/R value 83.17 mV and EC value 0.13 µS cm-1. However, the pH decreased to 3.7 in the 8% concentration solution, O/R and EC also increased to195.67 mV and 0.42 µS cm-1, respectively. These changes increased the antibacterial effect of solutions prepared from pomegranate peel powder. Pomegranate peel solutions showed similar effects to most of the standard antibiotics used. Also, by influencing the numbers of bacteria used, especially the 8% solution provided about 1.5-2 log reduction compared to the control. Consequently, while it was determined that 4% and 8% solutions of the powders obtained from this pomegranate peel could be used against these bacteria, it was understood that it would be beneficial for the food industry to conduct research against different microorganisms.

1.Introduction

Pomegranate is one of the fruits whose planting has become widespread in Türkiye recently due to agricultural support. Botanically, it is in the *Punicaceae* family, and both the plant and its fruit exhibit different characteristics from many fruits physically and chemically (Akarca and Başpınar, 2019; Akhtar *et al*., 2019).

This fruit, which is native to a wide region stretching from North India to Iran, is being cultivated in Türkiye, especially in the Aegean, Mediterranean and South-eastern Anatolia regions. The fact that it is not very selective in terms of soil properties, and that it is rich in tannins and many phenolic compounds with functional properties supports this increase in cultivation (Kurt and Şahin, 2013; Akhtar *et al*., 2019).

The parts of the pomegranate fruit such as the seed and peel can be shown as a source of different anthocyanins, hydroxycinnamic acids, hydroxy benzo acids, minerals, essential lipids,

complex carbohydrates, and tannins with hydrolysis ability. Most of these substances are compounds with antimicrobial or antioxidant properties (Tunç et al., 2013; Demir et al., 2019).

However, while the use of antioxidant and antimicrobial substances in the food sector is common, the cost of the use of synthetic materials, their allergic effects and carcinogenicity have led researchers to natural resources. In addition, the increase in the resistance of pathogenic microorganisms to drugs due to randomly used antibiotics can be counted among the reasons for this trend (Golge *et al*., 2018; Kilinççeker and Kurt, 2018; Akarca and Başpınar, 2019; Saeed *et al*., 2019).

As mentioned before, the functional compounds in the pomegranate have caused this fruit to be the subject of different scientific studies. In many studies, it has been revealed that pomegranate fruit or pomegranate peel can be used as antioxidant and antimicrobial material. Especially, while it is emphasized that the anthocyanin, ellagic acid, gallic acid, punicalagin and many other polyphenols in the pomegranate peel may have an antimicrobial effect, it has been stated that studies on this subject are insufficient and different studies are needed (Tunç *et al*., 2013; Morsy *et al*., 2018; Akarca and Başpınar, 2019; Karagecili *et al*., 2023).

Depending on what has been mentioned, the peel of the Zivzik pomegranate, which is native to Türkiye's Southeast Anatolian region (Siirt province), was studied in this study. Fruit inner grains of Zivzik pomegranate are larger and redder than other pomegranate varieties. Its acid rate is low and it can last for a long time without spoiling (Cetinkaya *et al*., 2013; Hallaç *et al*., 2022; Karagecili *et al*., 2023).

Although this pomegranate variety is a registered fruit, there is not much scientific study about the fruit parts. Therefore, after the peels obtained from this pomegranate were dried and pulverized, some biochemical properties of their solutions are prepared with water at different concentrations and their antibacterial effects against five different pathogens that are important in the food industry were investigated.

2.Materials and methods

The pomegranate peels used in the study were obtained from Zivzik pomegranates grown in Siirt province (Türkiye). The pomegranate peels were first washed with clean water and then dried with a clean cotton cloth. Then, the peels were dried in an oven at 70 °C for 1-2 days and ground. After this process, pomegranate powder solutions were prepared at the rates of 0% (control), 2%, 4%, and 8% with distilled water. While some physicochemical measurements mentioned below were made in these solutions, the antibacterial effects of some standard antibiotics and powder solutions were also determined.

The pH and O/R values of the solutions were measured using a pH-meter (Cemeroglu, 2013). Determination of electrical conductivity (EC) value Hanna HI2002 edge®, Romania brand device was used. The measurements were carried out by modifying the method used by Acir et al. (2019). The reading was made by dipping the probe into the samples diluted with distilled water, and the value found was determined as µS cm^{-1}.

2.1. Susceptibility of standard bacterial strains to standard antibiotics

The bacterial strains tested (*S. aureus* ATCC 29213, *E. coli* ATCC 25922, *B. cereus* ATCC 10876, *E. faecalis* ATCC 29242 and *P. aeruginosa* ATCC 8027) were obtained from Giresun University. The disk diffusion method was used to determine the resistance of bacteria to antibiotics (Temiz, 2010). Firstly, bacterial strains were reactivated in Tryptic Soy Agar (TSA, Merck) medium for18-24 hours at 37 °C. Pure cultures were adjusted according to the McFarland standard at a concentration of 0.5 ($1.5x10^8$ CFU) in test tubes containing physiological solution. Immediately after this process, 100 µL of bacterial solution were taken under aseptic conditions and spread on petri dishes with Mueller-Hinton (Merck) medium, and the solution was absorbed into the medium. Then, standard antibiotics (Erythromycin 15µg (Oxoid, E15), Streptomycin 10 µg (Oxoid, S10), Penicillin 10 µg (Oxoid, P10),

Amoxycillin/Clavulanic acid 30 µg (2:1; Oxoid, AMC 30) and Cephalexin 30 µg (Oxoid, CL 30)) were placed on the medium with a minimum distance of 2 cm according to the disc diffusion method. After this process, the petri dishes were incubated at 37 °C for 18-24 hours under aerobic conditions, and transparent zone diameters formed at the end of the incubation were evaluated by measuring with a digital calliper (Temiz, 2010).

2.2. Determination of the antibacterial effect of pomegranate peel

Well agar diffusion method was used for this analysis. In this method; each bacterial strain was inoculated into a Mueller-Hinton (Merck) medium, and after the bacterial solution was absorbed into the medium, wells were opened with a diameter of 0.5 cm and at least 2 cm between each well on the medium. 30 µL of each of the prepared pomegranate peel powder solutions were transferred to the wells and absorbed into the medium for approximately 20 minutes. Petri dishes were then incubated at 37 °C for 18-24 hours under aerobic conditions. The transparent zone diameters formed at the end of the incubation were measured with a digital calliper and evaluated (Ponce *et al*., 2003). Table 1 shows the values used for the interpretation of the antimicrobial effect depending on the zone diameter.

Table 1. Antimicrobial effect depending on zone diameter (Ponce et al., 2003)

Zone diameter (mm)	Antimicrobial effect	Determination
Diameter<8.00	Ineffective	-
9.00<Diameter<14.00	Low effect	+
15.00<Diameter<19.00	Effective	++
Diameter>20.00	Overly effective	+++

2.3.Counting of bacteria inoculated into pomegranate peel powder solutions

100µL of bacterial strains were transferred to each prepared pomegranate peel powder solution (0%, 2%, 4% and 8%) and homogenized. These solutions obtained as the main dilution were diluted up to 10^8 and other dilutions were prepared. Then, taking 100 µL from each dilution, they were inoculated on Baird Parker (Merck) for *S. aureus* (Tallent *et al*., 1998), Eosine-Methylene Blue (EMB, Oxoid) for *E. coli* (Feng *et al*., 1998), *Bacillus cereus* selective (BCS, Oxoid) for *Bacillus cereus* (Harrigan, 1998), Slanetz-Barley (Oxoid) for *E. faecalis* (Halkman, 2019), and Cetrimide (Merck) agar for *P. aeruginosa* (Harrigan, 1998). They were then incubated under aerobic conditions at 37°C for 18-24 hours. At the end of the incubation, the typical colonies that developed on the mediums were counted and evaluated.

The study was carried out in three replications and three parallels. Measurements were made from a single point in zones with proper shapes around the discs, and from 3 different points in zones that were not formed properly. The results of microbiological analyses were evaluated by taking their logarithms. Analysis of variance (ANOVA) was performed by taking the average of the measurements, and Duncan's multiple comparison test was applied when significance was found ($P<0.05$; SPSS 16.0, CHICAGO, IL, USA). The results were expressed as mean±standart deviation.

3.Results and discussions

The results of mentioned physicochemical attributes were presented in Table 2, and it is understood that as the ratio of pomegranate peel powder in the solution increased, the pH value decreases, while the O/R and EC values increased ($P<0.01$). While pH value was the lowest value as 3.75 in the solution containing 8% pomegranate peel powder, the value for O/R in this sample was higher as 195.67 mV than in the other. Additionally, the EC property is measured as 0.39 µS $cm^{-1}$and 0.42 µS cm^{-1}

higher in samples containing 4% and 8% powder than in other solutions (Table 2).

Table 2. Some physicochemical properties of pomegranate peel powder solutions at different concentrations

Concentration	pH	O/R (mV)	EC (µS cm^{-1})
Control	5.96±0.04^{a}	83.17±2.07^{d}	0.13±0.02^{c}
2%	3.95±0.01^{b}	185.93±0.35^{c}	0.33±0.03^{b}
4%	3.80±0.001^{c}	193.23±0.23^{b}	0.39±0.01^{a}
8%	3.75±0.001^{d}	195.67±0.21^{a}	0.42±0.01^{a}

$^{a-c}$ Different letters in the same column indicate significant differences among the concentration (P<0.05).

Table 3. Antibacterial effects of standard antibiotics on some food pathogenic microorganisms (mm)

Microorganism	Erythromycin	Streptomycin	Penicillin	Amoxycillin/ Clavulanic acid	Cephalexin
S. aureus ATCC 29213	30.33±0.58^{a}	18.00±0.0^{c}	30.33±0.58^{a}	27.33±0.58^{b}	29.67±0.58^{a}
E. coli ATCC 25922	11.33±0.58^{d}	21.33±0.58^{b}	0±0.00^{e}	20.33±0.58^{c}	22.33±0.58^{a}
B. cereus ATCC 10876	29.33±0.58^{a}	20.33±0.58^{b}	0±0.00^{d}	0±0.00^{d}	8.67±1.15^{c}
E. faecalis ATCC 29242	25.33±0.58^{b}	28.33±0.58^{a}	22.67±0.58^{c}	26.33±0.58^{b}	20.67±0.58^{d}
P. aeruginosa ATCC 8027	13.67±0.58^{c}	19.67±0.58^{b}	0±0.00^{d}	19.67±0.58^{b}	24.33±0.58^{a}

$^{a-c}$Different letters in the same row indicate significant differences among the standard antibiotics on bacteria(P<0.05).

In addition, selected bacteria are some important pathogens that can often be found in foods. The results showing the antimicrobial effects of the antibiotics used against pathogenic bacteria were presented in Table 3. According to the results, it was understood that these antibiotics have important effects on all pathogens used (P<0.01). While the large diameter of the disc formed indicates that the antimicrobial effect is high, Erythromycin, Penicillin, Amoxycillin/Clavulanic acid, and Cephalexin were found to be overly effective (+++) against *S. aureus*. Streptomycin, on the other hand, had an effective (++) force against this pathogen. Streptomycin, Amoxycillin/Clavulanic acid, and Cephalexin were overly effective (+++) against *E. coli*, while Erythromycin was low effective (+). Erythromycin and Streptomycin were overly effective (+++) against *B. cereus* whereas Cephalexin had a low effect (+). While all antibiotics were overly effective (+++) against *E. faecalis*, lastly antibiotics other than erythromycin and penicillin were found to be overly effective (+++) against *P. aeruginosa* (Table 3).

The results of well agar method measurement showing the antibacterial effect of solutions prepared from pomegranate peel powder, which is the subject of this study, against pathogenic bacteria were shown in Table 4. As can be seen from the table, the antimicrobial effects of all solutions against all pathogens were found to be significant at the level of P<0.01. While 2% and 4% solutions were effective (++) against *S. aureus* and *E. coli*, 8% solution was overly effective (+++). While all solution levels that contained powder were effective (++) against *B. cereus*, they showed an overly effective (+++) antimicrobial potential against *E. faecalis*. However, 4% and 8% of solutions were low effective (+) against *P. aeruginosa* whereas

other treatments were observed to be ineffective (-).

Table 4. Antibacterial effects of different levels of pomegranate peel powder solutions on selected food pathogen microorganisms (mm)

Microorganism	Control	2%	4%	8%
S. aureus ATCC 29213	0±0.0^{d}	15.33±0.58^{c}	19.33±0.58^{b}	20.67±0.58^{a}
E. coli ATCC 25922	0±0.0^{d}	14.67±0.58^{c}	16±1.00^{b}	19.67±0.58^{a}
B. cereus ATCC 10876	0±0.0^{c}	15±0.0^{b}	15.33±1.15^{b}	18.33±0.58^{a}
E. faecalis ATCC 29242	0±0.0^{c}	22.33±0.58^{b}	23.33±1.15^{b}	27.33±0.58^{a}
P. aeruginosa ATCC 8027	0±0.0^{c}	0±0.0^{c}	10.33±0.58^{b}	13.33±0.58^{a}

$^{a-c}$Different letters in the same row indicate significant differences among the antibacterial effects with pomegranate peel powder solutions ($P<0.05$).

Table 5. The effect of pomegranate peel powder solutions on the amounts formed as result of incubation of some food pathogenic bacteria (log CFU mL^{-1})

Microorganism	Control	2%	4%	8%
S. aureus ATCC 29213	7.12±0.60^{a}	6.18±1.03^{a}	5.45±0.36^{a}	5.11±0.18^{a}
E. coli ATCC 25922	8.40±0.03^{a}	8.12±0.07^{b}	7.35±0.01^{c}	6.33±0.06^{d}
B. cereus ATCC 10876	7.84±0.05^{a}	7.05±0.22^{b}	6.79±0.28^{b}	6.14±0.02^{c}
E. faecalis ATCC 29242	7.48±0.01^{a}	6.30±0.00^{b}	6.18±0.02^{b}	5.80±0.09^{c}
P. aeruginosa ATCC 8027	8.75±0.28^{a}	8.77±0.25^{a}	8.14±0.18^{ab}	7.69±0.25^{b}

$^{a-c}$Different letters in the same row indicate significant differences among the pomegranate peel powder solutions antibacterial effects ($P<0.05$).

The results of the analysis performed to determine the number of microorganisms formed in suitable media for each bacterium is presented in Table 5. Statistical analysis showed that pomegranate peel solutions were not significantly effective on *S. aureus* count ($P>0.05$), they were effective on *E. coli*, *B. cereus* and *E. faecalis* at a level of $P<0.01$whereas on *P. aeruginosa* count at the level of $P<0.05$. Generally, it was observed that the number of microorganisms decreased as the ratio of pomegranate peel powder in the solution increased in pathogenic bacteria except for *S. aureus*. While the counts of *S. aureus* were founded in the range of 5.11-7.12 log CFU/mL, the lowest microorganism numbers were determined as 6.33 log CFU/mL for *E. coli*, 6.14 log CFU/mL for *B. cereus*, 5.80 log CFU/mL for *E. faecalis* and 7.69 log CFU/mL for *P. aeruginosa* in the samples inoculated with 8% solution (Table 5).

3.1.Discussions

The pH value is one of the important parameters that affect the growth of microorganisms in a medium. Determination of acidic or basic properties is important in terms of properties such as quality, safety, and processing in foods (Kılınççeker *et al*., 2015; Kurt and Kılınççeker, 2011). In terms of microorganisms, moulds can grow in the pH range of 1.2-4.5, yeasts in the pH range of 1.5-

4.0 and bacteria in the pH range of 4.5-6.5, while it is known that pathogenic bacteria generally cannot grow at low pH (Temiz, 2015; Kim *et al*., 2018). In the study, as the concentration increased, the amount of organic acid passing from the pomegranate peel to the solution increased and decreased the pH values of the solutions (Table 2). Therefore, these low pH values in the solutions adversely affected the growth of microorganisms, as can be understood from Table 4 and Table 5. Like our results, Kennas *et al*., (2020) found the pH value of pomegranate peel powder to be 3.82, and Jalal *et al*., (2018), on the other hand, measured it as 3.83 and they emphasized that it is an acidic material.

The O/R potential value is one of the important internal factors that affect the development of microorganisms, such as pH. It has a positive value under aerobic conditions and a negative value under anaerobic conditions (Temiz, 2015). In this study, the O/R values of the solutions in the range of 83.17-195.67 mV are perceived as an indicator of the aerobic environment. For this reason, it was thought that especially the growth of anaerobic microorganisms might be adversely affected by the increase in the ratio of pomegranate peel powder in the solutions. Accordingly, the numbers of *S. aureus* and *E. coli* showing facultative anaerobic properties could be reduced, especially (Table 2).

The EC value is defined as the ability of a food or solution to conduct an electrical current. This value is a function of the type and amount of ingredients in foods, and electrolyte-containing materials such as salts, acids, gums, and thickeners have a significant effect on the electrical conductivity of foods (Singh and Heldman, 2015). It also shows a linear relationship between temperature and water/ion content (Jha *et al*., 2011). It is widely used to determine contaminants and microbial activity in water in the food industry (Kaptan and Kayisoglu, 2016). It was observed that the EC range in the investigated solutions increased depending on the increase in concentration, and this was attributed to the increase in the amount of substance or ionization. As can be seen in Table 2, an increase in the EC value was observed with the increase of the pomegranate peel concentration, suggesting that the transfer of antimicrobial substances into the solution increased, especially. Looking at the results, it is understood that the amount of pomegranate peel powder used is effective on the differences in pH, O/R and EC values in solutions and this also affects the antimicrobial strength.

According to results in Table 3 and Table 4, When the antibacterial effect of pomegranate peel powder is compared with standard antibiotics in terms of *S. aureus*; The solutions with 2% and 4% concentrations showed similarity with Erythromycin in that they were effective (++), while the 8% solution was overly effective (+++) as other antibiotics.

The antibacterial effect of 8% pomegranate peel powder solution in terms of *E. coli* was similar to that of Streptomycin, Amoycillin/Clavulanic acid and Cephalexin being overly effective (+++). Solutions containing 2% and 4% powder were determined as effective (++) compared to ineffective (-) Penicillin and low-effective (+) Erythromycin.

It was determined that all pomegranate peel powder solutions were effective (++) against *B. cereus*. However, they created a difference from standard antibiotics in that Penicillin and Amoxycillin clavulanic acid was ineffective (-), cephalexin was the low effect (+), and erythromycin and streptomycin were over effective (+++).

All solutions were overly effective (++) against *E. faecalis*, and these results were like that of standard antibiotics.

The ineffectiveness (-) of 2% pomegranate peel powder solution and Penicillin against *P. aeruginosa*, and the low effective (+) of 4% and 8% peel powder solutions were similar only to Erythromycin. However, they were also understood to be less effective (+) than other standard antibiotics.

Like our results in Table 4, Akarca and Başpınar (2019) stated that the zone diameters formed by the water extracts of the pomegranate peel in the disc diffusion method they applied

against seven pathogenic bacteria, were between 11.15-25.68 mm and the strongest effect was obtained especially against *S. aureus* and *B. cereus*. Balaban *et al*., (2021), when they apply pomegranate peel extracts as a biofilm at different densities, they found that the antimicrobial effect increased as the density increased, and the zone diameters formed were 9-17 mm for *B. cereus* and 0-13 mm for *E. faecalis*. In conclusion, they emphasized that pomegranate peel extracts can be used as an antimicrobial material for the food industry. Al-Zoreky (2009) observed that aqueous and ether extracts of pomegranate peel did not form an inhibition zone in a trial against eleven pathogens, but the water-methanol extract formed zones in the range of 12-20 mm and he said that these values were the result of a significant antimicrobial effect. Also, Dahham *et al*., (2010) measured the resulting disc diameters in the range of 18-25 mm in their study to determine the antimicrobial effect of pomegranate peel extract for seven bacteria. According to this result, while they said that pomegranate peel extract could be an important antimicrobial source, stated constituents such as phenols, tannins and flavonoids found in the extracts as the cause of this antimicrobial activity. In another study, it was said as a material with antimicrobial activity against many microorganisms due to components such as punicalagin in the pomegranate fruit peel structure (Rongai *et al*., 2019).

Lastly, examples of similar studies in which the pomegranate peel powders used in this study were effective at different concentrations and in support of the microorganism count results given in Table 5 can be given as follows. Rasuli *et al*., (2021) stored buffalo meats marinated with 0%, 0.5%, 1% and 1.5% pomegranate peel extract in cold storage. In the analyses made during the storage, they determined that the total viable counts in the samples decreased as the extract concentration in the sample increased. Morsy *et al*., (2018) added 0%, 1% and 1.5% pomegranate peel nanoparticles to meat patties and they applied cold storage for 15 days. In the microbiological analysis of meatballs, they said that the total bacteria, psychrophilic bacteria and lipolytic bacteria counts of the samples containing pomegranate peel nanoparticles were lower than control during storage and the lowest counts were at high concentrations (1.5%). Mahajan *et al*., (2015) determined that in cheeses prepared with pomegranate peel extract at 0%, 1% and 2% concentrations, the numbers of total viable, psychrophilic organisms and yeast-moulds in the samples containing the extracts decreased. They also stated that the increase in the ratio of pomegranate peel extract supports this decrease. As a result, it has been emphasized that the pomegranate peels used in these studies can be used as a natural antimicrobial agent for the food industry due to the anthocyanin, punicalagin, ellagic acid, gallic acid and some other phenolic compounds it contains. It can be said that the results of our study are in accordance with these studies. Solutions showed a reducing effect on the counts of pathogenic bacteria in our study depending on the functional components in the structure of the pomegranate peel used and the physicochemical properties given in Table 2.

4.Coclusion

As a result of the study, it was understood that the solutions prepared from the peel powders obtained from the Zivzik pomegranate can be used as an antibacterial material against *S. aureus, E. coli, B. cereus, E. faecalis* and *P. aeruginosa*. As the pomegranate peel concentration in the solutions increased, the amount of antibacterial substance transferred to the medium increased, decreased the pH values and increased the O/R and EC values. These changes increased the antibacterial effects of pomegranate peel powder solutions. It was thought that the antibacterial effect increased with the increase in the passage of bioactive substances and organic acids that decrease the pH value in the solution. As a matter of fact, when the 0% concentration is increased to 8%, it has been determined that there is a decrease in the number of microorganisms up to approximately 1.5-2 log levels. While it has been observed that solutions of different

concentrations have similar effects to most standard antibiotics used, it has been understood that the use of solutions with 4% and 8% concentrations can be recommended especially against these pathogens.

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EFFECT OF DIFFERENT DRYING METHODS ON NUTRITIONAL COMPOSITION, ANTIOXIDANT ACTIVITY AND PHYTOCHEMICALS OF *Enhydra fluctuans*

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https://doi.org/10.34302/crpjfst/2024.16.2.13

Article history:
Received: October 2nd, 2023
Accepted: May 4th, 2024

Keywords:
Antioxidant activity;
Drying techniques;
Enhydra fluctuans;
Flavonoid content;
Phenolic content.

ABSTRACT

Enhydra fluctuans is a common edible plant, showcases diverse biological advantages. This research investigates the effects of various drying methods (sun, oven, cabinet, vacuum, and freeze) on nutritional, antioxidant, and phytochemical attributes. By comparing outcomes with fresh leaves, we utilized five drying techniques, proximate composition, antioxidant activity, and phytochemical content (TFC, TPC). Findings reveal lowered level of ash and moisture, alongside elevated carbohydrate, fat, fiber, protein, antioxidant activity, Total Flavonoid Content (TFC), and Total Phenolic Content (TPC). While oven drying produces high levels of ash, fat, and fiber, sun drying records the highest moisture and lowest TFC. Vacuum drying yields lowest ash, fat, antioxidant activity and TPC. Freeze drying boasts highest protein (17.50±0.35%), carbohydrate (55.87±0.18%), antioxidant activity (488.21±1.25%), TPC (0.56±0.13mgQAE/g), and lowest fiber, moisture. Cabinet drying presents least carbohydrate. Oven drying has maximum energy (335.16±0.18 Kcal/100g), vacuum drying minimum. Statistically, moisture, protein, fiber, total energy, TFC, TPC, antioxidant activity are significant ($p<0.05$). However, dried sample's carbohydrate, ash, and fat content are statistically insignificant ($p>0.05$). In conclusion, among five dried samples, oven and freeze-dried exhibit notable significance as per the study's outcomes.

1. Introduction

Nature has provided medicinal substances for thousands of years, and numerous modern pharmaceuticals have been obtained from natural sources, some of which have historical evidence for their usage in traditional medicine. *Enhydra fluctuans*, a tropical herb, commonly known as helencha or harkuch, belonging to family Asteraceae, is gaining lot of importance for its therapeutic potentials (Rahman, 2015). This is an edible semi-aquatic herbaceous vegetable plant with serrate leaves, grows commonly all over the country. Common names for *Enhydra fluctuans* include "water crest" and "marsh herb." It is a separate-leaved, annual vegetable plant that is edible, semi-aquatic, and nonwoody. Tropical and subtropical areas are home to *Enhydra fluctuans* (Ali *et al.,* 2013). This plant is mostly found in Assam and the North-Eastern part of India (Chakraborty *et al.,* 2012). It is a widely consumed vegetable in Bangladesh and a nutrient-rich source of proteins, carbs, vitamins, and minerals (Sattar *et al.,* 2016).

In Bangladesh, the plant has also been used as a traditional medicine in addition to being used as food. Flavonoids, alkaloids, saponins, tannins, phenols, beta-carotene, protein, and carbohydrates are among the phytochemicals found in this medicinal plant (Sarma *et al.,* 2014;

Jayashree, 2013; Dewanji *et al.,* 1993; Hazra *et al.,* 2012; Satyajit, 2012; Kuri *et al.,* 2014). At least 35 distinct chemicals, mostly from the phytochemical groups of flavonoids, isoflavonoids, steroids, and terpenoids, have been found in *E. fluctuans*. According to (Barua *et al.,* 2021) the total phenolic content was found to be 60.67±0.083 g/ml GAE and 39.83±0.083 g/ml GAE for the ethanolic and aqueous extract, respectively. According to (Ghosh *et al.,* 2007), the plant's aerial portions have substantial antibacterial and anthelmintic properties. From this plant, terpenes (Krishnaswamy *et al.,* 1995), sesquiterpene lactones (Ali *et al.,* 1972), and carotene have all been recognized as chemical components. According to (Alfasane *et al.,* 2018), raw *Enhydra fluctuans* had 317.28 (kcal/100g) of energy, 14.00% ash, 18.20% protein, 1.14% fat, 11.50% fiber, and 56.60% carbohydrate.

Recently, the ability of crude extract and various fractions to scavenge free radicals was observed (Sannigrahi *et al.,* 2010). It has been noted that the leaves of E. fluctuans have hypotensive properties (Joshi and Kamat, 1972). It has many beneficial effects such as antioxidant activity (Uddin *et al.,* 2005); anti-cancer activity (Kumar *et al.,* 2012); antidiarrhoeal activity (Kumar and Khanum, 2012); hepatoprotective activity (Patil *et al.,* 2008); analgesic activity; neuroprotective potential (Alebiosu *et al.,* 2015); antidiabetic (Khan and Yadava 2010), anthelmintic and thrombolytic (Kuri *et al.,* 2014), Phagocytic and cytotoxic activity (Hassan *et al.,* 2015).

Drying is the process of removing extra water while preserving nutritional content, enhancing visibility, and preparing an item for usage. There are various methods of drying, including sun, vacuum, freeze, oven, and cabinet drying. Raw and dried samples have different nutritional compositions, antioxidant activities, and phytochemical components. A dried sample may have a lot or little in the way of nutritious components. At present the powder form of *Enhydra fluctuans* used as a drug for medicinal purposes that why it's necessary to know the effects of different drying on *E. fluctuans*. This study aims to investigate the impacts of various drying techniques on proximate composition, evaluating the antioxidant activity, determining the total phenol and total flavonoid content of dried *Enhydra fluctuans*. Therefore, our research on the antioxidant activity and functional potential of this plant will be helpful in both choosing plants as natural alternatives to medications for dietary supplements and in the development of antioxidant-based medications.

2. Materials and methods

The experiment was conducted in the department of Food Technology and Nutritional Science, Mawlana Bhashani Science and Technology University, Tangail. The study was carried out from January 2023 to March 2023.

2.1. Collection and preparation of sample

In this study, Enhydra fluctuans were collected from the local area of Tangail, Bangladesh. The foreign objects, rotten materials were removed from the collected sample and washed using lukewarm water to eliminate dirt and chemical stains. After washing, a brief period of rest was allowed for water drainage. The sample was then cut into appropriate sizes based on the chosen drying method.

2.2. Drying methods

The study employed five distinct drying methods. In sun drying, about 500 g samples were cleaned and chopped. These were laid in single layers on racks, rotated for uniform evaporation, and kept indoors at night. After around two days of drying, the samples cooled indoors before grinding. In vacuum drying, 400 g of samples were cut into pieces measuring 1 to 1.5 inches in length. Each tray of the vacuum dryer was loaded with a 100 g sample, spread thinly, and subjected to a temperature of 70°C. After 10 hours, the samples were removed from the vacuum dryer and left to cool. Following the cooling process, the samples were prepared for grinding. In terms of oven drying, a clean sample weighing 300 grams was obtained and subsequently divided into medium-sized pieces.

These fragments were then evenly distributed across the trays within a microwave dryer. After five hours, the sample was carefully extracted from the oven dryer. To ensure proper cooling, the sample was allowed to rest briefly before it could be subjected to the grinding process. For the freeze drying process, *Enhydra fluctuans* weighing 260 grams was meticulously divided into uniformly small pieces. These segments were then placed within a freeze dryer, operating at an approximate temperature of -68°C. Following a period of 7 to 8 hours, the sample was retrieved from the freeze dryer. After a short resting period, the sample was prepared for the subsequent grinding process. In the cabinet drying methods, a total of 300 grams of sample material was segmented into medium-sized pieces. These pieces were uniformly distributed among the trays within the cabinet drier. Upon completion of a one-day drying period, the sample was carefully removed from the cabinet drier.

Subsequent to each drying method, all the dried samples were subjected to grinding process using an electric grinder. The resulting powders were meticulously stored within separate plastic jars, each with a secure seal, in order to safeguard them against moisture absorption.

2.3. Proximate analysis of raw and dried *Enhydra Fluctuans*

The moisture, ash, carbohydrates, crude fat were determined by (AOAC, 2000) method. Protein content was determined by Kjeldahl method (Bradstreet, 1954). Crude fiber was determined by (AOAC, 1995).

2.4. Determination of phytochemical content

Total phenolic content (TPC) was determined by Folin-Ciacalteu method (Premathilaka, 2016) and Total flavonoid content (TFC) was determined by aluminum chloride colorimetric test (Kamtekar *et al.,* 2014). In the case of TPC, a Gallic acid curve was established using different dilutions (0.1, 0.01, 0.001, 0.0001, 0.00001 mg/ml) in methanol. Each dilution (100µl) mixed with water (500µl) and Folin-Ciocalteu reagent (100µl), stood for 6 min. Then, 7% sodium carbonate (1ml) and water (500µl) were added. Absorbance was measured at 760 nm after 90 min. The same was done with water extracts of three formulations.

Total phenolic content was calculated as mgGAE/g. All tests were triplicated. In the case of TFC, a quercetin calibration curve was established using dilutions (0.1, 0.5, 1.0, 2.5, and 5 mg/ml) from a standard 2 quercetin solution in methanol. For each dilution, 100 µl was mixed with 500 µl distilled water, then with 100 µl 5% Sodium nitrate, and stood for 6 minutes. Subsequently, 150 µl of 10% aluminum chloride solution was added and left for 5 minutes, followed by sequential addition of 200 µl 1M Sodium hydroxide solution. The mixture's absorbance was measured at 510 nm using a UV spectrophotometer. The same process was applied to all samples. Total flavonoid content was determined as mgQE/g. All steps were conducted in triplicate.

2.5. Determination of antioxidant activity

Antioxidant activity was determined by DPPH method (Brand-Williams *et al.,* 1995). To make the sample extract, 1g of the sample was put into 10ml of methanol in a beaker. It was stirred using a magnetic stirrer for 10-15 minutes and then filtered with filter paper. A new solution of DPPH (0.002%) was made in methanol and its absorbance was measured at 515 nm. Then, 50µl of the sample extract was mixed with 3ml of DPPH solution and left in the dark for 15 minutes. The absorbance was measured again at 515 nm.

% of inhibition = (Absorbance of DPPH-Absorbance of sample)/(Absorbance of DPPH)×100

2.6. Statistical analysis

The data were analyzed to know the mean and SD value and their statistical significance. SPSS Statistic software, version20.0 (SPSS Inc., Chicago, USA), was used for the statistical

treatment of the data. Analyses of variance (ANOVA) were carried out to evaluate whether there were significant differences ($p< 0.05$) amongst the samples.

3. Results and discussions

Table 1 provides a comprehensive overview of the basic nutritional components and energy content of raw *Enhydra fluctuans.* It demonstrates that raw *Enhydra fluctuans* had the following composition: 72.45± 0.69% moisture, 13.96±0.24% ash, 12.37±1.13% protein, 1.59±0.34%fat, 16.42±0.75% fiber, 54.98±4.55% carbohydrates, and 299.94±16.75 (kcal/100g) energy.

As per the findings of Datta *et al.,* (2019), the values for raw *Enhydra fluctuans* were as follows: 67.69±0.78% moisture, 15.15±0.44% ash, 8.00±0.06% protein, 1.10±0.01% fat, 15.37±0.21% fiber, 9.64±0.06% carbohydrates, and 80.53±0.16 (kcal/100g) of energy. Another study stated, raw Enhydra fluctuans contained 317.28(kcal/100g) of energy, 14.00% ash, 18.20% protein, 1.14% fat, 11.50% fiber, and 56.60% carbohydrates (Alfasane *et al.,* 2018). It became apparent that the findings of this study and those of other studies are nearly identical. But protein, carbohydrate, and calorie contents were slightly low; fiber content was high in comparison to other studies. The variations may result from the location of the crop's cultivation, the sampling techniques employed, the impact of the harvest season, etc.

Table 1. Proximate analysis of raw *Enhydra fluctuans*

Biochemical composition	Mean ± SD
Moisture (%)	72.45 ± 0.69
Ash (%)	13.96 ± .24
Protein (%)	12.37 ± 1.13
Fat (%)	1.59 ± .34
Fiber (%)	16.42 ± .75
Carbohydrates (%)	54.98 ± 4.55
Energy (Kcal/100g)	299.94 ± 16.75

Notes: Each value in the table was obtained by calculating the average of triplicate experiments (n=3) and data are presented as Mean ± Standard Deviation.

Table 2 presents the nutrient content and overall energy values of *Enhydra fluctuans* subsequent to the implementation of different drying techniques. It depicts that the sun dried sample had a moisture content of 10.81±0.19%, freeze dried sample had 3.82±0.26%, cabinet dried had 5.74±0.21%, vacuum dried had 8.22±0.10%, and the oven dried sample had 6.28±0.16%. According to this investigation, the sun dried sample had the highest moisture content, whereas the freeze dried sample had the lowest. Here, the moisture content of dried samples among five different drying were statistically significant ($p<0.05$). The amount of ash produced by Enhydra fluctuans dried using various drying methods were represented (Table 2). It depicts that the ash content were 13.38±1.79%, 13.05±0.93%, 13.62±1.18%, 12.34±0.58% and 13.63 ±1.01% for the sun, freeze, cabinet, vacuum and oven dried sample respectively. Though the differences among the values are not so notable, oven dried samples had the highest ash content of all the dried samples and vacuum dried had less. There is no significant significance ($p > 0.05$) were observed among them.

Table 2.Nutrients content and total energy of *Enhydra fluctuans,* after the application of various drying methods

Nutritional parameters	Drying techniques					P value
	Sun Mean ± SD	Cabinet Mean ± SD	Vacuum Mean ± SD	Oven Mean ± SD	Freeze Mean ± SD	
Moisture (%)	10.81±0.19	5.74±0.21	8.22±0.10	6.28±0.16	3.82±0.26	0.000
Ash (%)	13.38± 1.79	13.62±1.18	12.34± 0.58	13.6 ± 1.01	13.05±0.93	0.651
Carbohydrate (%)	55.61±0.49	55.53±0.56	55.64±0.27	55.57±0.75	55.87±0.18	0.923
Protein (%)	14.08± 0.28	15.36± 0.44	14.64± 0.13	13.23± 0.23	17.50± 0.35	0.000
Fat (%)	4.88 ± 0.73	4.04 ± 1.82	3.52 ± 1.21	6.20 ± 0.94	3.99 ± 0.41	0.100
Fiber (%)	20.38±0.77	16.06±2.50	21.02±2.46	23.86±2.95	7.55±1.21	0.000
Total energy (kcal/100g)	322.70±0.16	319.61±0.28	312.52±0.39	335.16±0.18	329.65±0.25	0.000

Notes: Each value in the table was obtained by calculating the average of triplicate experiments (n=3) and data are presented as Mean ± Standard Deviation. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p < 0.05$ level.

Table 3.Phytochemical composition of *Enhydra fluctuans*, following various drying techniques

Phyto-chemicals	**Raw and dried samples**						P value
	Raw Mean ± SD	Sun Mean ± SD	Cabinet Mean ± SD	Vacuum Mean ± SD	Oven Mean ± SD	Freeze Mean ± SD	
TFC (mgQE/g)	8.05±0.63	12.86±0.85	16.20±0.39	12.94±1.18	16.31±0.71	13.90±0.10	0.000
TPC (mgQAE/g)	0.15±0.02	0.37±0.03	0.55±0.09	0.28±0.36	0.46±0.05	0.56±0.13	0.005

Notes: Each value in the table was obtained by calculating the average of triplicate experiments (n=3) and data are presented as Mean ± Standard Deviation. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p < 0.05$ level. TFC =Total flavonoid content; TPC= Total Phenolic Content.

Table 4. Measured antioxidant activity of *Enhydra fluctuans* after the application of various drying methods

Biochemical parameter (%)	**Samples**	**Mean±SD**	**P value**
Antioxidant activity	Raw	19.91±1.67	0.000
	Sun	480.60±1.66	
	Freeze	488.21±1.25	
	Vacuum	372.86±5.77	
	Cabinet	480.60±1.87	
	Oven	481.77±1.05	

Notes: Each value in the table was obtained by calculating the average of triplicate experiments (n=3) and data are presented as Mean ± Standard Deviation. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p < 0.05$ level.

Table 2 presents the nutrient content and overall energy values of *Enhydra fluctuans* subsequent to the implementation of different drying techniques. It depicts that the sun dried sample had a moisture content of 10.81±0.19%, freeze dried sample had 3.82±0.26%, cabinet dried had 5.74±0.21%, vacuum dried had 8.22±0.10%, and the oven dried sample had 6.28±0.16%.According to this investigation, the sun dried sample had the highest moisture content, whereas the freeze dried sample had the lowest. Here, the moisture content of dried samples among five different drying were statistically significant ($p<0.05$). The amount of ash produced by *Enhydra fluctuans* dried using various drying methods were represented (Table 2). It depicts that the ash content were 13.38±1.79%, 13.05±0.93%, 13.62±1.18%, 12.34±0.58% and 13.63 ±1.01% for the sun, freeze, cabinet, vacuum and oven dried sample respectively. Though the differences among the values are not so notable, oven dried samples had the highest ash content of all the dried samples and vacuum dried had less. There is no significant significance ($p > 0.05$) were observed among them.

In the context of carbohydrate contents, the results showed the carbohydrate content of the sun, freeze, cabinet, vacuum and oven dried samples were statistically insignificant ($p>0.05$) which were accounted for 55.61±0.49%, 55.87±0.18%, 55.53±0.56%, 55.64±0.27% and 55.57 ±0.75% in the specified sequence. The sample that was freeze dried had the most carbohydrate (55.87±0.18%), whereas the sample that was cabinet dried had the least (55.53±0.56%), according to this experiment (Table 2). Furthermore, the protein concentration in dried *Enhydra fluctuans* obtained through various drying techniques exhibits notable variation. In this study, the protein content in the freeze-dried sample was higher (17.50± 0.35%) and lower (13.23 ± 0.23%) in the oven-dried sample. The protein content of all dried samples were statistically significant ($p<0.05$).

Table 2 displayed the variation in fat content among dried *Enhydra fluctuans* samples subjected to diverse drying techniques. The findings of this investigation highlight notable distinctions in fat content depending on the drying technique employed. Notably, the oven-dried samples exhibited the highest fat content (6.20±0.94%), while the vacuum-dried samples demonstrated the lowest fat content (3.52±1.21%) among the various techniques examined. In this instance, the fat content of various dried *Enhydra fluctuans* samples was not statistically significant ($p>0.05$).

The results from the table 2 shows varying fiber contents: sun-dried (20.38±0.77%), freeze-dried (7.55±1.21%), cabinet-dried (16.06±2.50%), vacuum-dried (21.02±2.46%), and oven-dried (23.86±2.95%). Notably, oven-dried samples had the highest fiber content (23.86±2.95%), while freeze-dried samples had the lowest (7.55±1.21%). Here, the fiber content of various dried *Enhydra fluctuans* sample has a p value of 0.00, which is less than 0.05. Therefore, this value is significant.

It was discovered that the oven-dried sample had the highest energy content (335.16±0.18 Kcal/100g) and the vacuum-dried sample had the lowest (312.52±0.39 Kcal/100g) (Table 2). Total energy of all dried samples were statistically significant ($p<0.05$).

Table 3 lists the total flavonoid content of raw and various dried *Enhydra fluctuans*. It exhibits that the total flavonoid content of the raw sample was 8.05±0.63 mgQE/g. The sun dried sample contained 12.86±0.85 mgQE/g, freeze 13.90±0.10 mgQE/g, cabinet 16.20±0.39 mgQE/g, vacuum 12.94±1.18 mgQE/g and oven 16.31±0.71QE mgQE/g of TFC. It stated that the raw sample's total flavonoid content was lower than that of the dried samples. Furthermore, among the dried samples, TFC was higher in the oven-dried sample (16.31±0.71mgQE/g) and sun dried contained less (12.86±0.85mgQE/g). The total flavonoid content of all dried samples were statistically significant ($p<0.05$). These significant variations may result from different genotypes or different location as indicated by Alam *et al.*, (2015). Total Phenolic Content (TPC) of raw and different dried *Enhydra fluctuans* was shown in Table 3.To start with,

total phenolic content of the raw sample was 0.15±0.02 mgQAE/g. Besides, the TPC of sun, freeze, cabinet, vacuum and oven dried sample were 0.37±0.03 mgQAE/g, 0.56±0.13 mgQAE/g, 0.55±0.09 mgQAE/g, 0.28±0.36 mgQAE/g, and 0.46±0.05 mgQAE/g respectively. It showed that the raw sample's total phenol level was lower than that of the dried samples. Additionally, among the dried samples, TPC was higher in the freeze dried sample (0.56±0.13) and vacuum contained less (0.28±0.36). In this case, the total phenolic content among five distinct drying samples were statistically significant ($p<0.05$).

Table 4 presents the antioxidant activity of raw and various dried forms of *Enhydra fluctuans*. The raw sample exhibited an antioxidant activity of 19.91±1.67%. Among the dried samples, sun-dried showed an antioxidant activity of 480.60±1.66%, freeze-dried had 488.21±1.25%, cabinet-dried resulted in 480.60±1.87%, vacuum-dried yielded 372.86±5.77%, and oven-dried displayed 481.77±1.05% antioxidant activity. It was observed that the raw sample's antioxidant activity was lower compared to the dried samples. Furthermore, within the dried samples, freeze-dried samples exhibited the highest antioxidant activity (488.21±1.25%), while vacuum-dried samples had comparatively lower activity (372.86±5.77%).

4. Conclusion

In this study, ash, fat, fiber, TFC contents were in the highest amount and moisture, protein content were in the lowest amount in oven dried sample. In vacuum drying, moisture content was the highest amount while ash, fat, TPC contents were the lowest. In freeze drying, protein, carbohydrate, and TPC were in the highest amount and fiber contents were in the lowest amount. TFC and carbohydrate contents were in the lowest amount in sun and cabinet dried sample respectively. Total energy was the highest in oven dried sample and the lowest in vacuum dried sample. This study outcome enables us to draw the conclusion that in comparison to five different dried samples the oven dried sample demonstrated the significant characterization.

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Acknowledgment

The authors acknowledge the infrastructure support, the resources and facilities provided by Food Technology and Nutritional Science Research Laboratory, Mawlana Bhashani Science and Technology University, Bangladesh.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

PASTA FORTIFICATION WITH TOMATO PEEL BY-PRODUCT: IMPACT ON TECHNOLOGICAL AND ANTIOXIDANT PROPERTIES

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https://doi.org/10.34302/crpjfst/2024.16.2.14

Article history:
Received: December 24th, 2023
Accepted: May 24th, 2024

Keywords:
Enriched pasta;
Antioxidant capacity;
Total polyphenols;
Colour;
Cooking quality.

ABSTRACT

Tomato peel is a by-product rich in bioactive compounds and dietary fibers, which are deficient in wheat pasta. The objective of this study was to investigate the impact of the addition of various levels (0, 5, 7.5, 10, 12.5 and 15%) of an industrial tomato peel by-product on selected properties of enriched pasta. The addition of tomato peel by-product significantly increased lipid, ash, pigments, total polyphenols content, and antioxidant capacity (ABTS and FRAP). In contrast, the enriched pasta showed a significant decrease in optimal cooking time and swelling index; the increase in cooking loss did not exceed the acceptable limit (8%). The tomato by-products can be successfully valorized in pasta-making because a 15% addition enhances the nutritional value of the final product without affecting the technological quality.

1. Introduction

Pasta is one of the most consumed foods in the world due to its low price, ease of preparation and long shelf life (Kamali Rousta *et al*., 2020). Currently, the increasing demand of the consumers for healthy food products rich in bioactive compounds with beneficial effects on human health and/or the reduction of chronic disease has encouraged food producers to develop various new functional food products (Bianchi *et al*., 2021; Mercier *et al*., 2016).

Pasta is commonly made from durum wheat semolina that has a high content in carbohydrates, but low contents in dietary fibers, minerals, proteins, vitamins and free phenolic compounds (Bouasla *et al.*, 2020; Brandolini *et al.*, 2011). However, the World Health Organization and the Food and Drug Administration consider the fortification of pasta with high-value-added ingredients of great nutritional importance because pasta can be a suitable carrier for the addition of healthy compounds (Bianchi *et al.*, 2021). Consequently, considerable efforts have been made towards the development of fortified pasta with various ingredients such as plant-based flours (e.g. cereals, germinated cereals, pseudo-cereals, pulses and dietary fibers), animal-based ingredients (e.g. egg products), protein concentrates and isolates, nutraceutical compounds, plants, microalgae and agro-industrial by-products (Bouasla *et al.*, 2022;

Ćetković *et al.*, 2022; Cota-Gastélum *et al*., 2019; Hidalgo *et al.*, 2020; Padalino *et al*., 2013).

Tomato (*Solanum lycopersicon*) is one of the most consumed crop in the world (Padalino *et al.*, 2017). Tomato is rich in many beneficial components such as carotenoids (lycopene, α-carotene and β-carotene), phenolic compounds (phenolic acids and flavonoids), organic acids, vitamins (ascorbic acid, vitamin A, and folic acid) and glycoalkaloids (tomatine) (Chaudhary *et al.*, 2018; Lu *et al.*, 2019). These health-promoting phytochemicals help in preventing various chronic degenerative diseases because they have antioxidant, anti-inflammatory, anti-proliferative, anti-mutagenic, and anti-atherogenic activities. Hence, the health promoting bioactivity of tomatoes make them useful ingredient for the development of functional foods (Chaudhary *et al.*, 2018). Tomato is consumed as fresh vegetable or in the form of various processed products (paste, sauce, juice, and ketchup) (Hidalgo *et al.*, 2017; Lu *et al.*, 2019). However, industrial tomato processing generates large amounts of by-products consisting of peels, pulp residues and seeds (Calvo *et al.*, 2007; Domínguez *et al.*, 2020), which can successfully be used in the formulation of new products (Betrouche *et al.*, 2022; Nakov *et al.*, 2022). Tomato peels are the most important part of these by-products because they are rich in bioactive compounds (lycopene and flavonoids) (George *et al.*, 2004).

Waste management as well as the promotion of health, well-being and sustainable lifestyles are among the goals of the 2030 United Nations Agenda (Bianchi *et al.*, 2021). In this context, many studies have been carried out on the valorization of by-products for their use in human food, cosmetics and pharmaceutical products. These new ingredients could be of great interest because their use could reduce industrial costs and justify new investments in equipment, while providing a correct solution to the problem of pollution linked to food processing (Calvo *et al.*, 2007; Padalino *et al.*, 2017). Furthermore, the conversion of industrial by-products into ingredients to produce new food is in the frame of circular economy and sustainability (Bianchi *et al.*, 2021).

Nevertheless, pasta fortification can impact the sensory attributes and the technological properties of pasta. Therefore, developing enriched pasta products with suitable quality is often a challenge and requires a compromise between processing ease, consumer acceptability and nutritional gain.

This study aims to valorize the tomato peel by-product (TPBP) in the production of enriched pasta evaluating the impact of TPBP on the technological and nutritional properties of durum wheat pasta.

2. Materials and methods

2.1. Raw materials

Durum wheat semolina was bought from a local market (Constantine, Algeria) and sifted to obtain semolina with particles size below 0.5 mm.

The tomato pomace was supplied by CAB Company (Guelma, Algeria). Seeds were removed by flotation as described by Padalino *et al.* (2017) and tomato peel by-product was dried at 40 °C in an air oven (Memmert, Schwabach, Germany) until a constant weight was reached. The dried tomato peel by-product (6% residual moisture) was then ground using a knife mill (Philips 2102, Drachten, The Netherlands) and sifted to obtain tomato peel by-product powder with granulation below 0.5 mm.

2.2. Preparation of pasta

The pasta was made according to the method described by Bouasla *et al.* (2022), with modifications. The control pasta (P0) was prepared by hydrating the durum wheat semolina with distilled water (48 mL/100 g) and kneaded manually for 15 min to obtain homogeneous, firm and non-sticky dough, which was rested for 10 min and then sheeted using the Marcato Ampia 150 pasta machine (Campodarsego, Italy) to obtain pasta sheets which were pre-dried in ambient temperature for 30 min. The pre-dried sheets were cut using the same pasta machine to produce fettuccine-type pasta with a 6.5 mm width, 1 mm thickness and

150 mm length. The pasta was dried at room temperature (25°C) for 24 h (moisture content less than 12.5%) then stored in hermetically sealed plastic boxes.

The enriched pasta was produced as described for the control pasta with three modifications: (i) durum wheat semolina was replaced by TPBP in the amounts of 5, 7.5, 10, 12.5 and 15% (w/w), and the pasta samples were coded P1, P2, P3, P4 and P5, respectively, (ii) before hydration, TPBP was mixed with durum wheat semolina for 5 min, (iii) the dough hydration level was gradually increased up to 67.5 mL/100 g.

2.3. Chemical composition

The proximate composition of TPBP and pasta samples was determined in duplicate according to AACC approved methods (AACC, 2000) for fat (n. 30-10) and ash (n. 08-01), and according to AOAC approved methods (AOAC, 2007) for proteins (n. 930.25). Fibers content was determined according to the standard method ISO 5498:1981 (ISO, 1981).

2.4. Antioxidant properties

2.4.1. Extract preparation

The extracts were obtained according to the method described by Bouasla *et al.* (2022). Samples of 1 g of ground dried cooked pasta were extracted three times with 20 mL of acetone (75%), stirred using a magnetic stirrer for 30 min and separated by centrifugation (1700 *g*, 5 min, 4°C). The combined extracts were concentrated under vacuum and the dry extract obtained was used for the evaluation of total polyphenols content (TPC) and antioxidant activities.

2.4.2. Total polyphenols content

The TPC was determined in triplicate in a microplate using the Folin-Ciocalteu method (Singleton and Rossi, 1965). A volume of 20 µL of dry extract prepared in methanol (1 mg/mL), 100 µL of Folin-Ciocalteu reagent diluted in distilled water (1:10), and 75 µL of Na_2CO_3 (7.5%) were mixed and left in the dark for 2 h, then the absorbance was read at 765 nm. A blank was prepared in the same way, replacing the extract with methanol. The TPC was expressed in mg gallic acid equivalent (GAE)/100 g dry matter (dm).

2.4.3. Quantification of pigments

The determination of the pigments of the TPBP and pasta samples was carried out according to Nagata and Yamashita (1992). A 1 g aliquot of the sample was weighed into a 200 mL amber flask. Analyses were performed in the dark to avoid carotenoids degradation and isomerization. A mixture of 10 mL of solvent (acetone/hexane 4:6) was added to the flask and sonicated continuously for 10 min (Misonix Ultrasonic Liquid Processor, USA). The resulting extract was transferred to a separating funnel and the optical density of the supernatant (the non-polar hexane layer containing the carotenoids) was measured at 663 nm, 645 nm, 505 nm and 453 nm and the values of lycopene, β-carotene and chlorophylls a and b were estimated in triplicate and the results are then expressed in mg/100 g dry matter:

$$\text{Lycopene (mg/100 mL)} = -0.0458 \times A_{663} + 0.204 \times A_{645} + 0.372 \times A_{505} - 0.0806 \times A_{453} \quad (1)$$

$$\beta\text{-carotene (mg/100 mL)} = 0.216 \times A_{663} - 1.22 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453} \quad (2)$$

$$\text{Chlorophyll a (mg/100 mL)} = 0.999 \times A_{663} - 0.0989 \times A_{645} \quad (3)$$

$$\text{Chlorophyll b (mg/100 mL)} = -0.328 \times A_{663} + 1.77 \times A_{645} \quad (4)$$

2.4.4. DPPH radical scavenging capacity assay

The scavenging capacity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method described by Yilmaz *et al.* (2015): 160 µL of a solution of DPPH (1 mM) in methanol were mixed with 40 µL of the extract (4 mg/mL). Absorbance was read at 517 nm after 30 min using a microplate reader (Enspire, PerkinElmer, MA, USA). A blank was prepared in the same way, replacing the extract with the methanol. The concentration was calculated using the equation obtained from a Trolox calibration curve. The test was repeated three times and the results were expressed in mg Trolox Equivalent Antioxidant Capacity (TEAC)/100 g dm.

2.4.5. Ferric reducing antioxidant power assay

The reducing power of iron (Fe^{3+}) was determined according to the method described by Oyaizu (1986) with modifications for a microplate protocol: 10 µL of the extract (4 mg/mL) were mixed with 40 µL of a phosphate buffer solution (pH = 6.6) and 50 µL of a solution of potassium ferricyanide $K_3Fe(CN)_6$ to (1%). The whole was incubated at 50°C for 20 min, then 50 µL of trichloroacetic acid at 10%, 40 µL of H_2O and 10 µL of the aqueous solution of $FeCl_3$ at 0.1% were added. The absorbance of the reaction medium was read at 700 nm against a similarly prepared blank, replacing the extract with methanol. The concentration was calculated using the equation obtained from a Trolox calibration curve. The test is repeated three times and the results were expressed in mg TEAC/100 g dm.

2.5. Color measurement

The color profile of uncooked pasta was measured in quadruplicate according to the method described by Zhou *et al.* (2015) with slight modifications, using a computer vision system (CVS), including a lighting system (a mini photo studio with a white background), a Cannon EOS-1200D digital camera (18 Mpx CMOS, 3x 18 –55mm f/3.5–5.6) and a computer with image processing software. An Adobe Photoshop CS4 system (Adobe Systems Inc., USA) was used to obtain the color values: lightness (L^*: 0 black to 100 white), greenness-redness (a^*: greenness when the values are negative and redness when the values are positive) and yellowness-blueness (b^*: yellow when values are positive and blue when values are positive), which were converted to CIE LAB values (L^*, a^* and b^*):

$$L^* = \frac{L}{2,5} \quad (5)$$

$$a^* = \frac{240 \times a}{255} - 120 \quad (6)$$

$$b^* = \frac{240 \times b}{255} - 120 \quad (7)$$

The total color difference (ΔE) was also calculated:

$$\Delta E = [(L^*_{sample} - L^*_{control})^2 + (a^*_{sample} - a^*_{control})^2 + (b^*_{sample} - b^*_{control})^2]^{0.5} \quad (8)$$

2.6. Determination of pasta cooking quality

The optimal cooking time (OCT, min) was determined in triplicate by cooking 25 g of pasta in 300 mL of boiling distilled water (cooking water/sample ratio of at least 10:1). Every 30 s during cooking, a strand of pasta was sampled and then pressed between two transparent glass plates. The OCT was recorded as the time when the dry core of the pasta disappeared (AACC, 2000).

The swelling index (SI) was determined in triplicate by cooking pasta (25 g) in boiling distilled water (300 mL) to the OCT. The cooked pasta was then rinsed with water, drained for 5 min and weighed. The SI was calculated by dividing the weight of cooked pasta by the weight of uncooked pasta (Bouasla *et al.*, 2022).

Cooking losses (CL) were determined in triplicate by evaporating the water from pasta cooking and rinsing to constant weight in an oven at 100 °C. The residue obtained was weighed and the cooking losses were calculated (AACC, 2000):

CL (%) = (weight of dry residue / weight of dry pasta) × 100 (9)

2.7. Statistical analysis

The data were subjected to one-way analysis of variance using the Statistica 10.0 software (StatSoft, Inc., Tulsa, OK, USA) and means were compared by *post hoc* Fisher LSD test at a significant 0.05 level. Correlation coefficients (r) between variables were also determined by Person correlation with a 95% confidence level using the same software.

3. Results and discussions

3.1. Chemical composition of TPBP and raw pasta

The chemical composition of the control pasta and the TPBP-enriched pasta samples is shown in Table 1. As expected, the incorporation of TPBP caused a significant increase in protein content, starting from 10% of TPBP (r=0.91), fibers content, starting from 5%

of TPBP (r=0.97), lipid content, starting from 12.5% of TPBP (r=0.84), and ash content, with 15% of TPBP (r=0.78). These increases are due to the content of TPBP in these nutrients, as shown by its composition: protein, 15.89 g/100 g dm; dietary fibers, 54.08 g/100 g dm; lipid, 3.40 g/100 g dm; and ash, 4.51 g/100 g dm. TPBP composition is in line with the results reported by Lu *et al.* (2019): 10.08-23.26 g/100 g dm for proteins, 1.63-5.50 g/100 g dm for lipid, and 1.04-25.64 g/100 g dm for ash.

Table 1. Chemical composition (g/100 g dm) of control pasta and enriched pasta.

Pasta	TPBP %	Protein	Fibers	Lipid	Ash
P0	0	10.59±0.12^{a}	0.60±0.01^{a}	0.12±0.01^{a}	0.76±0.01^{a}
P1	5.0	10.68±4.70^{a}	3.22±0.01^{b}	0.13±0.00^{a}	0.76±0.01^{a}
P2	7.5	10.86±0.12^{a}	4.65±0.03^{c}	0.16±0.01^{ab}	0.76±0.01^{a}
P3	10.0	11.34±0.06^{b}	6.97± 0.04^{d}	0.24±0.10^{abc}	0.95±0.27^{a}
P4	12.5	11.55±0.25^{b}	7.19±0.16^{de}	0.26±0.01^{bc}	1.13±0.01^{a}
P5	15.0	11.60±0.19^{b}	7.33±0.22^{e}	0.32±0.09^{c}	1.70±0.27^{b}

The results are expressed as mean value±standard deviation (N=2) and means with different letters in superscript within the same column are significantly different ($p<0.05$).

P0: control pasta (100% durum wheat semolina); TPBP: tomato peel by-product.

3.2. Antioxidant properties of pasta

Enriching pasta with TPBP brought a significant increase in TPC, passing from 51.80 mg GAE/100 g dm for control pasta (P0) to 109.56 mg GAE/100 g dm for P5 (r=0.97) (Table 2). Compared to the control pasta, the TPC increased 15.1%, 51.5%, 84.1%, 100.6%, and 111.5% for P1, P2, P3, P4, and P5 respectively. This increase could be due to the richness of tomato pomace in phenolic compounds such as flavonoids (kaempherol, naringenin, quercetin, rutin) and phenolic acids (caffeic acid, ferulic acid, gallic acid, syringic acid) (Betrouche *et al.*, 2022; Domínguez *et al.*, 2020; Nakov *et al.*, 2022; Valdez-Morales *et al.*, 2014). Indeed, Waqas *et al.* (2017) reported a TPC of tomato peels of 270.30 mg/100 g dm, while Betrouche *et al.* (2022) and Nakov *et al.* (2022) reported respectively total free-phenolics of 1137.8 mg/kg dm and 1211.4 mg/kg dm.

Table 2. Total polyphenol content (mg GAE/100 g dm) and pigments content (mg/100 g dm) of pasta samples.

Pasta	TPBP %	TPC	β-carotene	Lycopene	Chlorophyll a	Chlorophyll b
P0	0	51.80±0.92^{a}	2.76±0.03^{a}	1.97±0.01^{a}	3.72±0.01^{a}	5.90±0.01^{a}
P1	5.0	59.60±1.25^{b}	2.79±0.02^{a}	1.98±0.01^{a}	3.79±0.02^{b}	5.90±0.01^{a}
P2	7.5	78.47±1.68^{c}	2.79±0.01^{a}	2.00±0.01^{b}	3.86±0.01^{c}	5.93±0.01^{a}
P3	10.0	95.38±1.88^{d}	3.00±0.01^{b}	2.03±0.01^{c}	3.87±0.01^{c}	5.99±0.01^{b}
P4	12.5	103.92±0.90^{e}	3.15±0.01^{c}	2.13±0.02^{d}	4.03±0.01^{d}	6.14±0.04^{c}
P5	15.0	109.56±1.12^{f}	3.90±0.05^{d}	2.39±0.02^{e}	4.41±0.04^{e}	7.19±0.05^{d}

The results are expressed as mean value±standard deviation (N=3) and means with different letters in superscript within the same column are significantly different ($p<0.05$).

P0: control pasta (100% durum wheat semolina); TPBP: tomato peel by-product; TPC: total polyphenol content.

An increase in TPC was also observed for pasta enriched with different ingredients, such as spinach (Abrol *et al.*, 2017), buckwheat (Biney and Beta, 2014), carob fibers (Biernacka *et al.*, 2017), parsley leaves (Bouasla *et al.*, 2022), fibers from by-products of orange (Crizel *et al.*, 2015), okara (Kamble *et al.*, 2019), and onion skin (Michalak-Majewska *et al.*, 2020). The use of phenolic compounds from natural sources in foods is an interesting opportunity because of the biological activities of these compounds, in particular the antioxidant capacity (Crizel *et al.*, 2015). Phenolic compounds could potentially have a protective role against a wide range of diseases, including cancer and cardiovascular disease, as well as diabetes and Alzheimer (Cianciosi *et al.*, 2018). In addition, they are linked to anti-inflammatory, anti-allergic, antihypertensive and antimicrobial properties (Bhuyan and Basu, 2017).

The pigment contents of TPBP were 5.56 mg/100 g dm of β-carotene, 18.19 mg/100 g dm of lycopene, 25.70 mg/100 g dm of a chlorophyll and 30.84 mg/100 g dm of b chlorophyll. Tomato pomace is an excellent source of carotenoids, mainly in the form of lycopene and β-carotene (Azabou *et al.*, 2020; Betrouche *et al.*, 2022; Nakov *et al.*, 2022; Yagci *et al.*, 2022). Studies performed on tomato peels have reported lycopene yields ranging from 0.639 to 73.40 mg/100g depending on extraction method and type of raw material (Ho *et al.*, 2015; Kaur *et al.*, 2008; Knoblich *et al.*, 2005; Shi *et al.*, 2009).

Enrichment of pasta with TPBP resulted in a significant increase in the content of β-carotene (r=0.81), lycopene (r=0.80), chlorophyll a (r=0.86) and chlorophyll b (r=0.71).

The increase in antioxidant capacity is a primary goal of pasta fortification (Pasqualone *et al.*, 2016). The addition of TPBP significantly increased the reducing power from 38.78 mg TEAC/100 g dm for P0 to 86.51 mg TEAC/100 g dm for P5 (r=0.96) (Table 3). DPPH free radical scavenging capacity also increased ($p<0.05$) with increasing TPBP levels in pasta, growing from 6.62 mg TEAC/100 g dm for P0 to 15.37 mg TEAC/100 g dm for P5 (r=0.88).

Table 3. Antioxidant capacity of control and enriched pasta.

Pasta	TPBP %	FRAP (mg TEAC/100 g dm)	DPPH-SA (mg TEAC/100 g dm)
P0	0	38.78±0.98^{a}	6.62±0.12^{a}
P1	5.0	50.84±0.81^{b}	7.88±0.16^{b}
P2	7.5	72.29±1.80^{c}	7.92±0.08^{b}
P3	10.0	79.45±1.66^{d}	8.91±0.14^{c}
P4	12.5	86.02±1.55^{e}	11.32±0.24^{d}
P5	15.0	86.51±1.25^{e}	15.37±0.29^{e}

The results are expressed as mean value±standard deviation (N=3) and means with different letters in superscript within the same column are significantly different ($p<0.05$).

P0: control pasta (100% durum wheat semolina); TPBP: tomato peel by-product; FRAP: ferric reducing antioxidant power; DPPH-SA=1,1-diphenyl-2-picrylhydrazyl scavenging capacity; TEAC: Trolox equivalent antioxidant capacity.

According to Abrol *et al.* (2017), the antioxidant capacity depends on pigments (such as carotenoids and anthocyanin), ascorbic acid and total polyphenols of the product. So the increase in antioxidant capacity in enriched pasta could be linked to the richness of the tomato pomace in total polyphenols such as flavonoids and phenolic acids and in carotenoids (Betrouche *et al.*, 2022; Domínguez *et al.*, 2020; Nakov *et al.*, 2022). Indeed, FRAP and DPPH are strongly correlated with TPC (r=0.97 and r=0.84 respectively). Furthermore, lycopene is considered one of the most powerful natural antioxidants and acts by inhibiting free radicals (Rodriguez-Amaya, 2001; Silva *et al.*, 2019). Chlorophyll and its derivatives are also known

for their antioxidant capacity (Barros *et al.*, 2011).

Similar results have been reported by other authors by incorporating various ingredients for pasta fortification: spinach (Abrol *et al.*, 2017), carob fibers (Biernacka *et al.*, 2017), parsley leaves (Bouasla *et al.*, 2022), orange by-product fibers (Crizel *et al.*, 2015), soy okara (Mamble *et al.*, 2019) or onion skin (Michalak-Majewska *et al.*, 2020).

3.3. Color of raw pasta

The color profiles of the control pasta and the enriched pasta are shown in Table 4. Pasta color is the first property that the consumers evaluate when choosing a product in the market, and as such is a very important quality attribute that greatly influences consumer acceptance (Bouasla *et al.*, 2022). The control pasta showed higher *L** and lower *a** than the durum wheat pasta tested by Brandolini *et al.* (2018), because of the different drying temperatures, but the *b** values were similar. The lightness (*L**) of the pasta decreased ($p<0.05$) with increasing TPBP levels (r=-0.91), thus indicating that the enriched pasta became darker than the control. This decrease may be due to the dark color of TPBP as well as to the oxidation of carotenoid pigments resulting from the high oxygen permeability of enriched pasta (Mercier *et al.*, 2016).

Table 4. Color profile of control and fortified pasta.

Pasta	TPBP %	*L**	*a**	*b**	*ΔE*
P0	0	94.83±0.80^{c}	-7.25±0.28^{a}	16.39±0.31^{a}	
P1	5.0	87.46±0.88^{b}	-2.29±0.29^{b}	31.68±0.46^{b}	17.71±0.16^{a}
P2	7.5	86.30±1.71^{b}	-0.96±0.74^{c}	32.46±1.40^{b}	19.29±1.70^{ab}
P3	10.0	86.86±1.51^{b}	-0.21±0.53^{c}	34.62±0.91^{c}	21.15±1.04^{b}
P4	12.5	81.79±1.58^{a}	3.80±0.74^{d}	39.77±1.67^{d}	28.97±2.23^{c}
P5	15.0	82.59±0.73^{a}	4.88±0.47^{e}	43.63±0.42^{e}	32.24±0.58^{d}

The results are expressed as mean value±standard deviation (N=4) and means with different letters in superscript within the same column are significantly different ($p<0.05$).
P0: control pasta (100% durum wheat semolina); TPBP: tomato peel by-product.

Moreover, *a** values increased significantly with the increase in enrichment rate (r=0.98), varying from -7.25 for P0 to 4.88 for P5, thus indicating that the color of the enriched samples tends towards red. Similarly, the enrichment of the pasta with TPBP significantly increased the yellowness (*b**) from 16.39 for P0 to 43.63 for P5 (r=0.96). This was expected, since TPBP is characterized by its red color due to the presence of carotenoid pigments, in particular lycopene (18 mg/100 g in the TPBP used in the present study). Previous studies reported similar trends for pasta enriched with apple peel (Lončarić *et al.*, 2014) and carrot pomace (Gull *et al.*, 2015). Differently, the addition of carrot waste extract encapsulates produced a lighter pasta because of the presence of the encapsulate carrier while the high content of β-carotene increased *a** and *b** (Šeregelj *et al.*, 2022).The total color difference (ΔE) between control pasta and TPBP-enriched pasta increased ($p<0.05$) with increasing TPBP levels (r=0.94) ranging from 17.91 to 32.24. The color difference can be detected visually by an experienced observer when ΔE is greater than 3.5, and by an inexperienced observer when ΔE is greater than 5 (Bouasla *et al.*, 2022). Therefore, this indicates that the TPBP-enriched pasta samples are different from the control pasta and that significant differences could be visible to the naked eye.

3.4. Cooking quality of pasta

The incorporation of TPBP caused a significant reduction in OCT (r=-0.86) which

varied from 4.5 min for the P0 to 3 min for P5 (Table 5). This result agrees with previous reports on pasta enriched with tomato by-product (Padalino *et al.*, 2017), apple peel by-products (Lončarić *et al.*, 2014), soy okara (Kamble *et al.*, 2019), olive pomace (Simonato *et al.*, 2019), onion skin (Michalak-Majewska *et al.*, 2020), and persimmon by-product (Lucas-González *et al.*, 2020). A higher OCT was reported by Vimercati *et al.* (2020) in a thicker pasta enriched with 8.9% dried-tomato.

Table 5. Cooking quality of control and enriched pasta.

Pasta	TPBP %	Optimal cooking time (min)	Swelling index	Cooking loss (%)
P0	0	4.50±0.01^{c}	3.18±0.07^{c}	4.03±0.15^{a}
P1	5.0	3.50±0.01^{b}	2.98±0.17^{b}	4.75±0.07^{a}
P2	7.5	3.00±0.01^{a}	2.87±0.12^{b}	5.70±0.70^{b}
P3	10.0	3.00±0.01^{a}	2.83±0.03^{b}	5.73±0.67^{b}
P4	12.5	3.00±0.01^{a}	2.59±0.06^{a}	5.73±0.32^{b}
P5	15.0	3.00±0.01^{a}	2.90±0.08^{b}	6.80±0.26^{c}

The results are expressed as mean value±standard deviation (N=3) and means with different letters in superscript within the same column are significantly different ($p<0.05$).

P0: control pasta (100% durum wheat semolina); TPBP: tomato peel by-product.

The decrease in cooking time may be due to the addition of TPBP which could induce changes in the composition and microstructure of the pasta (Mercier *et al.*, 2016). Additionally, the amount of water required for starch gelatinization can be decreased with fortification by diluting the starch content of pasta. The incorporation of TPBP reduces the amount of durum wheat semolina and therefore decreases the glutenin fraction which has a higher molecular weight and a longer hydration time. In addition, the physical disruption of the gluten matrix by the fibers-rich fractions of TPBP facilitated water penetration into pasta containing non-traditional ingredients, resulting in shorter cooking time (Mercier *et al.*, 2016; Petitot *et al.*, 2010).

The swelling index provides information on the water absorption capacity. All the enriched pasta had a significantly lower SI (2.90-2.98) than the control pasta (3.18) (r=-0.70). Similar results have been reported for pasta enriched with tomato by-product (Padalino *et al.*, 2017) and persimmon by-product (Lucas-González *et al.*, 2020). The decrease in water absorption for the enriched pasta could be explained by the entrapment of the starch granules by the fibers particles, thus reducing their swelling during cooking. On the other hand, starch dilution can decrease the amount of water required for gelatinization and the amount of water absorbed during cooking (Bouasla *et al*., 2020). For pasta made from durum wheat, the appropriate weight of cooked pasta is about three times the dry weight (Sissons *et al.*, 2012) which was the case for all pasta samples.

Cooking loss is considered an important factor for the cooking quality of pasta (Mercier *et al.*, 2016) because provides information on the ability of pasta to retain its structural integrity during cooking (Kamble *et al*., 2019). The addition of 5% TPBP did not lead to a significant change in the CL compared to the control pasta, which had the lowest CL (4.03%). However, from 7.5% of TPBP, a significant increase in the CL, which ranged from 4.75% for P2 to 6.80% for the P5 paste (r=0.88), was noticed. This result agrees with Padalino *et al.* (2017), who found that the incorporation of tomato by-product (10 and 15%) brought about an increase in CL. Similar trends were also found for pasta enriched with plant-based by-products: mango peel, soy okara, onion peel, apple peel, and olive pomace (Ajila *et al.*, 2010; Kamble *et al.*, 2019; Lončarić *et al.*, 2014; Michalak-Majewska *et al.*, 2020; Simonato *et al.*, 2019). Vimercati *et al.*

(2020) reported a cooking loss around 7% in tomato enriched pasta.

The increased cooking loss with the incorporation of TPBP may be associated with structural changes in the protein network caused by the addition of dietary fibers from TPBP (Crizel *et al.*, 2015) which is a non-glutinous material and dilutes the gluten content, therefore disrupting the protein-starch matrix which is responsible for maintaining the physical integrity of the pasta during cooking. They also cause the unequal distribution of water in the pasta matrix due to the competitive hydration tendency of the fibers (high water absorption capacity). This leads to more solids leaching from pasta during cooking (Bouasla *et al.*, 2020; Mercier *et al.*, 2016; Padalino *et al.*, 2017). Moreover, the drying conditions applied in the present study could lead to the formation of micro-cracks that could be responsible for the high dry matter loss during cooking (Bouasla *et al.*, 2022). Indeed, Mercier *et al.* (2016) showed that the increase in cooking losses for enrichment less than 15% was doubled with low temperature drying (≤60°C) compared to high temperature drying (>60°C). According to the same authors, at low temperature, there is no coagulation of proteins that cause the strengthening of the gluten network. Although cooking losses increased with increasing enrichment level, all pasta samples were of good quality because cooking losses were less than 8% (Sissons *et al.*, 2012).

4. Conclusion

The incorporation of TPBP improved the nutritional quality of pasta by increasing the content of protein, fibers, ash and, more importantly, the content of pigments, total polyphenols and antioxidant capacity. However, the addition of TPBP caused a reduction in the optimal cooking time, the swelling index and an increase in cooking losses, without exceeding the acceptable limit. These results indicate that the incorporation of up to 15% TPBP in pasta improves its nutritional quality without penalizing the cooking quality and therefore presents a good material to enrich with bioactive components pasta or other food products.

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CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

INFLUENCE OF THERMAL PROCESSING ON THE NUTRITIONAL COMPOSITION, AMINO ACID PROFILE AND SENSORIAL CHARACTERISTICS OF BISCUITS PRODUCED FROM WHEAT-DEFATTED MELON SEED FLOUR BLENDS

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https://doi.org/10.34302/crpjfst/2024.16.2.15

Article history:
Received: August 12th, 2023
Accepted: April 12th, 2024

Keywords:
Egusi seed;
Defatting;
Amino acid;
Thermal processing;
Biscuits;
Nutritional properties.

ABSTRACT

The study was conducted to investigate the influence of thermal processing on the nutritional composition, amino acid profile and sensorial characteristic of biscuits produced from wheat flour-defatted melon flour blends from toasted and raw melon seeds. The melon seeds was toasted at 160^0 C for 20mins and the other portion left untreated. The treated and untreated melon seed was defatted using n-Hexane and grinded into flour. The flour from the defatted roasted and raw melon seed flour was incorporated with wheat flour at 4 percentage ratio (100:0, 95:5, 90:10, 80:20, 70:30) respectively. Nine (9) samples of biscuit was formulated with other ingredients to produce biscuits according to the standard method and subjected to statistical evaluation in complete randomized design using statistical Product for Social Services version 21. The means were separated using Duncan Multiple test range at ($p<0.05$). The inclusion of the defatted melon flour from the untreated (raw) and toasted melon significantly increased ($p<0.05$) the moisture (1.78-3.87 %), protein (13.47 to 21.05 %), ash (2.04 to 2.72 %), fat (9.34-12.73%) and reduced (74.92-56.86 %) carbohydrate content of the biscuits. The amino acid profile of the biscuits showed significant ($p<0.05$) difference. The amino acid values varied significantly ($p<0.05$) and showed increase with increase in the substitution of the defatted melon flour. The amino acid ranged from leucine (7.48 to 9.18 g/100 g), lysine (4.78-9.18 g/100g), Histidine (1.89-2.38 g/100g), Arginine (3.12-5.02 g/100g) and 10.48-12.80 g/100g in glutamic acid respectively. The physical analysis showed significant ($p < 0.05$) difference among the biscuit samples. The study showed that substitution of wheat flour with defatted raw and toasted defatted melon flour at a ratio of 95:5 and 90:10 yielded the most acceptable biscuits in all the sensory attributes assessed. This implies that biscuits produced from this composite blend contain sufficient amounts of protein, fat, fiber and carbohydrate. Hence, they can serve as relief for malnutrition.

1.Introduction

Biscuit is a term used for a diverse variety of baked products, commonly a flour based food product which is dry and flat and unleavened, usually savory and sweet, it could be combined with sugar, chocolate, honey etc. It is one of the ready-to-eat, cheap and convenience food product that is consumed among all age groups in many countries of the world (Iwegbue, 2012). This fact is reinforced by a research done by Nielsen which puts biscuits as the fourth most consumed snack in the world (Ferdman, 2019). Many of its, attractive features includes a wider consumption base, relatively long shelf life, greater convenience and good eating quality

makes it good for enrichment (Hooda and Jooda, 2005). The growing interest in this type of bakery product is due to their better nutritional properties and the possibilities. Currently, the production of baked product has been centered on wheat as a major raw material and it is considered nutritionally poor, as cereal proteins are deficient in essential amino acids such as lysine and threonine (Dhingra and Jood 2001). Biscuits are predominantly based on refined wheat flour (RWF) and the blending of RWF with oilseed such as defatted egusi seed flour might elevate the protein nutritional quality.

Wheat flour (*Triticum aestivum*. L) is the major ingredient for production of baked products such as biscuit and it contributes more than 50% of the entire calorie intake across the world (Masood et al., 2020). The entire grain is rich in nutrients such as minerals, protein (8–16%), fat (1–3%) and fiber (12–15%) (Senya, Kwaatermaa and Sitsofe, 2021). The cereal is uniquely rich in methionine and cysteine but lacks lysine and threonine (Peluola-Adeyemi *et al*., 2021). It is known that legumes like soybean, cowpea, African yam bean, bambara nut and velvet bean impart significantly the mineral, protein and B-complex vitamin needs of people in developing countries (Adeyanju et al., 2021). Thus, wheat flour supplementation with low-cost staples, from different legumes, cereals, roots and tubers will contribute in enhancing the nutritive quality of wheat products (Urigacha, 2020). Notably, the consumption of biscuits in many countries is increasing due to urbanization but is faced with the problem of being deficient in protein and certain micronutrients, and high cost of wheat importation in many countries resulting in the high cost of biscuits (Mudau et al., 2021).

Citrullus colocynthis commonly known as the Egusi melon is a member of the Cucurbitaceae family and the biological ancestor of watermelon, now found all over the world. Its origin is from West Africa. It's an important food crop in Nigeria and other Africa countries. It is grown for the seed which is rich in protein and used in preparing assorted dishes, especially soups. It contains 50% oil and 35% protein (Jack, 1972), the seeds have both nutritional and cosmetic importance. The seeds contain vitamin C and B2, minerals, riboflavin, fat, carbohydrates and protein (Lazos, 1986). .

The nutrient density of egusi seed which comprises nearly 50% w/w edible oil and 30% w/w pure protein makes it a functional food (Iwuoha and Eke, 1996). Traditionally, this oil seed has been processed by boiling in salted water, or roasting and then ground to be used as food thickener. Aside, its function as a thickener, the oil can be expelled and used for domestic purpose and also find application industrially. This oil are nutritious and expensive, and are used in homes for cooking and cosmetics purposes and of interest to pharmaceutical industries (Ayodele *et al*., 2013).

Locally, the residue from the oil after extraction is made into balls, fried to produce local snack, or is used as cattle feed (Schipper *et al*., 2000). In many parts of Africa, where farmers lack access to meat or dairy, the high oil and protein content can make an excellent dietary supplement (Jacob *et al*., 2015). The use of this locally available, nutritious feed stuff as a means of dietary diversification has been a continuous effort by researchers in combating the menace of micronutrient deficiencies and functional composition of the food crops. Nonetheless, information of the nutritive value of such local foodstuffs and ingredients is also necessary in order to encourage the consumption and its cultivations (Manpreet and Sharma, 2017).

Food product enrichment is a reasonable method for treating appropriate dietary deficiencies, which improves health and prevents chronic diseases. Numerous studies have been carried out around the world on how to maximize the nutritional value of food products while keeping costs down (Achu *et al*., 2005; El-Adawy *et al*., 2001; Rangel *et al*., 2003). Significant consideration has been given to enrich wheat flour products with high proteins food from different protein sources like pulses, legumes and oilseed flour (Hoover, 1979).

Nevertheless, having these effects, indigenous people from different groups who grow the egusi melon seed have limited the utilization of this highly nutritious and proteinous feedstuff only to pepare egusi soup. The Egusi oil, which contains a large amount of omega-6 fatty acids, likewise its defatted meal which is rich in protein has not found so much application in the food production (Bankole *et al*., 2005). There is therefore need, to assay the nutrient composition, amino acid profile and sensorial characteristics of wheat-defatted melon seed flour in baked goods like biscuits. This will improve its nutritive value, create variety and make it more affordable to consumers and to generate new information on wheat-defatted melon biscuits which is largely lacking.

2.0 Materials and methods

2.1. Material

Refined wheat flour (RWF-Honeywell ltd) and the Dehulled egusi seeds (*Citrullus Colocynthis sub spp mucosospermus*), shortening, baking powder, milk powder, salt, flavours and other general ingredients were procured from Ogige market in Nsukka Local Government Area of Enugu State. All chemical reagents used were procured from a pure analytical grade dealer.

2.1.1 Samples preparation

Dehulled egusi seeds (10 kg) were sorted to remove the foreign materials, chaff and damaged seeds as well as stones. The seeds were cleaned with water to further remove the dirts, dried in an oven at 50^0C for 24 hours to remove the moisture content. The clean and dried Egusi seeds were divided into 2 portion sizes of 5 kg each, and one portion was subjected to toasting and the other left as raw (control).

2.2. Methods

2.2.1 Processing of Toasted Egusi Flour (TEF)

The sorted cleaned dehulled Egusi seed (5 kg) were poured in a sauce pan and subjected to an open air heating at 100 - 140^0C for 30 minutes. The melon seed was stir until a golden colour was obtained using a wooden spoon to prevent burning. After toasting, the seeds were allowed to cool, milled using attrition milling machine into fine flour, packaged in a polyethylene bag and then kept in a cool dry place for further analysis.

2.2.2. Processing of Raw Egusi Flour (REF)

The finely cleaned melon egusi seed (5 kg) were grinded and packed in a polyethylene bag prior to use.

2.2.3. Processing of Defatted Egusi Flour

The method of Oniyeike *et al*. (1994), were adopted with slight modification. The toasted and raw egusi flour samples were defatted using solvent extraction apparatus for eight hours refluxing with n-hexane. After the extraction, the oil were collected in an air tight plastic container and the defatted flour were dried in an oven, milled into flour and stored in an airtight bag as defatted Toasted egusi flour and defatted Raw egusi flour. These were kept in a cool dry place for further analysis.

Table 1.Formulation of wheat-defatted melon flour.

Sample	Wheat	Defatted melon flour
A	95	5
B	90	10
C	80	20
D	70	30
E	95	5
F	90	10
G	80	20
H	70	30
I	100	0

2.3. Formulation of the Flour Blends

The Wheat and Defatted melon flour samples were formulated on the percentage ratio of (95:5; 90:10; 80:20; 70:30) for wheat with toasted and raw defatted melon flour respectively While the refined wheat flour (RWF) was used as control sample as shown in Table 1 below.

2.4.Recipe for the Biscuit Production

The recipe of Aliyu and Sani (2009) was adopted for the biscuit production. The raw materials used include refined wheat flour and defatted egusi flour (100 g), sugar (10 g), margarine (30 g), salt (2 g), sodium bicarbonate (1 g), water (50 g), milk (10 g), vanilla flavour (2 g). These were weighed appropriately and all the ingredients except flours were mixed thoroughly in a Kenwood mixer (a 3-speed hand mixer), it was then transferred to a bowl. The flours and baking powder were added with continuous mixing for 15 min until smooth dough was obtained. The dough were cut, placed on a clean platform then rolled out using rolling pin until the desired uniform texture and thickness is obtained. Cookies cutter were used to cut the sheet of the dough into required shapes and sizes. They were transferred on to a greased (with margarine) baking tray. The baking were done at a temperature of 180 - 200^{o} C, allowed to bake for 30 - 45 minutes. After baking, the hot cookies were removed from the pan and placed on a clean tray to cool down. The cookies were allowed to cool, the samples was then stored in polyethylene bag and kept in a shelf for further analysis and sensory evaluation.

2.5. Proximate Composition of the Biscuits

The moisture, crude protein, total ash, fat, crude fiber was determined described by AOAC (2012) and carbohydrate content was determined by difference as summarized : % Carbohydrate = 100 – (% moisture + % protein + % Ash + % Crude fibre). The energy value (kcal/100 g) was determined according to the method of Marero *et al.* (1998) was calculated by application of the thermal coefficients of Atwater and Rosa (1899). with 4 calories for 1g of proteins; 9 calories for 1 g of lipids and 4 calories for 1 g of carbohydrates.

The energy value (kcal /100 g) = (4 x Protein %) + (4 x Carbohydrate %) + (9 x fat %)

2.6. Determination of Amino Acid Composition

The amino acids composition of the samples was measured on hydrolysates using amino acids analyzer (Sykam-S7130, Tokyo, Japan) based on high performance liquid chromatography technique. Sample hydrolysates were prepared following the method of Moore and Stain (1963). About 200 mg of the sample was taken in a hydrolysis tube. Then 5 ml of 6 N HCl was added to the sample and the tube tightly closed and incubated at 110 ^{o}C for 24 h. After incubation, the solution was filtered and 200 ml of the filtrate was evaporated to dryness at 140oC for 1 h. The hydrolysates after dryness were diluted with 1.0 ml of 0.12 N citrate buffer (pH 2.2). Aliquot of 150 of the sample hydrolysate was injected in an action separation column at 130 ^{o}C. Ninhydrin solution and an eluent buffer (solvent A, pH 3.45 and solvent B, pH 10.85) were delivered simultaneously into a high temperature reactor coil (16 m length) at a flow rate of 0.7 ml/min. The buffer ninhydrin mixture was heated in the reactor at 130^{0} C for 2 min to accelerate chemical reaction of amino acids with ninhydrin. The products of the reaction mixture were detected at wavelengths of 570 and 440 nm on a dual channel photometer. The amino acids composition was calculated from the areas of standards obtained from the integrator and expressed as gm/100 gm protein.

2.7. Determination of the physical characteristic of the biscuits

Weight:

The weight of the cookies was determined according to the method of Ayo, Ayo, Nkama, and Adeworie (2007). The weights of cookie samples were determined with the aid of a weighing balance (model) immediately after cooling.

Diameter:

The diameter (D) of the cookies was determined according to the method of AACC (2000). Four cookies were placed edge to edge and their total diameter was measured with the aid of a ruler. The cookies were rotated at angles of 90° for duplicate reading. The experiment was repeated twice and average diameter was recorded in millimeter.

Thickness:

The thickness of the cookies was determined according to the method of Ayo et al. (2007). The cookies thickness was measured with the aid of a digital vernier caliper with 0.01 mm precision.

Spread ratio

Spread ratio of the cookie samples was determined according to the method of Gomez et al. (1997). For spread ratio, two rows of four well-formed cookies were made and the height measured. They were arranged horizontally edge to edge and the sum of their diameters measured. The spread ratio was calculated as diameter divided by height.

Breaking strength

The breaking strength was determined according to the method described by Okaka and Isiehs (1997). Cookies of known thickness (0.4 cm) were placed centrally between two parallel metal bars (3 cm apart) and weights were applied until the cookies snapped. The least weight that caused the breaking of the cookies was regarded as the break strength of the cookies.

2.8. Sensory evaluation of the cookies

Sensory evaluation of the cookies was carried out according to the method described by Retapol and Hooker (2006). A panel of twenty members consisting of students and members of staff in Food Science and Technology Department, University of Nigeria, Nsukka was chosen based on their familiarity and experience with wheat-based cookies for sensory evaluation. Cookies produced from each flour blend, along with the reference sample were presented in coded form on white plastic plates and were randomly presented to the panelists. The panelists were provided with portable water to rinse their mouth between evaluations. However, a questionnaire describing the quality attributes (colour, taste, flavour, crispiness and overall acceptability) of the cookies was given to each panelist. The panelist assigned scores for each parameter as against the maximum score of 9. Each sensory attribute was rated on a 9-point hedonic scale (1 = dislike extremely and 9 = like extremely).

2.9. Statistical analysis

Data obtained were subjected to appropriate statistical analysis (ANOVA) using a statistical package for the Social Sciences, SPSS (version 16). Mean separation was done using Duncan multiple range test and significance difference was accepted at 5% confidence level.

3.Result and Discussion

The result of the proximate composition of the wheat-defatted melon seed biscuits is shown in Table 2. Moisture, protein, fat, ash and carbohydrate contents of the flour samples ranged from 1.78-3.87 %, 11.13 – 13.19 %, 1.74 -2.72 %, 11.14 -21.05 % and 56.86 – 74.92 % respectively. The moisture content of the biscuits samples incorporated defatted raw and toasted melon flour with wheat showed a significant ($p<0.05$) difference among the samples. The significant increase was observed in the moisture level with increase in the incorporation of the defatted flour sample from raw and toasted melon. Sample D had the highest value (3.87 %) while sample E has the least moisture value (1.78 %). The moisture value as observed in the study was lower when compared with the moisture value (6.25 – 10.0 %) reported by Peter-Ikechukwu *et al*. (2016) for biscuit from wheat blend with toasted watermelon flour. The moisture content of the samples were all within the acceptable range of (<14 %) for shelf stability. Sample D (70:30 % wheat: defatted raw melon flour) had the highest value of protein 21.05 % while sample E (95:5 % wheat:defatted toasted melon flour) has the least (11.74 %) protein content. There was a significant ($p<0.05$) increase in the protein content with incorporation of the defatted melon seed flour. This is expected as defatted melon flour has been reported to contain 60 % dry weight basis of protein (El-Adawy and Taha, 2001; Ojieh et al., 2007).

The highest ash content of the biscuits (2.72±0.03 %) were record in sample D (wheat 70 % + defatted raw melon flour 30%) and the lowest ash content (1.74±0.01 %) was recorded for sample F (wheat 90% + 10 % defatted toasted melon flour). The lower percentage of ash content as observed with the biscuits from

the blends of defatted toasted biscuits could be attributed to toasting treatment effect.

Table 2. Proximate composition of wheat- defatted raw and toasted melon seed flour blend biscuits

SAMPLE	MC (%)	ASH (%)	FAT (%)	PROTEIN (%)	CRUDE-FIBRE (%)	CHO (%)	ENERGY (Kcal)
A	1.91± 0.05^{e}	2.52± 0.05^{b}	11.13± 0.08^{e}	14.29± 0.13^{e}	0.99± 0.13^{e}	69.18± 0.08^{c}	434.03± 0.11^{d}
B	2.21± 0.01^{d}	2.16± 0.06^{d}	11.36± 0.06^{e}	16.55± 0.19^{c}	1.22± 0.02^{d}	66.52± 0.04^{e}	434.44±. 05^{d}
C	3.29± 0.03^{b}	2.07± 0.04^{d}	11.64± 0.05^{d}	17.08± 0.08^{b}	1.33± 0.04^{c}	64.60± 0.06^{f}	431.44± 0.33^{e}
D	3.87± 0.11^{a}	2.72± 0.10^{a}	12.66± 0.16^{b}	21.05± 0.23^{a}	2.85± 0.07^{a}	56.86± 0.06^{h}	425.54± 0.21^{g}
E	1.78± 0.04^{f}	1.74± 0.01^{e}	9.34±0. 10^{f}	11.14± 0.08^{g}	1.09± 0.01^{e}	74.92± 0.13^{a}	428.28± 0.69^{f}
F	2.36± 0.02^{c}	1.83± 0.04^{e}	11.82± 0.16^{d}	11.29± 0.04^{g}	1.11± 0.01^{e}	71.61± 0.25^{b}	437.94± 0.55^{b}
G	2.39± 0.08^{c}	2.36± 0.06^{c}	12.73± 0.10^{b}	14.32± 0.16^{e}	1.30± 0.01^{c}	66.92± 0.23^{d}	439.51± 0.58^{a}
H	3.19± 0.03^{b}	2.50± 0.03^{b}	13.19± 0.18^{a}	15.98± 0.12^{d}	1.64± 0.05^{b}	63.51± 0.35^{g}	436.65± 0.72^{c}
I	3.22± 0.04^{b}	2.04± 0.04^{d}	12.32± 0.12^{c}	13.47± 0.12^{f}	0.11± 0.06^{f}	68.86± 0.06^{c}	440.12± 0.86^{a}

Values are means ± SD of duplicate analysis. Means in the same column having same superscript are not significantly different ($p>0.05$). Key. Sample A 95:10 % Wheat:Raw defatted melon seed; Sample B 90:10 Wheat: Raw defatted melon seed,; Sample C 80:20 % Wheat :Raw defatted melon seed, Sample D 70:30 % Wheat :Raw defatted melon seed, Sample E 95:10 % Wheat:toasted defatted melon seed; Sample F 90:10 Wheat: Toasted defatted melon seed,; Sample G 80:20 % Wheat :Toasted Defatted melon seed, Sample H 70:30 % Wheat :Raw defatted melon seed, sample I 100% wheat flour watermelon.

Fat contents of the biscuits were observed to be increasing with increase percentage of the defatted melon flour and were significantly different ($p<0.05$) from the control sample I (100% wheat flour). The fat contents range 11.13 - 13.19 % obtained from this study was lower than the fat contents ranges of 25.08 to 30.79 % reported by Bolarinwa et al (2016) for malted sorghum-soy biscuits and (19.32 – 21.50 %) presented by Orafa et al. (2023) for acha-based biscuits incorporated with defatted melon seed, but were higher than the fat content (5.20 – 6.36 %) of cookies produced from cocoyam, sorghum and pigeon pea as reported by Okpala and Okoli (2011). The increase in fat with increase defatted melon flour shows that it is a good source of energy to human body. Fat and oil provides twice as carbohydrate on weight-weight basis (Iwe 2000). Increase in the level of flour inclusion increased the crude fibre content and were significantly ($p<0.05$) different.

The crude fibre content increased from 0.99 to 1.64 %. This increase could be attributed to the relative fibre content in defatted melon seed flour as Ojieh et al. (2007) reported a fibre content of (12.0 %) in melon seed. A decrease in the carbohydrate content (CHO) from 74.92 to 56.86 % in biscuits from wheat:defatted raw and toasted melon flour blend respectively. This observation was similar to the report of Orafa et al. (2023).

The biscuit with 30 % defatted raw melon flour substitute has the lowest carbohydrate content (56.86 %) while the biscuit with 5 % defatted toasted melon flour sample had the highest (74.92 %) carbohydrate content. This shows that defatted melon seed flour is low in carbohydrate content. Carbohydrate are macronutrients such as starch, sugar and fibre

found in food, provides the body with energy (Ojieh et al., 2007)

3.1. Physical properties of wheat- defatted raw and toasted melon seed flour biscuits

The results of the physical characteristic of the biscuits were reported in Table 3 below. The diameter, spread ratio, weight, diameter and break strength were significant ($p<0.05$). The diameter ranges from 7.02 – 9.02 mm respectively in sample blends from wheat: defatted raw and toasted melon flour. The sample E (95:5 % wheat flour + defatted toasted melon flour) had the highest diameter value of (9.02 mm) and the least diameter value (7.02 mm) was recorded in the sample A (95:5 wheat+ defatted raw melon flour) and where significantly different from the sample I (control :100 % wheat flour) of 7.18 mm. The biscuits showed a progressive decrease with increasing percentage of the defatted flour in the diameter of the biscuit. The diameter of the biscuits were increasing with increased substitution of the defatted melon flour. The resultant effect could be attributed to the reaction of the gluten network during dough handling causing a stretch in the dough and the spring back after baking, thereby resulting to a thicker biscuits with smaller diameter. The report of the diameter observed in this study were relatively lower with the ranges of (50.2 – 57.3 mm) reported by Ikuomola et al. (2017) for cookies from malted barley bran blends. Gernah et al. (2010) also reported a similar increasing trend for the diameter (38.90–40.20 mm) of cookies made from wheat-brewers spent grain flour blends. The increase in the diameter of biscuit could also be attributed to the soluble fibre content of the flour in its correlation to the water absorption capacity of the flour, because the higher WAC observed in this flour blends could suggest percentage of soluble fibre in the blends causing the intake of water and reduced release of water for sugar breakdown during baking.

Table 3. Physical parameter of wheat: defatted raw and toasted melon seed flour biscuits

Sample	Height (cm)	Diameter (cm)	Spread Ratio	Break strength (kg)	Weight (g)
A	7.02 ± 0.01^{e}	16.55 ± 0.71^{e}	2.26 ± 0.03^{d}	2.05 ± 0.07^{b}	6.89 ± 0.07^{d}
B	7.85 ± 0.04^{d}	20.06 ± 0.04^{d}	2.70 ± 0.21^{c}	2.20 ± 0.00^{ab}	12.62 ± 0.18^{c}
C	8.23 ± 0.07^{c}	21.01 ± 0.01^{c}	2.56 ± 0.02^{c}	2.28 ± 0.11^{a}	14.68 ± 0.29^{b}
D	7.60 ± 0.08^{d}	19.05 ± 0.01^{d}	2.51 ± 0.04^{c}	2.32 ± 0.18^{a}	11.99 ± 0.59^{c}
E	9.02 ± 0.06^{a}	17.04 ± 0.01^{e}	1.88 ± 0.01^{d}	1.68 ± 0.04^{c}	16.65 ± 0.00^{a}
F	8.65 ± 0.33^{b}	21.55 ± 0.70^{b}	2.49 ± 0.01^{c}	1.64 ± 0.01^{c}	16.47 ± 0.52^{a}
G	8.24 ± 0.05^{c}	24.53 ± 0.69^{a}	2.98 ± 0.10^{a}	1.60 ± 0.00^{c}	16.88 ± 0.01^{a}
H	7.93 ± 0.22^{d}	21.56 ± 0.71^{b}	2.73 ± 0.16^{bc}	1.61 ± 0.01^{c}	16.63 ± 0.10^{a}
I	7.18 ± 0.04^{e}	21.01 ± 0.01^{b}	2.93 ± 0.01^{ab}	2.17 ± 0.08^{ab}	14.31 ± 0.21^{b}

Values are means ± SD of duplicate analysis. Means in the same column having same superscript are not significantly different ($p<0.05$).

The spread ratio (S.R) of the biscuits ranged from 1.88 to 2.98 and were significantly different ($p<0.05$) among the varied samples from blends. Sample E (95:5 % wheat-defatted toasted melon flour) had the lowest S.R value of (1.88), while sample G (80:20 % wheat-defatted toasted melon flour) had the highest S.R value (2.98), when compared with the value of (2.93) for the control sample I (100 wheat Flour). The incorporation of the defatted melon flour caused a significant ($p<0.05$) increase in the S.R values of the biscuits. The S.R values was inconsistent in the trend with increase of the defatted melon flour. This was also in line with the observations of Gbadamosi et al. (2011) who also reported an inconsistent trend in the spread ratio values of cookies

produced from blends of wheat flour and African oil bean seed flour.

The Break Strength of the biscuits ranges from 1.60 – 2.32 kg in blends of wheat-defatted melon respectively. Sample D (70:30 % wheat-defatted raw melon flour blend) have the highest break strength while sample H (70:30 wheat-defatted toasted melon flour blend) had the least breaking strength (1.60 kg), when compared with the B.S value of (2.17 kg) recorded with the control sample. This study reveals that the processing affect the break strength of the biscuits as observed with biscuits with defatted toasted melon seed flour when compared to the biscuits from the wheat:defatted raw melon seed flour. These significant reduction as observed with samples from toasted blends could be attributed to the carbohydrate/starch content of melon flour been affected by the thermal processing, thereby resulting to the biscuit not to be as hard/strong like that of wheat.

3.2. Sensory score of the biscuits from wheat defatted raw and toasted melon seed flour blends

The result of the sensory score from the biscuits produced from blends of wheat and defatted raw melon seed is shown in **Table 4** below. There were significance differences ($p<0.05$) among the biscuit samples in their sensory attributes of colour, flavor, taste, crispness and overall acceptance.

The colour score ranged from 4.90 - 7.50 with sample H (containing 70% wheat flour: 30% defatted toasted melon flour) having the lowest colour of (4.90) and sample I (100% wheat flour control) having the highest colour score (7.50) among the samples. The values obtained in the colours for the biscuit were above the poor rating when compared with the control sample. Colour is an important attribute in consumption of food and a significant parameter in judging well baked biscuits. It reflects the suitable raw material used for the preparation and also provides information about the formulation and quality of the product (Oyet and Chibor, 2020). There was a significant low score in colour observed for biscuits from blends with 30 % defatted raw and toasted melon seed flour substitution. This shows that at 30 % inclusion the biscuit was not acceptable by the panelist.

The flavour score of biscuits from defatted melon of raw and toasted melon flour and 100% wheat flour shows significant ($p<0.05$) difference. The flavor score ranged from 4.25 in sample containing sample D and 7.05 for sample I (100 % wheat flour). Increase in the percentage level of the flour reduced the flavor attribute. The 30 % inclusion of the defatted melon seed flour did not have a high acceptance in the flavor.

Table 4. Sensory score of wheat- defatted raw and toasted melon seed flour biscuits

Sample	colour	Flavour	Taste	Crispiness	General Acceptance
A	6.80 ± 1.01^{ab}	6.70 ± 1.56^{ab}	6.65 ± 2.13^{bc}	6.35 ± 1.69^{ab}	7.00 ± 1.38^{ab}
B	6.70 ± 1.63^{ab}	7.05 ± 1.23^{a}	7.25 ± 1.71^{a}	7.10 ± 1.48^{ab}	7.75 ± 1.41^{as}
C	6.50 ± 1.43^{abc}	5.65 ± 1.53^{bc}	6.20 ± 1.74^{abc}	6.60 ± 0.94^{ab}	6.60 ± 1.60^{ab}
D	5.45 ± 1.96^{cd}	4.25 ± 1.86^{d}	4.50 ± 2.04^{d}	5.10 ± 1.97^{c}	5.05 ± 2.09^{c}
E	6.95 ± 1.61^{ab}	6.10 ± 2.05^{abc}	6.85 ± 1.93^{ab}	7.25 ± 1.37^{a}	7.15 ± 1.63^{ab}
F	7.05 ± 1.47^{ab}	6.05 ± 2.04^{abc}	6.50 ± 1.99^{ab}	6.40 ± 1.73^{ab}	6.85 ± 1.50^{ab}
G	5.95 ± 1.64^{bcd}	5.55 ± 1.70^{bc}	5.55 ± 1.88^{bcd}	6.75 ± 1.71^{ab}	6.00 ± 1.56^{bc}
H	4.95 ± 2.24^{d}	4.90 ± 2.45^{cd}	4.95 ± 2.42^{cd}	6.05 ± 1.82^{bc}	5.30 ± 2.32^{c}
I	7.60 ± 1.47^{a}	6.80 ± 1.91^{bc}	7.00 ± 2.25^{a}	6.95 ± 1.64^{ab}	7.40 ± 1.47^{a}

Values are means ± SD of duplicate analysis. Means in the same column having same superscript are not significantly different ($p<0.05$).

Table 5. Amino acid composition of biscuits from wheat: defatted melon flour

	Samples							
Amino acids (g/100g)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Leucine	8.18±0.01^{c}	8.57±0.04^{d}	8.61±0.00^{d}	9.18±0.02^{e}	7.48±0.04^{a}	7.78±0.31^{b}	7.95±0.07^{b}	8.85±0.07^{d}
Lysine	3.45±0.01^{a}	3.94±0.01^{c}	3.95±0.03^{c}	4.33±0.04^{d}	3.67±0.04^{c}	3.67±0.04^{b}	3.96±0.06^{c}	4.78±0.04^{e}
Iso- leucine	3.09±0.01^{a}	3.19±0.01^{a}	3.09±0.01^{a}	3.47±0.01^{b}	3.93±0.04^{d}	3.56±0.06^{c}	4.16±0.34^{d}	4.08±0.03^{d}
Phenylanin	3.20±0.01^{a}	3.39±0.02^{b}	3.39±0.02^{b}	3.46±0.00^{b}	3.86±0.06^{d}	3.15±0.06^{a}	3.67±0.05^{c}	4.00±0.01^{e}
Tryptophane	0.80±0.01^{a}	0.90±0.01^{b}	0.88±0.01^{b}	0.98±0.04^{c}	0.99±0.02^{c}	0.87±0.04^{b}	1.03±0.01^{c}	1.21±0.01^{d}
Valine	3.21±0.02^{a}	3.70±0.14^{d}	3.53±0.03^{b}	3.81±0.01^{d}	3.68±0.04^{c}	3.08±0.01^{a}	3.60±0.01^{c}	3.97±0.03^{a}
Methonine	1.29±0.01^{a}	1.46±0.02^{b}	1.36±0.02^{a}	3.50±0.07^{g}	2.17±0.04^{e}	1.68±0.03^{c}	2.04±0.01^{d}	2.29±0.02^{e}
Arginine	4.23±0.03^{c}	4.44±0.05^{d}	4.42±0.04^{d}	3.12±0.03^{a}	4.85±0.07^{e}	4.08±0.03^{b}	4.77±0.05^{e}	5.02±0.04^{f}
Trytosine	2.91±0.01^{a}	2.92±0.02^{a}	3.35±0.35^{b}	3.20±0.14^{ab}	4.17±0.05^{c}	3.46±0.02^{b}	3.79±0.01^{c}	4.40±0.14^{d}
Histidine	1.89±0.01^{a}	2.07±0.03^{c}	2.00±0.00^{b}	2.17±0.04^{d}	2.16±0.02^{d}	1.91±0.01^{a}	2.27±0.05^{e}	2.38±0.03^{f}
Cysteine *NEAA	1.18±0.04^{a}	1.29±0.02^{bc}	1.23±0.03^{ab}	1.37±0.05^{d}	1.29±0.02^{bc}	1.21±0.01^{ab}	1.40±0.01^{d}	1.56±0.06^{e}
Alanine	3.23±0.04^{a}	3.50±0.01^{b}	3.30±0.00^{a}	3.97±0.04^{c}	3.65±0.07^{b}	3.21±0.02^{a}	3.86±0.04^{c}	4.27±0.18^{d}
Proline *NEAA	3.17±0.02^{b}	3.37±0.02^{c}	3.37±0.02^{c}	4.86±0.06^{e}	2.97±0.04^{a}	3.18±0.04^{b}	3.38±0.04^{c}	3.53±0.04^{d}
Methonine	1.29±0.01^{a}	1.46±0.02^{b}	1.36±0.02^{a}	3.50±0.07^{g}	2.17±0.04^{e}	1.68±0.03^{c}	2.04±0.01^{d}	2.29±0.02^{e}
Glutamic acid	12.80±0.01^{d}	12.25±0.08^{c}	12.07±0.04^{c}	13.31±0.19^{e}	10.48±0.04^{a}	10.50±0.14^{a}	11.39±0.26^{b}	11.43±0.10^{b}
Glycine	3.10±0.01^{a}	3.26±0.06^{b}	3.07±0.04^{a}	3.43±0.04^{c}	4.05±0.01^{f}	3.59±0.02^{d}	3.90±0.01^{e}	4.29±0.06^{g}
Threonine	2.58±0.04^{a}	2.96±0.06^{b}	2.94±0.05^{b}	3.03±0.01^{b}	3.72±0.40^{c}	3.19±0.02^{b}	3.68±0.11^{c}	3.78±0.04^{c}
Serine	3.58±0.04^{b}	3.65±0.35^{b}	3.26±0.06^{a}	3.79±0.01^{bc}	3.77±0.05^{bc}	3.56±0.06^{ab}	3.85±0.06^{bc}	4.02±0.03^{c}

Values are means ± SD of duplicate analysis. Means in the same column having same superscript are not significantly different ($p<0.05$).

Key. Sample A 95:10 % Wheat:Raw defatted melon seed; Sample B 90:10 Wheat: Raw defatted melon seed,; Sample C 80:20 % Wheat :Raw defatted melon seed, Sample D 70:30 % Wheat :Raw defatted melon seed, Sample E 95:10 % Wheat:toasted defatted melon seed; Sample F 90:10 Wheat: Toasted defatted melon seed,; Sample G 80:20 % Wheat :Toasted Defatted melon seed, Sample H 70:30 % Wheat :Raw defatted melon seed,

Flavor attributes is a pleasant desirable sensation by the panelist from every food produced. The flavor score of all the samples both the raw boiled and toasted samples showed a high score expect those with 30% inclusion of the flour.

The taste score shows significant ($p<0.05$) differences among the biscuits samples. Sample M (containing 100 % wheat flour) had the highest and most acceptable taste score of (7.25) and sample D (containing 30% raw defatted melon flour) had the least score of 4.50.

All the samples had a good taste rating score except for sample D (containing 30 % defatted raw melon flour) and sample L (containing 30 % defatted toasted melon flour) having 5.0 score rating. This shows that 30% inclusion of this flour affected the taste score of the products on preferential scaling but was not totally poor in acceptance. Taste is the primary factor that determines the acceptability of any product which has the highest impact as far as market success of product is concerned (Feyera, 2019).

3.3.Amino acid composition of biscuits

Table 5 shows the amino acid composition of biscuits from wheat:defatted raw and melon seed flour

There were significant ($p<0.05$) difference in the amino acid profile with biscuits sample. The leucine content ranged from (7.48 g/100g) in sample E to (9.18 g/100g) in sample D and was significantly ($p<0.05$) higher than other samples. Sample H (70:30 wheat-defatted toasted melon seed flour) had the highest lysine content (4.78 g/100g) while sample A (95:5 wheat-defatted raw melon flour) had the least Lysine value (3.45 g/100g). The high percentage of lysine recorded with sample from wheat blend with defatted toasted melon flour reveals that toasting treatment improved the lysine content when compared to the sample from defatted raw melon flour, and where significantly ($p<0.05$) increasing with increase in the defatted melon flour. Lysine is essential for children as its critical for bone formation, production of hormone, also helps to lower the serum triglyceride levels (Gersten, 2013). The value of lysine reported was relatively closer to the values of 3.35-5.07 g/100g reported by Oyet and Chibor (2020). The phenyl-lanine content of the composite biscuit showed an increase with substitution and ranges from 3.15 -4.00 g/100g. There was a significant ($P<0.05$) increase in the Arginine content and ranges from 3.12-5.02 g/100g, tyrosine (2.92 – 4.40 g/100g), Histidine content of the biscuits samples, 1.89- 2.38 g/100g). In this study, glutamic acid was observed to be the most abundant amino acid ranges from 10.48 to 12.80 g/100g. There was a significant ($p<0.05$) decrease in the glutamic acid of biscuits toasted, while there was observed increase with percentage increase of the raw defatted flour. The high value of glutamic acid observed shows that citrullus coloythnis seed is abundant, when compared with other amino acid value and this is in agreement with Ogunmodimu *et al*. (2015) that plant-base foods are most predominant in this amino acid. The effect of processing like toasting and defatting did not actually affect the amino acid profile of the biscuit but rather enhanced it.

4.Conclusion

The results vividly showed that it could be possible to produce nutritious and acceptable cookies through the substitution of wheat flour with defatted melon flour. The high protein, ash and fibre contents of cookies made from wheat: defatted raw and toasted melon seed flour as well as the acceptability of the composite cookies attested to this fact. The amino acid profile of the biscuits were improved and were significantly high in the essential amino acids. The processing of toasting did not actual affect the quality of the biscuits nutritionally. The results also showed that substitution from defatted raw and toasted melon seed flour did not alter the physical characteristics and consumer acceptability of the cookie samples especially at 5 – 20 % substitution level were preferred both in colour, Taste, flavor and general acceptabilty. Conclusively, the study actually reveals that producing a biscuits from the wheat defatted

melon seed could actually be adopted and incorporating at 5 – 20 % could help to substantially reduce foreign exchange on wheat importation and reduce wastage of the by-product, while improving the nutritional status of consumers.

5. References

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CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

EFFECT OF SUPPLEMENTING YOGURT WITH ESSENTIAL OIL OF LEMON LEAVES ON PHYSICOCHEMICAL AND RHEOLOGICAL PROPERTIES

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https://doi.org/10.34302/crpjfst/2024.16.2.16

Article history:
Received: January 1st, 2024
Accepted: June 8th, 2024

Keywords:
Lemon leaves;
Essential oil;
Elasticity;
Viscosity;
Rheology.

ABSTRACT

Citrus limon is a flowering plant belonging to the Rutaceae family. Citrus fruits constitute one of the main valuable sources of essential oil used in foods and medicinal purposes. This study was designed to investigate the effect of adding the essential oil of lemon leaves in yogurt on its rheological and physicochemical parameters in comparison with potassium sorbate. Five batches of yogurt were prepared with different concentrations of the essential oil (0, 1250, 2500, 3750 µg/ml), one batch was dedicated to potassium sorbate (0.1%). Essential oil of lemon leaves was analyzed by gas chromatography/mass spectrometry (GC/MS), yogurts formulated were analysed by the rheometer. The GC/MS analysis allowed the identification of 27 compounds accounted for total percentage of 99.76%. The two dominant compounds were Limonene (57.96%) and β-pinene (16.29%). The essential oil exhibited an excellent DPPH scavenging activity with a half maximal inhibitory concentration an (IC50) of 2,41± 0,04 µg/ml comparable to that of ascorbic acid with an IC50 of 5,87± 0,75 µg/ml. pH values of yogurts with essential oil were significantly the highest after those of yogurts with potassium sorbate ($p<0.05$), which improves the acidity stability of yogurts during storage.The thixotropic index recorded for yogurts with essential oil was the greatest (3789 Pa/s for the highest concentration), while that with potassium sorbate was the lowest (2280 Pa/s) compared with the control (3329 Pa/s). The viscoelastic behaviour of the control yogurt and the essential oil yogurts were almost the same, the addition of essential oil did affect significantly the thixotropic behaviour of yogurts ($p<0.05$).

1.Introduction

Yogurt, is one of the most consumed dairy products by all age groups, rich in calcium, proteins, lipids, vitamins and minerals, it confers various health benefits along with pleasant taste (Tamime and Robinson, 2007). It is often used for weight management, lactose intolerance, immunological effects, gastro-intestinal or respiratory tract diseases and dental health (Das et *al*., 2019).

The contamination of yogurt and other dairy products with undesirable microorganisms, especially yeasts and moulds, during shelf life is a serious problem (Tamime and Robinson, 1985; Plockova et al*.,* 1997) . Some methods are used in order to increase the shelf life of yogurt. These strategies include addition of gas, restriction of oxygen, cold storage, heat treatment and the use of antimicrobial agents (Karagül-Yüceer et *al*., 2001). Food preservatives are specific additives used to

prevent deterioration from enzymes, microorganisms, and exposure to oxygen. All chemical preservatives must be nontoxic and readily soluble, not impart off-flavors, exhibit antimicrobial properties over the pH range of the food, and be economical and practical **(**Mac Donald and Reitmeier, 2017). Sorbic acid and its potassium salt are commonly used as preservatives for food (Akbari-adergani et *al.,* 2013). In the code of federal regulation under the provision set forth by the FDA (US Food and Drug Administration) on potassium sorbate in 1999 is declared that if food additives are generally recognised as safe and declared on the label, they can be used in foods (JECFA, 1973) . Potassium sorbate prevents microbial growth and spoilage by inhibiting mould and yeast, slows changes in color, texture, and flavor **(**Mac Donald and Reitmeier, 2017).

During the last years, consumers are more aware and lean more and more towards a healthy and organic food, the trend in the market for functional foods or nutraceuticals has increased, particularly products that include in their formulation functional and natural food additives like essential oils, vitamins, minerals, carotenoids, flavonoids, anthocyanins, etc. (Torres-Giner et *al*., 2010). Essential oils are aromatic compounds found in great quantities in oil sacs or oil glands present at different depths in the fruit peel, mainly flavedo part and cuticles (Mahato et *al*., 2019).

Citrus are the most important crops in the world in terms of production according to the Food and Agricultural Organisation (FAO). The essential oil of lemon has previously been reported to possess antibacterial, antioxidant and fungicidal properties and is on the « Generally Recognized As Safe» list fully approved by the Food and Drug Administration FDA (2018) (Yazgan et *al*., 2019). Thus, it is applied in food industries as a preservative or flavoring agent (Sharma and Tripathi, 2008). After extracting the juice from citrus fruits, residues from different parts as the peels, leaves and flowers constitute a very important source of EO (Viuda-Martos et *al*., 2009).

Food rheology is the study of deformation and flow of food materials. Milk gels are visco elastic, thus yogurt's rheological properties can be characterized using both the viscous and elastic components. Visco-elastic indicates that the material has some of the elastic properties of an ideal solid and some of the flow properties of an ideal (viscous) liquid (Lee and Lucey, 2010).

According to the literature, there is no study on rheology of yogurt supplemented with lemon leaves essential oil. For this, the objective of this work is to formulate a naturally flavoured yogurt by essential oil of Algerian lemon leaves and investigate the effect of this supplementation on the acidity stability and the rheological behaviour of the yogurt in comparison with synthetic food preservative.

2. Materials and methods

2.1. Materials

In this study, we used whole milk powder (28% fat, protein 34.0%, carbohydrate 53.2%). The essential oil of lemon leaves was purchased from a local producer and supplier. Yogurt culture, *Lactobacillus delbrueckii subsp bulgaricus* and *Streptococcus thermophilus* obtained from Chr. Hansen Denmark was used as starter culture for yogurt preparation.

2.2. Yogurt preparation

The yogurt was prepared according to the method of Mihoubi et *al*. (2017). Aliquots of 13.7 g of whole milk powder and 12.5 g of sugar were dissolved into 100 mL of distilled water by stirring for 5min. After that, the reconstituted milk was heated to 95°C for 5min in a thermostatically controlled boiling water bath followed by cooling to the incubation temperature (42 to 43°C) and inoculated with 0.03g of yogurt culture (*Lactobacillus delbrueckii subsp bulgaricus* and *Streptococcus thermophilus*). After stirring, the essential oil of lemon leaves was added at different concentrations: (YEO1) 1250µg/ml, (YEO2) 2500µg/ml, (YEO3) 3750µg/ml. In order to be able to study the stability of acidity of yogurts with EO we have studied in parallel the effect of a synthetic preservative, for this, another trial of

yogurt with potassium sorbate at a rate of 0.1 g / 100 g (YPS) was prepared (Codex Alimentarius, 2019). YC corresponds to the sample control. The different yogurt samples were then incubated at 45°C until pH 4.6 (within 5h). After fermentation, yogurts were transferred to a refrigerator at 4 °C overnight to reduce post-acidification. After stabilization, each yogurt sample was stored for 0, 1,7, 14, 21 and 28 days at 4°C in a refrigerator to evaluate the physicochemical characteristics. Each batch of yogurt was prepared in triplicate.

2.3. Physicochemical analysis

2.3.1. Gas Chromatography - Mass Spectrometry Analysis of the essential oil

The chemical composition of the EOLL was investigated by a gas chromatograph (Hewlett–Packard Co, Model 6890) coupled with a mass spectrophotometer (Hewlett–Packard Co, Model 5973, Palo Alto, CA, USA), with HP-5Ms capillary column (30.0m×0.25mm×0.25 µm). The carrier gas, the Helium, was used at 1.2 ml/min flow rate. The initial temperature was 60 ºC for 8 minutes that subsequently elevated to 200 ºC at a rate of 2ºC/min. The chromatograph was equipped with a split/split less injector used in the split mode, and 2 µL of essential oil was always injected. The compounds of essential oils were identified on the basis of GC-MS retention times, their Kovats indices (calculated using a homologous series of C10-C22 n-alkanes injected at the same conditions), and mass spectra (authentic chemicals and NIST 05 spectral library collection).

2.3.2. Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical-scavenging activity of EOLL was measured using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method of Kirby and Schmidt with some modifications (Kirby and Schmidt, 1997). Briefly, 500 µl of sample solutions (various concentrations of the EOLL) were mixed with 1ml DPPH solution (4% (w/v) in methanol) (Sigma-Aldrich, Germany). The mixture was mixed and incubated at room temperature for 30 min in the dark. Scavenging capacity was read spectrophotometrically by monitoring the decrease of the absorbance at 517 nm. DPPH solution was served as negative control and ascorbic acid was used as a positive control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. DPPH radical scavenging activity (%), expressed as percentage inhibition of DPPH, was calculated according to the formula:

$$\mathbf{DPPH\ RSA}(\%) = [(\mathbf{A0} - \mathbf{At})/\mathbf{A0}] \times \mathbf{100} \quad (1)$$

Where:
RSA: radical scavenging activity
A0: the absorbance of the control reaction (blank) containing all reagents except the tested compound,
At: the absorbance value of the tested sample.
Extract concentration providing 50% inhibition (IC50) was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate, and the results are expressed as the mean ± standard deviation.

2.3.3. pH and Titratable Acidity

The pH values of yogurt samples were measured using a digital pH meter (Nick, 776, Jena, Germany). The titratable acidity of yogurts was determined according to IDF standard (IDF/ISO/AOAC). According to which, 10g of yogurt with a few drops of phenolphthalein were titrated with a solution of NaOH (0.1N). It is expressed in grams of lactic acid per 100 g of product and is defined by the following equation:

$$\mathbf{At} = \frac{\mathbf{Vx0.9}}{\mathbf{M}} \quad (2)$$

Where:
At: titratable acidity
V: volume (ml) of 0.1 M sodium hydroxide;
M: mass (g) of the sample;
0.9: conversion factor of the lactic acid.
All samples were measured in triplicate.

The results are expressed in degree Dornic (°D).

2.4. Rheological measurements

The rheology tests were carried out using a constrained rotary rheometer (AR2000, TA Instruments). It is fitted with a coaxial cylinder module (standard size DIN) with a cylindrical rotor: height of the immersion cylinder (42 mm), diameter of the external cylinder (14 mm), diameter of the internal cylinder (15 mm).

A fixed protocol, established during previous work on yogurt (Koskoy and Kilic, 2004), was applied for sample preparation.

Yogurts were stored before analysis in a refrigerator at 4°C for15 days (Cayot et *al*., 2003). In order to homogenize the content and eliminate the possible presence of serum on the surface, each pot of yogurt was gently mixed three times from bottom to top using a small spoon, while turning the pot from left to right. The temperature of the samples is adjusted to 10°C and is kept constant throughout the analysis. The yogurt sample (10 ml) is placed in the space between the inner cylinder and the outer cylinder. The samples remained in the system for 10 to 15 min until the temperature stabilized (10° C).

The rheological measurements are carried out by stress scanning and under harmonic conditions. A ramp of shear stresses between 0.1 Pa and 100Pa, with a frequency of 1 Hz was exerted. The stress distribution was logarithmic, with a step of 100.6 Pa (50 points). A controlled shear speed test was also performed to determine the curves and the type of flow of the four yogurts.

Thixotropic index refers to the area between the upward and downward shear stress curves. The Herschell- Bulkley model was used for fitting data (Karsheva et *al*., 2013):

$$\sigma=\sigma_0+k\dot{\gamma}^n \quad (3)$$

Where:

σ is the shear stress (Pa),

σ_0 is the yieldstress (Pa),

k is the consistency index (Pa.sn),

$\dot{\gamma}$ is the shear rate (s^{-1}), and n is the flow index (dimensionless).

The apparent viscosity, η, was described as the ratio of shear stress, σ, to shear rate, $\dot{\gamma}$.

The speed was varied linearly from 0 to 300 1/s for 5 min (increasing curve), then in the opposite direction from 300 to 0 1/s (decreasing curve) for 5 min. The results of the shear stress and viscosity are measured every 6 s. The viscosity in the flow curve corresponding to the shear rate of 50 1/s is taken as the apparent viscosity of the yogurt. This value is taken as the viscosity in the mouth: the value of the shear speed in the mouth is approximately 50 1/s (Bourne, 2002).

Dynamic oscillation tests were carried out following the evaluation of the flow behavior to characterize the viscoelastic properties of the yogurt.

The stress scans were increasing from 0.1 to 15 Pa to determine the linear viscoelastic range (LVR) of the yogurt samples at a constant frequency of 1 Hz (6.28 rad / s). Frequency sweeps of 0.05 to 100 Hz were then caused in the LVR with a constant shear stress of 1 Pa to obtain the storage module G', the loss module G" and the angle Delta δ (Delta degrees= Tanδ), which represents the ratio of G" to G'. When the material is more like a solid, the G' dominates and therefore Tanδ becomes <1.0. The analysis was repeated in triplicate.

2.5. Statistical analysis

The physiochemical and rheological properties were expressed as mean ± standard deviation (SD) of three replicates using SPSS (version 25.0, SPSS Inc.). Analysis of variance test and Duncan's multiple range tests were used to examine the significant difference. A *P-value* of less than 0.05 was considered as highly significant.

3.Results and discussions

3.1. Chemical composition of the essential oil

The components of EO are important, as their qualitative and quantitative composition determines the characteristics of the oils and subsequent effect on its biological activities.

The analysis by (GC /MS) of EOLL identified 27 compounds accounted for total percentage of 99.76%. The identified components, with their relative percentages and the retention time, are given in Table 1.

Table 1. Chemical composition of Lemon leaf EO analyzed by GC/MS.

N°	Rt[a] (min)	KI[b]	Components	%
1	10.35	939	α-Pinene	4.773
2	11.22	953	Camphene	0.020
3	13.19	994	β-Pinene	16.29
4	14.12	999	Mycrene	0.405
5	17.52	1038	Limonene	57.96
6	18.29	1051	β-Ocimene	0.030
7	18.97	1062	γ-terpinene	0.511
8	21.97	1075	Cis-sabinene hydrate	0.016
9	22.26	1088	Terpinolene	0.054
10	23.70	1103	Linalool	0.070
11	24.21	1184	Terpinen-4-ol	0.086
12	25.77	1162	Citronellal	1.027
13	31.85	1223	(Z)-carveol	0.036
14	32.10	1248	Cis Citral	0.152
15	34.22	1272	Neral	0.252
16	40.40	1362	Neryl acetate	0.478
17	41.64	1381	Geranyl acetate	0.232
18	43.42	1399	(E)-β-Farnesene	0.030
19	43.81	1410	(Z)-α-Bergamotene	0.291
20	46.15	1415	β-Caryophyllene	0.067
21	47.78	1481	(Z)-β-Farnesene	0.020
22	48.30	1490	Viridiforene	0.025
23	48.98	1498	(Z)-α-Bisabolene	0.024
24	49.35	1504	β-Bisabolene	0.375
25	53.68	1575	(−)-Caryophyllene oxide	0.175
26	55.95	1642	δ-Cadinene	0.019
27	75.29	1685	α-Bisabolol	0.032
			Monoterpenes	83.068
			Sesquiterpenes	2.156
			Aldehydes	7.463
			Alcohols	3.192
			Ester	3.710
			Others	0.175
			Total	99,764

[a]*Rt: retention time*

[b]*KI :Kovats Indices on HP-5MS Capillary Column in reference to C10-C22 n-alkanes injected in the same conditions*

The volatile compounds of EOLL are classified into 6 groups including monoterpenes, sesquiterpenes, alcohols, aldehydes, esters and others. Monoterpenes are the major components, accounting for 83.06% of the total oil. Limonene (57.96%) is the predominant component of monoterpenes, followed by β-pinene (16.29%), and α-pinene (4.77%). Aldehydes are the second group with Neral (6.25%) and citronellal (1.02%) as principal

components. In addition, two esters: neryl acetate (1.47 %) and geranyl acetate (2.23%) were found in EOLL. The sesquiterpenes (2.15%) included β-caryophyllene (1.36%), β-bisabolene (0.38%) and (Z)-α-bergamotene (0.29%). the only sesquiterpene oxide detected was caryophyllene oxide (0.17%). The main alcohol detected was linalool (3.07%). Previous studies conducted on *C. grandis* leaf EO; it has been reported that the main compounds of the EO were sabinene (5.2%), β-pinene (8.0%), limonene (35.9%), β-Ocimene (7.4%), linalool (22.1%), and citronellal (15.6%) (Rowshan and Najafan, 2013).

In this study, limonene (57.96%) represents the most important component of the EOLL, it has been reported that EOLL of various origin is characterized by high concentrations of limonene (Kirbaslar and Kirbaslar, 2004). In previous study, limonene represented (3.2–75.2%) and (1.5 to 63.4%) of the total components of leaf EO of 43 taxa of lemons and limes respectively (Lota et *al*., 2001) this component represents (62%) of the composition of EO of *Citrus aurantium L.* leaves **(**Gholivand and Piryaei, 2013) .Previous studies also reported that the various EOLL had different volatile compound compositions and the main volatile compounds of the EOLL were found including limonene, β-pinene, sabinene and β-ocimene (Lan-Chi et *al*., 2019). The concentration of citral (Neral (6.25%) and citronellal (1.02%)) is the most important factor in determining the commercial value of the EOLL. This component contributes significantly to the quality of lemon flavor and aroma (Gramshaw and Sharpe, 1980).

3.2. DPPH radical scavenging activity

The major process of food deterioration is initiated by auto-oxidation process of lipids (Tepe et *al*., 2005). The sensory and nutritional qualities of fat-based foods (dairy products and meat or fried foods) are lost during this process. The use of synthetic and natural antioxidants reduces the oxidative degradation of foods. For this reason, the importance of antioxidants for human health and food industry is evident**.** The toxicological studies performed in the past have shown that some synthetic antioxidant compounds were involved in liver damage and carcinogenesis (Gülcin et *al*., 2004). Therefore, the antioxidant activity of natural products is of increasing interest to researchers and consumers**.** For this reason, the scavenging activity of EOLL was estimated using the DPPH assay by comparing with the activity of the ascorbic acid as a known antioxidant. The Radical scavenging activity of EOLL is shown in Fig 1.

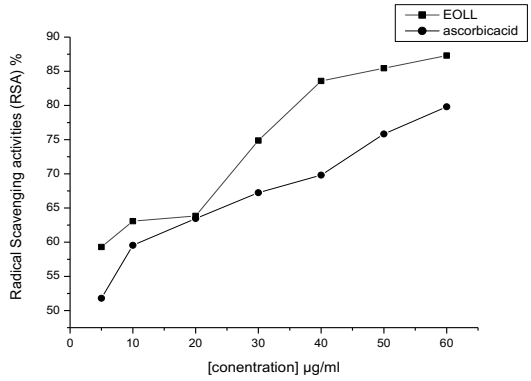


Figure 1. Scavenger effect of EOLL at different concentrations on the stable 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH)

The radical-scavenging activity (RSA) of the EOLL and positive controls ascorbic acid increased with increasing concentration. The EOLL showed 73.91% ± 11.82 inhibition of

DPPH while ascorbic acid (as positive control) exhibited 66.78% ± 9.56 activity, showing their significant difference ($p<0.05$).

EOLL exhibited an excellent DPPH scavenging ability with a IC50 of 2,41±0,04 µg/ml comparable to that of ascorbic acid with a IC50 of 5, 87± 0,75 µg/ml (Fig 2).

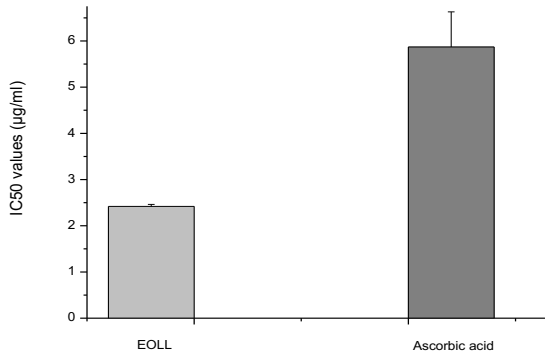


Figure 2. Antioxidant capacity (IC50) of EOLL by DPPH method

Table 2. Variation of pH and titratable acidity values of yogurts samples during storage at 4°C

Yogurt samples	Storage period (days)					
	0	**1**	**7**	**14**	**21**	**28**
pH						
YEO1	4.66±0.02^{b}	4.63±0.02^{b}	4.48±0.01^{b}	4.44±0.01^{b}	4.44±0.04^{a}	4.36±0.01^{a}
YEO2	4.64±0.02^{b}	4.59±0.02^{c}	4.43±0.02^{bc}	4.41±0.02^{bc}	4.35±0.02^{b}	4.32±0.02^{b}
YEO3	4.51±0.01^{d}	4.44±0.02^{d}	4.39±0.06^{c}	4.36±0.01^{cd}	4.31±0.01^{c}	4.21±0.02^{c}
YPS	5.13±0.01^{a}	5.07±0.02^{a}	4.67±0.01^{a}	4.57±0.02^{a}	4.48±0.01^{a}	4.35±0.02^{a}
YC	4.55±0.03^{c}	4.45±0.01^{d}	4.38±0.01^{c}	4.34±0.06^{d}	4.21±0.02^{d}	4.14±0.01^{d}
Titratrable acidity(D°)						
YEO1	90.00±1.00 ^{c}	90.33±0.57 ^{c}	94.00±1.00 ^{b}	96.00±1.00 ^{bc}	97.00±1.73 ^{d}	102.00±2.00 ^{c}
YEO2	91.33±0.57 ^{bc}	92.00±1.00 ^{b}	95.66±0.57 ^{b}	97.00±1.73 ^{b}	101.00±1.00^{c}	102.66±0.57 ^{c}
YEO3	92.66±0.57 ^{ab}	96.00±1.00 ^{a}	98.66±1.15 ^{a}	101.66±1.52 ^{a}	104.33±0.57^{b}	107.66±0.57 ^{b}
YPS	72.00±1.73 ^{d}	87.00±1.00 ^{d}	90.00±1.00 ^{c}	93.66±1.52 ^{c}	94.66±1.52 ^{e}	100.66±0.57 ^{c}
YC	93.33±0.57 ^{a}	95.33±0.57 ^{a}	100.33±1.52 ^{a}	100.66±0.57 ^{a}	108.00±1.00 ^{a}	112,00±1.00 ^{a}
ANOVA	**<0.001*** **	**<0.001*** **	**<0.001*** **	**<0.001*** **	**<0.001*** **	**<0.001*** **

The same superscript letter in each column shows no significant difference between values ($p < .05$). Data was provided as mean ± standard deviation (n=3).

YEO1= yogurt with1250µg/ml of essential oil; YEO2= yogurt with 2500µg/ml of essential oil; YEO3= yogurt with 3750µg/ml of essential oil; YPS= yogurt with 0.1 g / 100 g of potassium sorbate; YC= yogurt control with no additive.

This result is in agreement with those obtained by Frassinetti et *al*. (2011), demonstrating the scavenging abilities ranging from 20 to 70% of Citrus spp. EOs. The important antioxidant activity of the EOLL can be explained by the presence of monoterpenes, particularly limonene and γ-terpinene, which have been reported to have a good antioxidant activity (Conforti et *al*., 2007).The other components of the EOLL (caryophyllene, citral) are also responsible of the antioxidant properties **(**Dawidowicz and Olszowy, 2014). It is so difficult to explain the antioxidant activity pattern of EOs due to their complex mixtures constituted by several components. That is why; many reports on the antioxidant aptitude of the essential oils often refer to concepts such as synergism, antagonism and additivity **(**Ben Hsouna et *al*., 2017). In general, citrus leaf oil had higher antioxidant activity than peel (El-hawary et *al*., 2013).

3.3 Evolution of yogurts acidity during storage

According to the findings of the current research, acidity enhanced in the control, lemon EO and potassium sorbate yogurt samples during refrigerated storage over 28 days ($p<0.05$). On the other hand, pH decreased in all the samples within 28 days of storage, at the temperature of 4ºC ($p<0.05$) (Table 2).

The results below are in line with the findings of Wolfschoon (1983), Yeganehzad et *al*. (2007), Ahari et *al*. (2020) and Massoud and Sharifan (2020), they reported a decrease in pH of yogurt during refrigerated storage. For each day of storage, a significant difference was recorded for the pH of the five yogurt samples. The pH values of control YC yogurt were the lowest followed by those of yogurts with the essential oil YEO1, YEO2, YEO3, while the yogurt with potassium sorbate YPS, marked the highest pH during almost the 28 days of storage. This suggests a higher rate of organic acid production in the lemon leaves essential oil samples than those with potassium sorbate.

The reason the pH decreased was the acidity, which increased during the storage period following the conversion of lactose to lactic acid (Tamime and Robinson, 1985). During fermentation time, lactic acid production increases with the growth of the starter culture *S. thermophilus and L. bulgaricus.*

In this study, the change from an EO of lemon leaves concentration from 1250µg/ml to 3750µg/ml promoted the production of lactic acid by yogurt starter. Conflicting results have being reported by Massoud and Sharifan (2020), who observed a prevention of the growth of starter cultures and the production of lactic acid, by the *Rosmarinus Officinalis* essential oil.

According to table 2, the pH values obtained with essential oil yogurts at a concentration of 1250µg/ml are the closest to those obtained with potassium sorbate. However, the lemon aroma is not perceived at this concentration, for this reason a higher concentration of EO, which is 2500µg/ml, is necessary to flavour the yogurt and ensure a stability of acidity close to that of the synthetic preservative. From this, it can be assumed that the essential oil of lemon leaves slightly affects the development of yogurt cultures, unlike potassium sorbate, this synthetic preservative significantly reduced the acidity of yogurt throughout storage ($p<0.05$).

3.4. Rheological properties

3.4.1. Flow behaviour

The flow behaviors of the five yogurts produced are represented by the flow curves illustrated in fig 3. For all yogurts, the viscosity decreases as the shear rate is increasing. Massoud and Sharifan (2020) found the same viscosity trend in improved yogurt with *Rosmarinus officinalis* essential oil. Wang et *al*. (2020) have reported similar results in a study on stirred yogurt with apple pomace. The strength and the number of bonds between casein micelles in yogurt affect apparent viscosity, as well as their structure and spatial distribution (Lucey and Singh, 1998). According to Horne (1998), this can occur due to the physical destruction of the weak bonds between the molecules of the product and the decrease in the energy of interaction between them. This is the behaviour of a non-

Newtonianfluid. It appears that the addition of essential oil slightly increased the apparent viscosity of yogurts, in particular YEO3 in comparison with the control, however, at yogurt YPS the lowest apparent viscosities were recorded.

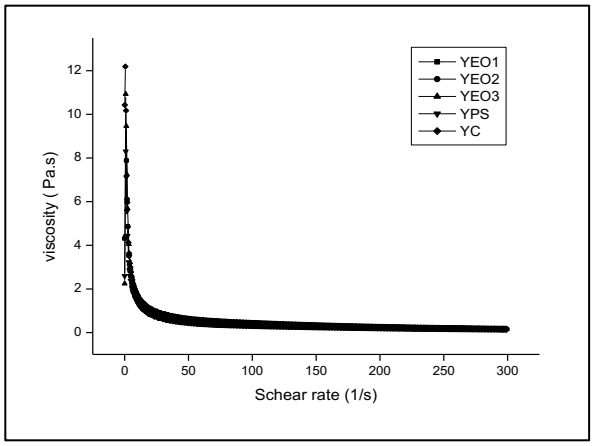


Figure 3. Flow curves of yogurt samples. Squares: YEO1, Circles: YEO2, up triangles: YEO3, down triangles: YPS, diamonds : YC. YEO1= yogurt with1250µg/ml of essential oil; YEO2= yogurt with 2500µg/ml of essential oil; YEO3= yogurt with 3750µg/ml of essential oil; YPS= yogurt with 0.1 g / 100 g of potassium sorbate; YC= yogurt control with no additive.

With the aim of objective characterization of the behaviour of the various yogurts, the experimental values were adjusted to the Herschel–Bulkley rheological model. This model is suitable for characterizing rheological behaviour in the pseudoplastic zone, for intervals of shear rate neither very high nor very low, and presented good determination coefficients ($R^2 \geq 0.99$).

$$\tau = K\gamma^n + \tau_0 \quad (4)$$

With:
τ: shear rate (Pa);
τ_0: initial shear rate (yield stress) (Pa);
K: consistency coefficient (Pa .s^n);
γ: shear rate (1/s);
n: flow index.

Table 3. Herschel Bulkley model parameters.

Yogurts	Apparent vicosity (Pa.s)	Thixotropy (Pa/s)	Yiel stress τ_0 (Pa)	Index (n)
YEO1	20.21 ^{d}	3088 ^{d}	-16.64 ^{a}	0.2134 ^{b}
YEO2	22.43 ^{c}	3245 ^{c}	-19.38 ^{c}	0.2008 ^{c}
YEO3	30.89 ^{a}	3789 ^{a}	-26.16 ^{d}	0.1688 ^{e}
YPS4	6.997 ^{e}	2280 ^{e}	-32.052 ^{e}	0.3374 ^{a}
YC	23.22 ^{b}	3329 ^{b}	-18.96 ^{b}	0.1933 ^{d}

The same superscript letter in each column shows no significant difference between values ($p < .05$). Data was provided as mean ± standard deviation (n=3).
YEO1= yogurt with1250µg/ml of essential oil; YEO2= yogurt with 2500µg/ml of essential oil; YEO3= yogurt with 3750µg/ml of essential oil; YPS= yogurt with 0.1 g / 100 g of potassium sorbate; YC= yogurt control with no additive.

Table 3 shows the parameters of the Herschel Bulkley model. This model is designed to characterize the behaviour of fluids with a fluency threshold. The model incorporates an independent term, τ_0, to the Ostwald de Waele model, which represents the value of the minimum force that must be applied to the sample in order for it to begin to flow.

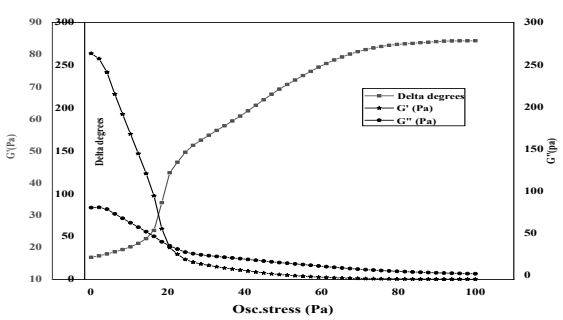
Since milk composition was kept constant in the present study, the differences observed were mainly due to change in the food preservative type. The addition of EO of lemon leaves in the yogurt significantly influences the flow parameters (table 3). All values of n (flow index), which measures the deviation degree from a Newtonian fluid, are less than 1, confirming the pseudoplastic behavior of yogurts, as already reported (Teles Et Flôres, 2007; Fischer et *al*., 2009).The addition of lemon leaves EO has significatly increased the apparent viscosity in yogurt YEO3 with (30.89 Pa.s), however the use of the synthetic preservative, potassium sorbate, considerably decreased the viscosity (YPS: 6.99 Pa.s) in comparison with the control YC: 23.22 Pa.s. These results are in disagreement with those obtained by Ahari et al. (2020), who found the lowest viscosity of yogurt with the highest content of cumin essential oil. Increasing the concentration of essential oil in yogurts significantly reduced the initial shear rate τ0 (yield stress) compared to the control. YPS, showed the lowest τ0, this indicates that a higher shear stress is needed to initiate the flow in this yogurt.

According to the table 3, all samples exhibited thixotropic characteristics, due to the differences in tension and viscosity between the ascending and descending curves. This phenomenon results from the breakage of the gel when a shear force is used (Bourne, 2002).

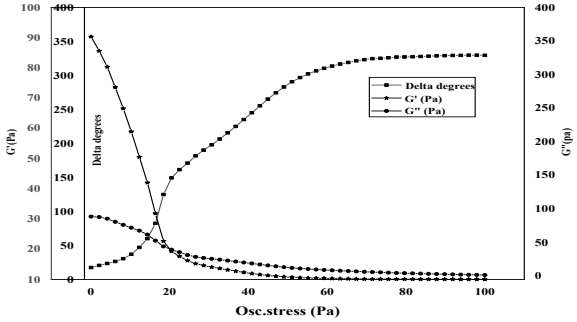
The thixotropic index recorded for YEO3 yogurt (3789 Pa/s) was the greatest, while that of YPS yogurt was the lowest (2280 Pa/s) compared with the control (3329 Pa/s). The addition of the EOLL or potassium sorbate, visibly affected the surface values of the hysteresis. These measures indicate that the yogurt YEO3 was more susceptible to structural breakdown under external force and less capacity to recover to its original structure. (Ahari et *al*., 2020) found a higher thixotropy in yogurt control compared to yogurt with cumin essential oil.

3.4.2. Viscoelastic properties of yogurts

The understanding of the rheology and the microstructure of food gels is an important tool for improving consumer satisfaction (Ahmed et *al*., 2017). The storage modulus, G′, represents the elastic behavior of the network and strength of the structure contributing to the 3D network. The loss modulus, G″, represents the viscous behavior of a sample and characterizes interactions not contributing to the 3D network (Tabilo-Munizaga and Barbosa-C´anovas, 2005). Therefore, if G′ is higher than G″, the solid-like properties are dominant over the liquid-like ones, as it is the case of gel-like materials.



YC



YEO1

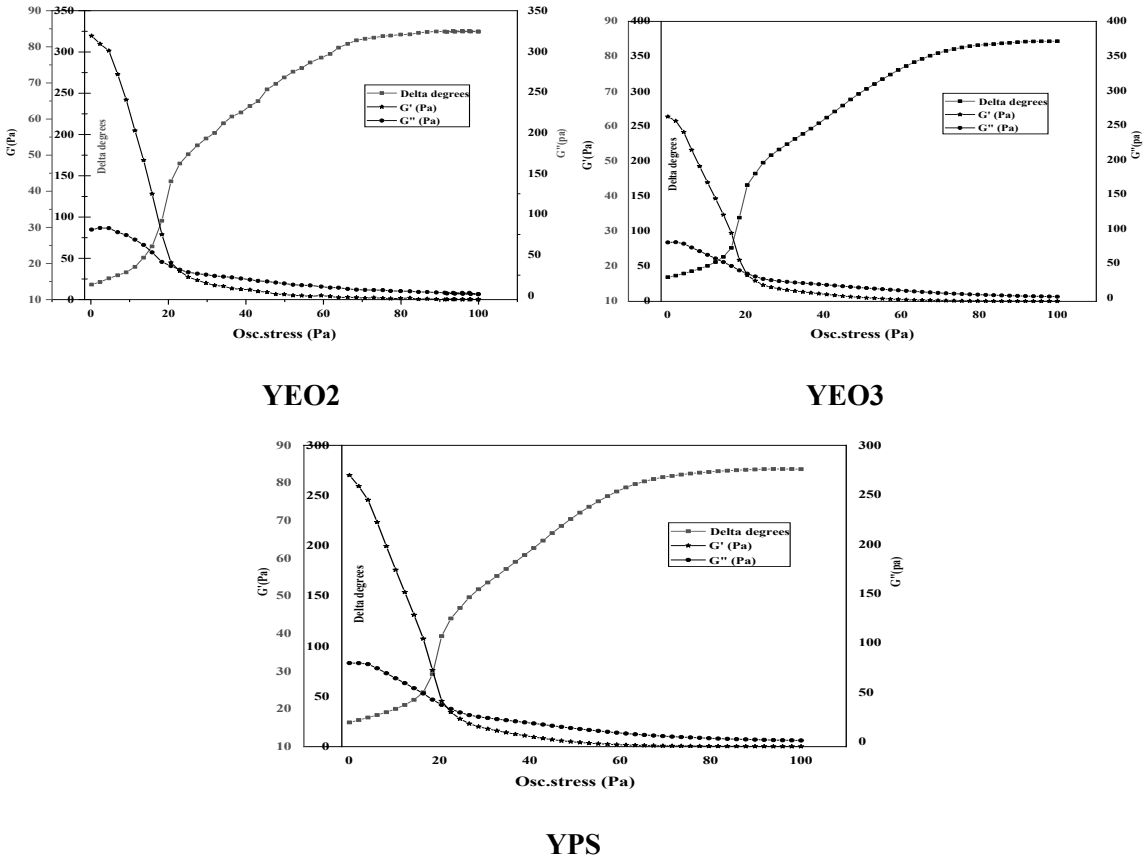


Figure 4. Viscoelasticity curves of yogurt samples. Squares: Delta degrees, Circles: G′, stars: G″.YEO1= yogurt with1250µg/ml of essential oil; YEO2= yogurt with 2500µg/ml of essential oil; YEO3= yogurt with 3750µg/ml of essential oil; YPS= yogurt with 0.1 g / 100 g of potassium sorbate; YC= yogurt control with no additive

As shown in Fig 4, storage modulus (G′) and loss modulus (G″) for all the yogurts samples are frequency dependent. All yogurts exhibit viscoelastic characteristics. Higher G′ and lower tan δ values were observed at oscillation stress, less or equal to 22.53 Pa, 24.56 Pa, for (YEO1, YEO2, YEO3, YC) and YPS respectively, indicating a predominant solid-like behavior of the yogurt gel. Nevertheless, at higher oscillation stress, a cross-over between these parameters occurred, with behavior shifting to a predominant liquid-like one, indicating breakdown of the yogurt structure. The viscoelastic behavior of the control yogurt and the essential oil yogurts were almost the same.

4.Conclusions

This study demonstrated the effect of adding the essential oil of lemon leaves to low-fat yogurt on its physicochemical and rheological properties. The highest pH and lowest acidity rates belonged to groups with the potassium sorbate followed by the essential oil at a rate of 1250µg/ml, a concentration of 2500µg/ml of EO is maintained to improve the stability of acidity of yogurts during storage and to benefit from the natural aroma of the essential oil. The addition of EO did not significantly affect the viscoelastic behaviour of low-fat yogurts; the maximum viscosity was obtained with a rate of 3250µg / ml of EO. The addition of EO did affect significantly the thixotropic behaviour of low-fat yogurts ($p<0.05$).

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POTENTIAL OF NISIN LOADED LIPID NANOPARTICLES ON INHIBITION OF *ENTEROBACTER CLOACAE* BIOFILM FORMATION

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https://doi.org/10.34302/crpjfst/2024.16.2.17

Article history:
Received: January 11th, 2024
Accepted: June 19th, 2024

Keywords:
Enterobacter cloacae;
Food borne pathogen;
Biofilm formation;
Nisin loaded liposomes;
Food grade nanoparticles;
Encapsulation efficiency.

ABSTRACT

The food borne pathogen *Enterobacter cloacae* contribute to food borne illness in humans. Biofilm formation in *Enterobacter cloacae* makes them more resistant to antibiotics. The main goal of the research is to prevent biofilm-forming *Enterobacter cloacae* by encapsulating nisin in liposomes using nanotechnology. The isolate was identified by 16S rRNA gene sequencing, and the biofilm-formed were characterized. Nisin was selected based on sensitivity testing. A microvesicle encapsulation method was used to encapsulate nisin in liposomes. Bacterial control was determined by colony forming units in an in vitro bioassay. Inhibition and eradication of *Enterobacter cloacae* was investigated using a microbial biofilm high-throughput antimicrobial susceptibility test using the Calgary biofilm apparatus. The food borne pathogen *Enterobacter cloaca was* isolated from the skin of grapes. After characterizing the biofilm formation on the isolate, the results showed that *Enterobacter cloacae* has the highest biofilm formation in tryptic broth (TSB) and brain heart infusion medium (BHI). In the antibiotic susceptibility test, the isolate is inhibited by antibiotics when presented in high concentrations. High-throughput analysis was performed using the Calgary biofilm apparatus, and the results showed that the nisin-loaded liposome exhibited good inhibition compared to antibiotics. One mM concentration of nisin (3.3 mg/10 mL) was used to encapsulate them in liposomes using a microvesicle encapsulation method. The results showed a tremendous inhibition of the food borne pathogen *Enterobacter cloacae* by the colony-forming units. This liposomal encapsulation of nisin promises high inhibition and can also be used for food safety.

1.Introduction

An *Enterobacter cloaca is* a food borne pathogen responsible for about 80% of all food borne illnesses. They tend to cause food borne illness by eating different foods (Shaker *et al.,* 2007). Despite the use of modern preservation techniques in food production, the number of food borne illnesses has increased in recent years. The use of chemicals such as preservatives to improve the shelf life and safety of food is a concern. However, the use of these chemical preservatives can harm human health (Anana *et al.,* 2020).

Bacterial biofilms fuse with a matrix of extracellular polymeric substances consisting of polysaccharides, proteins, lipids and extracellular DNA and adhere to the target surface (Zhong *et al.,* 2017). *Enterobacter cloacae* can form biofilms and is also more resistant to antibiotics. Because *Enterobacter cloacae* is able to form biofilms, they cannot be

prevented using low concentrations of antibiotics; therefore, inhibition is common with high doses of antibiotics (eg, 100 micrograms), which in turn can cause negative human health effects. Therefore, an alternative solution that can save us from the problem is nanotechnology.

Nanomaterials are specifically designed for incorporation into foods as nanoparticles for use as delivery systems or to modify optical, rheological or flow properties (Mc Clements & Xiao, 2017). Food-grade nanoparticles are used to encapsulate hydrophobic bioactive molecules such as phospholipids and lipids as an alternative to oral administration of vitamins, nutrients, and nutrients that can be used in the food industry.

Lactobacillus lactis produce an antimicrobial peptide Nisin, which consists of 34 amino acids and belongs to the lantibiotic family. They have been used as preservatives since ancient times and approved by the World Health Organization (Prombutara *et al.,* 2012). 'Nisin consists of unusual and distinct post-translationally modified amino acids: thioether-linked lanthionine and 3-methyllanthionine and unsaturated 2, 3-didehydroalanine and 2,3-didehydrobutyrine' (Breukink *et al.,* 1999). Nisin has several antimicrobial effects: it binds to peptidoglycan and lipid II precursors and inhibits cell wall biosynthesis. 'Nisin is widely used in foods such as cheeses, salads, canned soups, ice to preserve fish, baby food, shakes and baked goods' (Samelis *et al.,* 2005). Nisin can be used as a preservative because it is high in protein and non-toxic because the digestive system can convert protein into amino acids. Several dietary components such as 'glutathione, proteases, sodium metabisulfite and titanium dioxide' can damage nisin (Quintavalla & Vicini, 2002). Nisin, on the other hand, is typically incorporated directly into food systems as commercial products to avoid contamination with Listeria bacteria, an application in which activity declines over time as a result of enzyme degradation and interaction with food ingredients such as protein and lipids. (Jung *et al.,* 1992). Nanoparticulate systems can significantly enhance the nisin's controlled release and distribution. It seems that liposomes are appropriate vehicles for nisin's regulated movement and action within the cheese matrix. Nisin's distribution, stability, and availability within the cheese matrix are all enhanced by retention in liposomes. 'Both membrane-bound and encapsulated nisin, which can have both short-term (release of encapsulated nisin) and long-term (desorption of membrane-immobilized nisin) antibacterial activity, can be advantageously incorporated to this system' Benech *et al.* (2002). According to Benech *et al.* (2002), this technique can help reduce undesirable bacteria in foods that are kept for a long time, like cheese. Nisin can be encapsulated utilizing the microvesicle encapsulation method in liposomes, such as phosphatidyl and linolenic acids. The primary study's objective was to incorporate nisin into liposomes.

2. Materials and methods

2.1.Materials

2.1.1. Microbial Medium compositions

- Brain Heart Infusion Broth (g/L) **(**agar-15.0, brain extract -7.8, dextrose- 2.0, disodium phosphate- 2.5, heart extract- 9.7, peptone- 10.0, sodium chloride-5.0)
- Luria Bertani broth (g/L) (sodium chloride-0.5, tryptone-10, yeast extract- 5, agar-15)
- Tryptic Soy Broth (g/L) (Peptone from casein-17.0, Peptone from soymeal -3.0, D(+)-Glucose monohydrate- 2.5, Sodium chloride - 5.0 g/L, di-Potassium hydrogen phosphate - 2.5)
- Muller-Hinton agar (g/L) (agar- 17.0, beef infusion solids - 2.0, casein hydrolysate - 17.5, starch - 1.5)
- Congo red agar (g/L) (Brain heart infusion broth -37, sucrose -50, agar - 10, congo red stain -0.8)

2.2.Methods

2.2.1. Isolation and screening of Food borne Pathogen

Enterobacter cloacae, a food borne and hospital-acquired pathogen, was isolated from the skin of grapes. The isolate was identified as *Enterobacter cloacae* by 16s rRNA and the NCBI Genome Repository accession number is MK61597. Morphological and biochemical characterizations of the bacterial strain were

performed. Bacterial strains were grown at 37ºC and maintained on nutrient agar plates at 4 ºC.

2.2.2. Growth curve of food borne pathogen Enterobacter cloacae

The growth curve of the food borne pathogen *Enterobacter cloacae* was evaluated with a 1% stock culture of the foodborne pathogen. *Enterobacter cloacae* was inoculated into eight different growth media such as Brain Heart Infusion Broth, Brain Heart Infusion Modified Broth -1 and 25 g/L Protease Peptone, Brain Heart Infusion Modified Broth -2 and 4 g/L Protease Peptone, Feed Broth, Luria Bertani Broth, Tryptic Soy Broth, Tryptic Soy Broth Modified -1 with an additional 25 g/L tryptone, and Tryptic Soy Broth Modified - 2 with an additional 25 g/L Glucose and turbidity was analyzed every half hour with a spectrophotometer for six hours. The optical density values of the food pathogen *Enterobacter cloacae* cultured every 30 minutes were observed and graphed.

2.2.3 Antibiotic resistance test

The food borne pathogen *Enterobacter cloacae* was inoculated overnight in nutrient broth. Muller-Hinton agar plates were prepared and the food borne pathogen Enterobacter cloacae was swabbed with sterile cotton swabs. 20 different antibiotics were prepared at a high concentration (100 mcg/ml) and placed on swab plates. The plate was kept at 37°C overnight for incubation. The formation of groups indicated that they were sensitive, and the absence of groups indicated that they were resistant to that particular antibiotic.

2.2.4. Characterization of biofilm

2.2.4.1. Tube assay method

This method is mainly used to assess the biofilm formation in glass tubes. Cells were pre-grown in eight different growth mediums, and 1% of the culture was used to inoculate 10 ml of eight different growth mediums in a test tube. The tubes were incubated, and biofilm formation was monitored by crystal violet staining. This assay was carried out over 24- and 48-hour incubation periods.

2.2.4.2 Congo red agar (CRA) method

The food-borne pathogen *Enterobacter cloacae* was streaked on the CRA plate and incubated aerobically at 37ºC for 24 h. Production of black or red colonies with a dry, crystalline consistency resulted in a positive result (Panda *et al.,* 2016).

2.2.4.3. Microtiter plate (MTP) method

In this method, 96-well plates were used to evaluate biofilm formation and optimize the formation of biofilm by the food-borne pathogen *Enterobacter cloacae* in eight different growth media. 10µl of the overnight culture was taken and diluted with 990µl of the fresh sterile eight different growth mediums and incubated for 24, 48, 72, and 96 h. Following a one-hour incubation period, a 15-minute staining period with 1% crystal violet, and a rinse with distilled water to eliminate any remaining stain, the planktonic cells were eliminated using phosphate buffered saline (PBS). Optical density (OD) was measured in the ELISA reader plate at 650 nm after the wells were filled with 33% glacial acetic acid.

2.2.4.4. Biofilm formation in different substrates

Different substrates were chosen, including glass, polyvinyl pyrrolidone, plastic, steel, and wood. They were then cleaned with soap, rinsed with distilled water, and allowed to air dry. Following an overnight culture inoculation, the dry substrates were incubated for 1 – 3 days at 37˚C. Following the incubation period, sterile distilled water was used to wash the substrates after they were taken out of the broth culture. Following a 15-minute staining period with 1% crystal violet, the residual adhering bacteria were fixed using methanol. With the faucet running, the extra crystal violet was removed. Following resolubilization with 33% glacial acetic acid, the adhering bacteria were quantified at 570 nm versus optical density.

2.2.5. Screening for nisin that inhibit the growth of bacterial pathogen

The inhibition of the growth of the food-borne pathogen *Enterobacter cloacae* was carried out by the use of nisin. Muller-Hinton agar plates were prepared, and food-borne pathogens *Enterobacter cloacae* were swabbed with sterile cotton swabs. Nisin was introduced to the wells at varying amounts after the wells were perforated. The plates were stored at 37°C for an overnight incubation period. At various

concentrations, the zone of inhibition was seen.

2.2.6. Preparation of liposome encapsulated nisin

2.2.6.1 Nisin encapsulated linolenic acid

A stock solution was prepared by adding 12.5 mM alpha-linolenic acid to 27.5 mM NaOH. Nisin stock [3.3g of nisin (1 mM) in 0.02 N HCL]. Encapsulation of nisin was done by addition of 1.39 g of alpha-linolenic acid, which was dissolved in 0.25 mM chloroform in 50 ml of water. 0.5 ml of nisin was added to alpha-linolenic acid and stirred at 50 °C until the chloroform was completely evaporated. 5 ml of 27.5mM NaOH was added to the evaporated lipid, sonicated for 5 cycles (1 cycle = 10 sec), and kept in an ice bath for 3 min. The solution is filtered using a syringe filter and stored at room temperature.

2.2.6.2. Nisin encapsulated phosphotidyl choline

A stock solution was prepared by adding 76 mg phosphotidyl choline to 10 mM phosphate buffered saline. Nisin stock was prepared by dissolving 3.3g of nisin in 0.02 N HCL. Encapsulation of nisin was done by adding 76 mg of phosphotidyl choline, in 100 ml of ethanol, and 0.5 ml of nisin stock to the phosphotidyl choline stirred at 50°C until the ethanol completely evaporated. 5 ml of 10 mM phosphate buffer saline was added to the evaporated lipid, sonicated for 5 cycles (1 cycle = 10 sec), and kept in an ice bath for 3 min. The solution is filtered using a syringe filter and stored at room temperature.

2.2.6.3. Quantification of nisin using spectrophotometer

Nisin present in the encapsulated liposomes were quantified by protein estimation by Lowry's method and the OD was taken at 660nm and the graph was plotted.

2.2.6.4. DL% (Drug loading) and EE % (Encapsulation efficiency) of nisin

$$\textbf{Active Loading (DL \%)} = \frac{\text{Entrapped drug}}{\text{Nanoparticle weight}} \text{ X } 100 \quad (1)$$

Where entrapped drug is the weight of nisin-loaded nanoliposomes in Lowry's protein estimation and nanoparticle weight is the weight of nisin-loaded nanoliposomes.

$$\textbf{Encapsulation Efficiency(EE\%)} = \frac{C_{total} - C_{out}}{C_{total}} \textbf{ X 100} \quad (2)$$

Where C_{out} - liposome suspension Diluted with water and filtered and C_{total} - hot ethanol (70ºC) diluted liposome suspension to break up the liposomes and release the drugs contained in the solvent.

2.2.6.5 Calgary biofilm device method for testing of microbial biofilm

Step 1: Inoculation on modified MBEC Assay plate

Step 2: Setting up the antimicrobial challenge plate

Step 3: Neutralization and recovery

Step 4: Determination of MIC and MBEC

2.2.6.6 Susceptibility testing of nanoliposomes

The least dilution of the nanoliposomes that inhibit the organism was determined using the minimum inhibitory concentration. The *Enterobacter cloacae* from the overnight TSB modified-1 broth was added to each well in the 96-well plates at a concentration of 100µl. 50µl of the nisin-loaded nanoliposomes (linolenic acid and phosphatidylcholine) and fresh broth were added. The plate was incubated at 37 °C overnight. The OD recorded at 570 nm.

2.2.6.7 In vitro *liposome biological activity assay*

The bioavailability of the liposomes loaded with phosphotidyl choline and Linolenic acid was determined by CFU (Colony-Forming Units). The assays were performed after incubation with a 1% v/v suspension of food-borne pathogens (TSB modified 1) for 24 h (37 ºC) with nisin loaded liposomes, at 3% concentration. The planktonic *Enterobacter cloacae* were also assayed.

2.2.7 Dynamic Light Scattering Zeta-Potential Analysis (DLS-Zeta Potential)

DLS determines the size of the liposome and Polydispersity Index (PDI). DLS was performed using a standard Brookhaven Instruments Goniometer, BL-200M (Biomolecular

Biomolecular Analyzer, BI- 9000AT Digital Correlation). DLS was used to determine the Zeta potential after dilution (1 mM NaCl) of nisin (nisin) and nisin (encapsulated) liposomes (Zetasizer®, nano ZS (ZEN 3600).

3. Result and discussion

3.1. Identification of *Enterobacter cloacae*

The food-borne pathogen *Enterobacter cloacae* was isolated from the grape peel of the grape fruit and observed by streaking on nutrient agar plates. The isolate was characterized as negative for Gram's staining and positive for catalase, oxidase, Voges Proskauer, citrate utilization, and nitrate reduction.

3.2 Cell growth analysis of *Enterobacter cloacae*

Cell growth was investigated in eight different growth media as shown in Figure 1 (NB, LB, BHI, BHI-1, BHI-2, TSB, TSB-1, and TSB-2). *Enterobacter cloacae* were able to grow for different periods of time, ranging from half an hour to six and a half hours. Among the eight different growth media, BHI media showed good growth in the logarithmic phase.

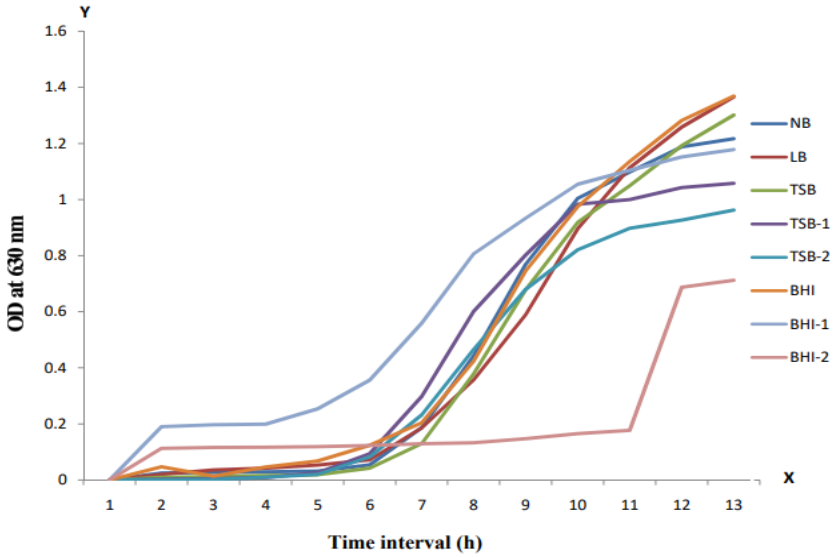


Figure 1. Analysis of growth curve of *Enterobacter cloacae* in different growth media (*BHI: Brain Heart Infusion broth, BHI-1: Brain Heart Infusion modified broth -1 BHI -2: Brain Heart Infusion modified broth- 2, NB: Nutrient broth, LB: Luria Bertani broth, TSB :Tryptic Soy broth, TSB-1: Tryptic Soy broth modified-1, TSB-2: Tryptic Soy Broth modified -2)* at a various period of time.

Figure 2 (A): 24 h

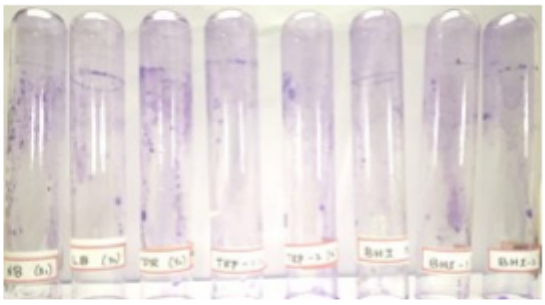


Figure 2(B): 48 h

Figure 2 (A): The violet color ring shows that the biofilm formation of food borne pathogen *Enterobacter cloacae* at a time period of 24 h.

Figure 2 (B): The violet color ring shows that the biofilm formation of food borne pathogen *Enterobacter cloacae* at a time period of 48 h.

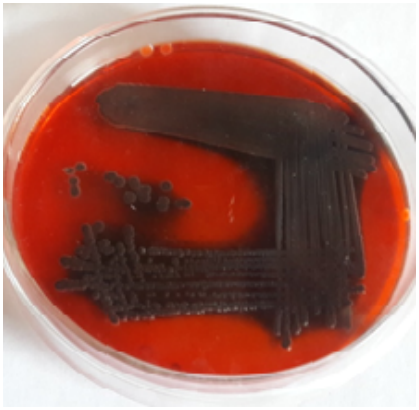


Figure 2(C): The Black Crystalline Colonies produced by *Enterobacter cloacae* showing strong biofilm formation streaked in Congo Red Agar Plate.

3.3. Antibiotic resistance of *Enterobacter cloacae*

The antibiotic resistance pattern of the foodborne pathogen *Enterobacter cloacae* was observed using the filter paper method. The absence of the zone shows that the food-borne pathogen *Enterobacter cloacae* was resistant to the antibiotics used, and the presence of the zone indicates that the same were sensitive to the antibiotics**.** When lower concentration antibiotics are provided, the isolate shows resistance to most of the antibiotics and is sensitive to Roxithyromycin and Azithromycin, whereas it is multidrug sensitive to all the antibiotics in the tests except for Erythromycin, Nystatin, and Bacteriocin when given at higher concentrations. *Enterobacter cloacae* are a multiresistant organism that shows resistance against most of the antibiotics when lower concentrations are provided. The spread of zone in antibiotics with the food-borne pathogen *Enterobacter cloacae* was earlier explained in a study (Mezzatesta *et al.,* 2012).

3.4 Characterization of biofilm formation of *Enterobacter cloacae*

3.4.1 Biofilm formation by tube assay method

The biofilm formation in the tube assay method was carried out for different time periods of 24 h and 48 h. 1% of the overnight culture was inoculated in the eight different growth media and incubated statically. After staining with the crystal violet stain and a half-hour incubation, they result in a violet color ring (Figure 2(A,B) around the tube after staining with crystal violet, which shows the attachment of biofilm formation on the foodborne pathogen *Enterobacter cloacae* at a time period of 24 h and 48 h. The more visible the ring around the walls of the glass, the more biofilm formation occurs in different media at different periods of time.

3.4.2 Biofilm formation by Congo red agar (CRA) method

The Congo Red Method (CRA) was carried out after an incubation period of 24 h. The overnight culture from the broth was streaked on BHI medium containing Congo red stain. After the incubation time, the organism showed black colonies on the streaked line on the Congo red agar plate (Figure 2(C)). Mirriam *et al.,* (2013) in their work demonstrated that *Enterobacter cloacae* readily forms biofilms on microtiter plates and the study also indicated the suitability fo BHI and TSB medium for the cultivation of the *Enterobacter cloacae* biofilm. In the study done by Kim *et al.* (2012), twenty two clinical isolates of *Enterobacteriaceae* were investigated for the biofilm forming ability by Congo Red staining and calcofluor staining methods and the highest biofilm forming ability was seen among the isolates of *Enterobacter cloacae* when compared to the isolates of *Enterobacter aerogenes.*

3.4.3 Biofilm formation by Microtiter plate method

The microtiter plate was carried out to evaluate the relationship between the biofilm

formation of *Enterobacter cloacae* in different media and at different time intervals, as shown in Table 1.

Table 1. Relationship between biofilm formations of *Enterobacter cloacae* in static microtiter plates in different media at different time intervals

Different Media	Time in Hours			
	24 hours	48 hours	72 hours	96 hours
NB	0.0050 ± 0.003 [b,c]	0.0095 ± 0.0035 [d,e]	0.0295 ± 0.0035 [b,d,f]	0.799 ± 0.0707 [c,e,f]
LB	0.0285 ± 0.0085	0.011 ± 0.0042 [d,e]	0.031 ± 0.0085 [d]	0.06 ± 0.0212 [e]
TSB	1.771 ±0.101 [a,b,c]	0.026 ± 0.0085 [a,d,e]	0.0715 ± 0.0078 [b,d,f]	1.151 ± 0.0707 [c,e,f]
TSB-1	0.188 ± 0.025 [a,b,c]	0.0265 ± 0.0078 [a,d,e]	0.0615 ± 0.0106 [b,d]	2.2075 ± 0.0912 [c,e]
TSB-2	0.199 ± 0.033 [a,b,c]	0.010 ± 0.0042 [a,d,e]	0.058 ± 0.0226 [b,d,f]	0.6545 ± 0.0742 [c,e,f]
BHI	0.108 ± 0.001 [a,b,c]	0.0065 ± 0.0078 [a,d,e]	0.048 ± 0.0156 [b,d,f]	0.169 ± 0.0071 [c,e,f]
BHI-1	0.0915 ± 0.0055 [a,b,c]	0.032 ± 0.0141 [a,e]	0.0305 ± 0.0092 [b,f]	0.5175 ± 0.0078 [c,e,f]
BHI-2	0.077 ± 0.011 [b,c]	0.0485 ± 0.012 [d,e]	0.387 ± 0.0071 [b,d,f]	0.829 ± 0.0566 [c,e,f]

The values are obtained by measuring the OD_{650} at four different time intervals and expressed as mean ± SD at significance $p<0.05$.

[a] the significant relationship between the formation of biofilm at 24 h and 48 h in different mediums used
[b] the significant relationship between the formation of biofilm at 24 h and 72 h in the different mediums used.
[c] the significant relationship between the formation of biofilm at 24 h and 96 h in the different mediums used.
[d] the significant relationship between the formation of biofilm at 48 h and 72 h in different mediums used
[e] the significant relationship between the formation of biofilm at 48 h and 96 h in different mediums used.
[F] the significant relationship between the formation of biofilm at 72 h and 96 h in the different mediums used.

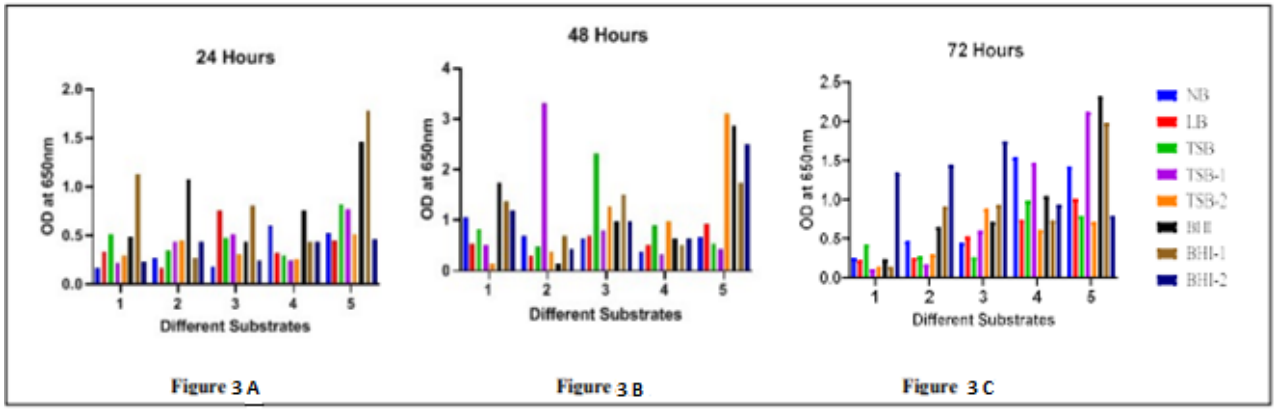


Figure 3 A Figure 3 B Figure 3 C

Figure 3(A): Biofilm formation of *Enterobacter cloacae* on different substrates in eight different media over a 24-hour period of time.
Figure 3(B): Biofilm formation of *Enterobacter cloacae* on different substrates in eight different media over a 48-hour period of time.
Figure 3(C): Biofilm formation of *Enterobacter cloacae* on different substrates in eight different media over a 72-hour period of time.

The cultures were loaded into a 96-well plate and incubated for1 to 4 days. Followed by the addition of crystal violet and glacial acetic acid. OD_{650} recorded to determine how the biofilm is adhered to the surface of the 96-well plates. *Enterobacter cloacae* form good biofilm formation and show a significant relationship between the time intervals in modified TSB-1 broth when compared to the other different growth media. A study conducted in 2013

demonstrated that *Enterobacter cloacae* readily forms biofilms on microtiter plates, in BHI and TSB medium. However, temperature and incubation time significantly affected biofilm formation by these bacteria (Nyenje *et al.,* 2013).

3.4.4. Biofilm formation and attachment on different substrates

After overnight incubation of the media along with the culture and substrates (glass, PVC, plastic, steel, and wood), are subjected to crystal violet staining. The biofilm attachment in the substrate is measured by taking an optical density value at 650nm. The substrate attachment was higher in wood at 24 h and 72 h and produced good attachment in plastic at 48 h (Figure 3 (A, B, C)**.** Mohana Priya and her colleagues reported that different substrates, such as glass, wood, teflon, steel, and plastic, showed biofilm attachment in *Myroides odorarimimus.* Among the five substrates, teflon and plastic were found to support high biofilm formation in food flavobacterium media (Mohana Priya *et al.,* 2018). Crystal violet staining at 650nm (A_{650}) on a 96-well plate reader at three different time intervals (1 to 4 days) determines the biofilm formation. The cell concentration of 6.57 x 10 cfu/ml was used. The mediums used were NB, LB, TSB, TSB-1, TSB-1, BHI, BHI-1, and BHI-2, and the substrates used were glass (1), PVC (2), plastic (3), steel (4), and wood (5).

3.5. Encapsulation efficiency of nisin-loaded liposomes

Screening of nisin, the zone of inhibition was observed at a high concentration (3.3 mg/10 ml). Nisin was encapsulated in the liposomes by the microencapsulation vesicle method. The encapsulation of nisin was evaluated by protein estimation. Nisin showed higher encapsulation efficiency and loading capacity at pH 7 in linolenic acid and pH 8.5 in phosphotidyl choline (Figures 4 (A) and 4 (B)). In a study conducted by Tomoko and Fumiyoshi in 2005, 'the encapsulation efficiency of various drugs with three kinds of egg yolk lecithin namely purified egg yolk lecithin (PEL), partially hydrogenated purified egg yolk lecithin (R-20) and completely hydrogenated purified egg yolk lecithin (R-5) with different iodine values were done. Among the three kinds of lecithin PEL tendered to show a higher encapsulation efficiency than R-20 and R-5'.Microencapsulation of nisin Z in lipid vesicle made of high melting point phospholipids was successfully achieved using liposome prepared with a high EE of 47% (Lardi *et al.,* 2003). This study indicated that 'the nisin loaded vesicles or nisin immobilized on the unlamellar membranes may provide a powerful tool for controlling spoilage and pathogenic organisms in food and can improve nisin stability, efficacy and distribution in food matrices'.

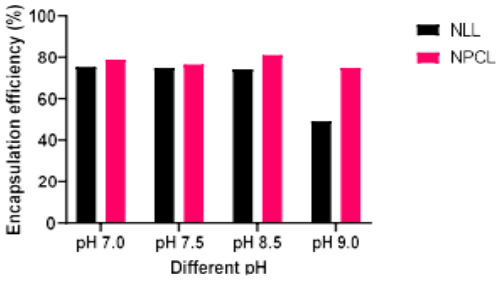


Figure 4 (A)

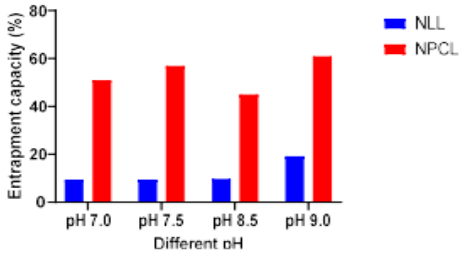


Figure 4 (B)

Figure 4(A): Encapsulation efficiency of the nisin loaded phosphatidyl choline and nisin loaded linolenic acid were analysed and the results showed higher encapsulation efficiency pH 7 in linolenic acid and pH 8.5 in phosphatidyl choline.

Figure 4(B): Loading capacity of the nisin loaded phosphatidyl choline and nisin loaded linolenic acid were analysed and the results showed higher loading capacity in pH 9 in linolenic acid and phosphatidyl choline.

3.6. Susceptibility testing of *Enterobacter cloacae* in nisin and nisin loaded liposomes

Minimal Inhibitory Concentration of *Enterobacter cloacae* for nisin and nisin loaded liposomes were done in 96 well plates as in table 2. Overnight culture was added to the well plate along with media and nisin, nisin loaded liposomes. The minimum inhibitory concentration was evaluated at an optical density of 650nm. The concentration ranging from 10µg/ ml to 0.017 µg/ ml are used. From this, 0.156 µg/ ml is enough for inhibition of *Enterobacter cloacae* nisin linolenic acid, 2.5 µg/ ml showed Minimal Inhibitory Concentration for nisin phosphotidyl choline liposome and 5 µg/ ml showed Minimal Inhibitory Concentration for nisin.

Table 2. Susceptibility test of *Enterobacter cloacae* for Nisin and Nisin liposomes.

Concentration (µg/ml)	Nisin	Nisin Phosphatidyl choline Liposome	Nisin linolenic acid Liposome
10	-	-	-
5	0.0545 ± 0.060	-	-
2.5	0.0695 ± 0.040	0.2315 ± 0.302	-
1.25	0.0885 ± 0.039	0.2660 ± 0.342	-
0.625	0.0955 ± 0.060	0.2990 ± 0.356	-
0.312	0.1200 ± 0.063	0.3205 ± 0.181	-
0.156	0.1425 ± 0.055	0.3475 ± 0.181	0.0795 ± 0.078
0.007	0.1695 ± 0.052	0.3745 ± 0.183	0.1010 ± 0.033
0.035	0.1985 ± 0.048	0.4745 ± 0.179	0.1410± 0.062
0.017	0.2815 ± 0.101	0.6520 ± 0.203	0.2240 ± 0.061

The values given are obtained by measuring the turbidity at 650nm $_{(A650)}$ on a 96-well plate reader and expressed as mean ± SD.

3.6.1. In vitro biological activity assay

The *in vitro* nisin and nisin liposome activity was tested by colony-forming units. The fresh broth was inoculated with inoculums, nisin, and nisin-loaded lipid nanoparticles.

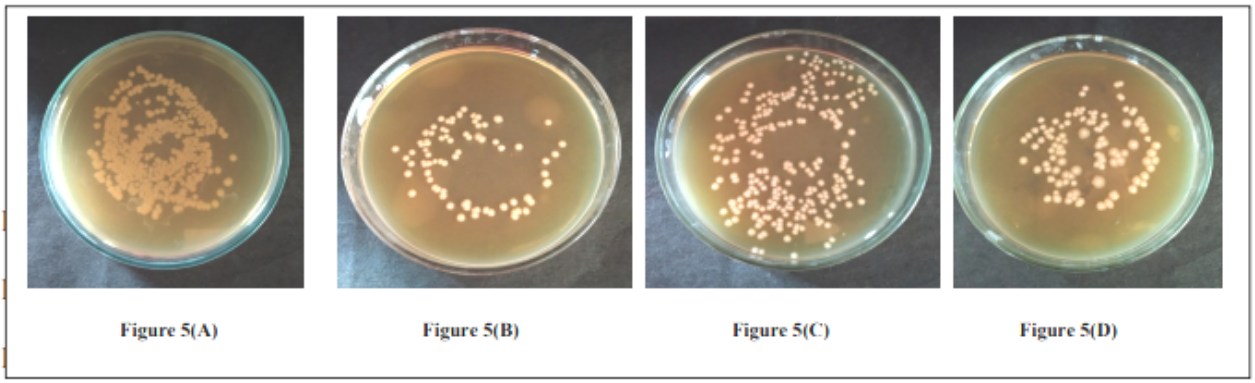


Figure 5(A): *Enterobacter cloacae* 6.57 x 10^8 CFU/ml
Figure 5(B): Nisin loaded linolenic acid 7.4 x 10^7 CFU/ml
Figure 5 (C): Nisin 1.54 x 10^8 CFU/ml
Figure 5(D): Nisin loaded phosphatidyl choline 1.02 x 10^8 CFU/ml

Enterobacter cloacae was kept as a control without adding any inhibitors for colony-forming units, which produced 6.54 x 10^8 CFU/ml (Figure 5(A)), and for liposomes added

at the same concentration of nisin, a nisin-loaded linolenic acid liposome, and a nisin-loaded phosphatidylcholine liposome. We observed 7.4 x 10^7 CFU/ml (Figure 5(B)) in nisin-loaded linolenic acid liposomes, 1.54 x 10^8 CFU/ml (Figure 5(C)) in nisin, and 1.02 x 10^8 CFU/ml (Figure 5(D)) in nisin-loaded phosphatidylcholine liposomes. It is observed that the numbers of colony-forming units were lower in liposome-loaded nisin compared to nisin and control. This shows the growth inhibition of liposomes. In a previous study, the biological activity and nisin demonstrated that nisin was entrapped in the nanoparticles in an active form, and hot homogenization did not substantially deteriorate the nisin activity (Prombutara *et al.*, 2012).

3.7. High-throughput assay of antibiotics, nisin, and nisin-loaded lipid nanoparticles

High-throughput assays were performed to evaluate the minimum inhibitory concentration and the minimum eradication biofilm concentration, as shown in Table 3.

Table 3. MIC and MBEC of antibiotics, Nisin, and Nisin liposomes when treated on a preformed biofilm of *Enterobacter cloacae*

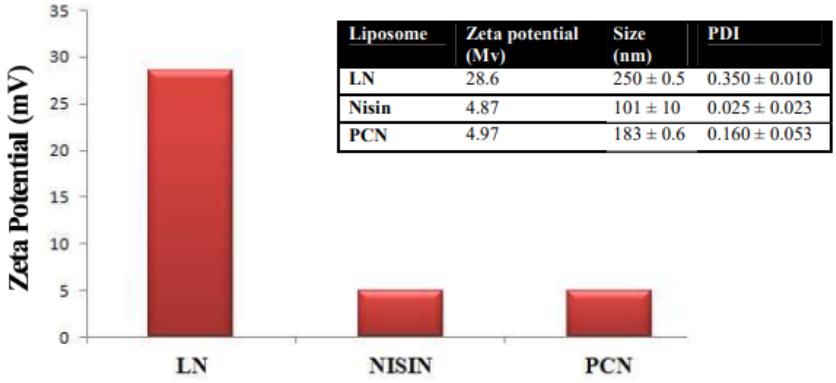
S.No	Antibiotics / Nisin / Liposomes	MIC in µg/ml	MBEC in µg/ml
1.	CM	>25	>100
2.	NM	>0.78	>12.5
3.	TET	>0.39	>100
4.	STR	No inhibition	>25
5.	AMP	>0.39	>100
6.	KAN	>100	>25
7.	ERY	>0.39	>6.25
8.	CAZ	No inhibition	>100
9.	NYS	>0.39	>100
10.	CHL	>0.39	>100
11.	CRO	>0.39	>100
12.	VAN	>3.125	>0.195
13.	BAC	>6.25	>100
14.	AMX	>0.195	>25
15.	CIP	No inhibition	>0.195
16.	CAR	>6.25	>50
17.	P	>0.39	>6.25
18.	CEF	No inhibition	>1.56
19.	CPH	>0.39	>3.125
20.	HYG	No inhibition	>100
21.	NISIN	>5	>0.625
22.	Nisin linolenic acid Liposome	>0.312	>0.035
23.	Nisin Phosphatidylcholine Liposome	>2.5	>0.017

The minimum inhibitory concentration of different antibiotics to inhibit the foodborne pathogen *Enterobacter cloacae* at a concentration of 25 g/ml is enough. But for the eradication of the foodborne pathogen *Enterobacter cloacae,* a higher concentration of 100 µg/ ml is needed. For nisin encapsulated linolenic liposomes, the Minimal Inhibitory Concentration is greater than 0.312 µg/ ml and the eradication, the minimal concentration is greater than 0.035 µg/ ml. For nisin encapsulated phosphotidyl choline liposomes, the Minimal Inhibitory Concentration is greater than 2.5 µg/ ml and the eradication, the minimal concentration is greater than 0.017 µg/ ml. Similar descriptive studies in other Gram negative shows indulgences of proton motive force in membranes of *E. coli* when AgNPs are exposed in various concentrations (Lok *et al.,*2007).

Minimal Inhibitory Concentration and Minimal Biofilm Eradication Concentration were evaluated for 21 different antibiotics and nisin-loaded liposomes using the Calgary device, and the minimum inhibition concentration and minimal biofilm eradication concentration were noted. Yamakami *et al.,* (2014) stated that 'acyl chain length of phosphatidyl choline mediates the sustained bactericidal activity of encapsulated nisin. Liposomal nisin composed of distearoyl phoshatidylcholine (DSPC) exerted the highest bactericidal activity as compared to those containing dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoyl phoshatidylcholine (DSPC)'. The results might suggest that DSPC-based liposomes are capable of preserving nisin's bioactivity.

3.8. Zeta Potential of Nisin and Nisin-Loaded Lipid Nanoparticles

The zeta potential of nisin-loaded linolenic acid liposomes (LN), nisin, and nisin-loaded phosphatidyl choline liposomes (PCN) is represented in the Zeta potential analysis (Figure 6).



Liposome	Zeta potential (Mv)	Size (nm)	PDI
LN	28.6	250 ± 0.5	0.350 ± 0.010
Nisin	4.87	101 ± 10	0.025 ± 0.023
PCN	4.97	183 ± 0.6	0.160 ± 0.053

Figure 6. Graph representing the zeta potential of nisin loaded linolenic acid liposome (LN), nisin and nisin loaded phosphatidyl choline liposomes (PCN). Nisin loaded phosphatidyl choline showed an effective zeta potential value when compared to nisin and nisin loaded linolenic acid liposomes. Polydispersity index (PDI).

Nisin-loaded phosphatidyl choline showed `effective zeta potential value when compared to nisin and nisin-loaded linolenic acid liposomes. The zeta potential value of nisin is 4.87 Mv, nisin-loaded linolenic lipid nanoparticles are 28.6 Mv, and nisin-loaded phosphatidylcholine lipid nanoparticles are 4.97 Mv. When compared to nisin-loaded linolenic lipid nanoparticles, nisin-loaded phosphatidyl choline lipid nanoparticles show good and efficient zeta potential values. Previous studies reported that 'the incorporation of freshly prepared nisin into solid lipid nanoparticles (SLNs) resulted in a slight increase in the average particle size with a slight decrease in zeta potential. Whereas after 28 days of storage at 30°C, nisin-loaded solid lipid nanoparticles had significantly increased their average sizes with an overall decrease in

zeta potential' (Prombutara *et al.,* 2012).

Finally, to overcome the hindrances associated with nisin as a food preservative, the use of nanotechnology for the synthesis of nisin-loaded or coated nanoparticles has been introduced (Silva, 2010). Nisin inhibits food-borne pathogens and the prevention of spore germination. For nisin's antimicrobial activity adsorption of nisin molecules onto the bacteria's surface and disruption of the membrane is very important. Nisin efficacy in food could be improved through incorporation or encapsulation in natural or synthetic polymers (Silva, 2010).

Adsorption of bacteriocins to various surfaces with retention of activity may be successfully achieved (Bower *et al.,* 1995a; Bower *et al.,* 1995b; Daeschel *et al.,* 1992; Ming *et al.,* 1997). Membrane-immobilised nisin or surfaces could provide several advantages as a nisin delivery system, such as reducing the amount of nisin that would be used and improving its stability (Cutter & Siragusa, 1997; Siragusa & Cutter, 1999). Nisin adsorbed to polyethylene used for meat packaging has been shown to be more stable and active against gram-positive pathogens and the food spoilage organisms *L. monocytogenes* and *Brochothrix thermosphacta* than nisin applied directly in a free form (Bower *et al.,* 1995a; Bower *et al.,* 1995b; Cutter & Siragusa, 1996; Cutter & Siragusa, 1997). Previous studies have shown that nisin adsorbed to lipid membranes can retain its antimicrobial activity and may have potential for use as a food-grade antimicrobial agent (Bower *et al.,* 1995a; Bower *et al.,* 1995b; Cutter & Siragusa, 1996; Scannel *et al.,* 2000). The desorption of nisin from lipid membranes occurs on contact with bacterial cells (Daeschel *et al.,* 1992).

It appears that liposomes are the appropriate carriers for the controlled delivery and action of nisin in the chevre matrix. The binding of nisin to liposomes enhances the stability, availability and distribution of nisin within the chevre. The presence of encapsulated as well as membrane-related nisin may provide an additive effect to this system, providing short-term antibacterial action (release of encapsulate nisin) as well as long-term antibacterial activity (desorption of nisin membrane-imobilised). This technique could improve the management of undesirable bacteria in goods (like cheese) that are kept in storage for long periods of time.

According to Daghastanli *et al.,* (2004) with regard to 'the efficiency of liposomal nisin, the selection of the acyl chain length of phospholipids is quite evident from our findings'. It has been postulated that liposome stabilisation is increased with long acyl groups, which explains why DSPC-nisin releases nisin more slowly than DPPC-nisin or DMPC-nisin (Daghastanli *et al.,* 2004). The current review shows that by varying the lipid arrangement, nisin-discharge profiles from the liposomes can be made depending upon the length of the acyl chain of phospholipids. The revelations appear that the hydrophobic collaborations between determinedly charged nisin and the acyl chain of phospholipids activate supported bactericidal development.

4. Conclusions

The food-borne pathogen *Enterobacter cloacae* Biofilm activity was observed for the samples nisin, nisin-loaded linolenic acid liposomes, and nisin-loaded phosphatidylcholine liposomes against control *Enterobacter cloacae*. The results showed that the sample in nisin-loaded liposomes had more inhibition against the control Enterobacter cloacae. So therefore, the study concluded that nisin-loaded nanoliposomes had better biofilm inhibition activity in a high-throughput assay.

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