CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

Vol. 15(4)
2023

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. 15, Nr.(4) 2023

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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POSSIBILITIES FOR PARTIAL REPLACEMENT OF PORK MEAT IN COOKED SAUSAGES BY MEALWORM FLOUR

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https://doi.org/10.34302/crpjfst/2023.15.4.1

Article history:
Complete by editor

Keywords:
Alternative proteins;
Meat products;
Texture;
Quality;
Meat replacement.

ABSTRACT

Dried and grinded "mealworms" or the larvae of *Tenebrio molitor* in form of flour contain up to two thirds of protein and fat. Therefore, the objective of this study was to investigate the possibility for partial replacement of pork meat by mealworm flour expressed through the changes in color, texture, microbiological status and morphology of cooked sausages. The experimental cooked sausages were prepared by substituting 1.5% (CM1), 2% (CM2) and 3% (CM3) pork meat with mealworm flour. It was found that up to 3% substitution of pork meat with mealworm flour (CM3) decreases the free water in the cooked sausages. The lack of difference in a_W after replacement of pork meat with mealworm flour does not compromise the shelf life and safety of the produced cooked sausages. As the amount of added mealworm flour increases the structural strength, plastic strength and springiness of experimental cooked sausages decreases. The substitution of 1.5% pork meat with mealworm flour (CM1) was the most appropriate for sausage processing without negative color changes. The appropriate pH of mealworm flour, together with the formed stable emulsions and good water binding capacity both before and after cooking, confirmed the potential of suitable meat substitute in meat industry. The microbial status of the experimental cooked sausages (CM1, CM2 and CM3) increased during storage, but still in the regulated limits for cooked meat products.

1.Introduction

In recent years, demand for animal protein has increased due to the exponential growth of the human population and changes in consumption habits brought about by the globalization of the market. These factors led to the development of industrial production systems, resulting in negative environmental effects (FAO, 2013). Many researches have been conducted to explore the feasibility of using non-meat ingredients as proteins of plant origin or milk proteins to promote a healthier meat sausage product, emphasizing the physicochemical properties and sensory characteristics in relation to the addition of new ingredients and substitution of animal fats (Syuhairah *et al.*, 2016).

Insects, as an alternative source of proteins for the food and feed industry, have a number of advantages over raw materials of plant origin (Cruz-López *et al.*, 2022). The consumption of water and energy for the cultivation of insects is minimal. Other positive environmental aspect is the opportunity to feed the insects with organic waste (Vlahova-Vangelova *et al.*, 2022). According to data from the Food and Agriculture Organization, agricultural production annually generates 27% waste (FAO, 2013) worth 750 billion dollars (The Economist, 2014), which is not utilized at the moment. In the light of circular economy development strategy this saved energy and water resources, would be used in the cultivation of cereals (Van Huis *et al.*, 2013). In addition, insects contain

high quality protein and essential amino acids and are a good source of fatty acids and minerals (Talens *et al.,* 2022).

Edible insect proteins have the potential to become meat replacers in cooked sausage processing due to their appropriate technological characteristics, such as pH and emulsion capacity (Kim *et al.,* 2016). Even more, the insect flours are rich in microelements and unsaturated fatty acids and can be used as an additional nutritional component with potential health benefits (Van Huis *et al.,* 2013). Due to their rearing some authors suggest they have a higher microbial load and potentially compromise food safety (Vandeweyer *et al.,* 2017).

The term "mealworm" refers to the larvae of *Tenebrio molitor*, Family *Tenebrionidae* (dark beetles). According to EFSA, the larvae of Tenebrio *molitor* larvae are extremely rich in protein, fat and fiber and they can be used raw, dried and ground in the form of flour. Dried yellow mealworms have excellent potential as an alternative to plant sources in feed. Their protein content varies between 41 and 66%, and at the same time is a source of high-quality protein (Turck *et al.,* 2021).

The overall objective of this study was to investigate the possibility for partial replacement of pork meat by 1.5%, 2% and 3% mealworm flour (MF) expressed through the changes in color, texture and microbiological status of in cooked sausages.

2. Materials and methods

2.1. Materials

The sausages were prepared using chilled pork shoulder (pH 6.10) and chilled pork bacon (pH 6.00) purchased from the local market (48 h *post mortem*).

The mealworm flour was obtained from live larvae of *T. Molitor* (70 days hatched), provided by the manufacturer "VI BI EF PRO" LTD (Petrich, Bulgaria). After 48 h fasting, live larvae were conventionally frozen (chilled at -20°C, QUIGG, UFT-Plovdiv, Bulgaria) for 72 h, then blanched in boiling water for 45 s, drained out, dried using conventional drying rack (60°C, 6 h, LUMEL) and finally grounded in a blender. The Proximate composition of mealworm flour is presented in Table 1. Mealworm flour pH was pH - 6.90 and color parameter: L* - 49.78, a* - 5.24 and b* - 8.44.

Table 1. Proximate composition of mealworm flour (*T.Molitor* larvae)

Parameters	
Proteins (%)	62.46±1.32
Fats (%)	14.04±0.67
Ash content (%)	3.00±0.20
Carbohydrates (%)	19.48±0.75
Moisture (%)	1.03±0.06

2.2. Sausages processing

The refrigerated (0-4°C, 48 h post mortem) pork meat was cut on pieces to approximately 50 g, and mixed with nitrite salt, phosphates and ice in cutter machine with a final temperature of batter 12-14 °C (Table 2).

Table 2. Formulations of the cooked sausages

Ingredients, %	**Samples**			
	C	**CM1**	**CM2**	**CM3**
Pork shoulder	60.00	59.25	59.00	58.05
Pork bacon	40.00	39.25	39.00	38.50
Mealworm flour (MF)	0.00	1.50	2.00	3.00
Ice	10.00	10.00	10.00	10.0
Sodium tripolyphosphate	0.20	0.20	0.20	0.20
Nitrite salt (0.55% $NaNO_2$)	2.00	2.00	2.00	2.00

The filling mass (batter) was separated of four equal portions (3 kg each). The experimental sausages were prepared by substituting 1.5% (CM1), 2% (CM2), 3% (CM3) meat with mealworm flour (MF). The filling mass (batter) was filled in gas and

moisture impermeable polyamide casings and the sausages (appr. 250 g each) were cooked at 86°C until core temperature of 72°C was reached. After cooking, the sausages were cooled in an ice bath and stored in refrigerator (0-4°C). For the part of analyses a batter was used. The batter measurements were carried out up to 2 h after obtaining.

2.3. Analysis methods

2.3.1. Instrumental color determination

Konica Minolta colorimeter CR-410 (Konica Minolta Holding, Inc., Ewing, New Jersey, USA) was used to evaluate the lightness (L* value), the redness (a* value) and the yellowness (b* value) of the color (Smarzyński *et al.,* 2019).

2.3.2. pH measurement

The pH value of the samples was measured electro-potentiometrically with a portable Meat pH meter HI99163 (Hanna instruments Inc., 270 George Washington Hwy Smithfield) (Pereira *et al.,* 2011).

2.3.3. Free water

The free water of the batters and cooked sausages was determined by drying at 105°C (Vandeweyer *et al.,* 2017).

2.3.4. Free water

Water activity of the cooked sausages was measured using a Novasina AG CH-8853 (Zurich, Switzerland) (Vandeweyer *et al.,* 2017).

2.3.5. Texture profile analysis (TPA)

The texture profile (TPA) was conducted by the method, described by Bourne (2002) with certain modifications (Vassilev *et al.,* 2012). For evaluation of the texture profile the following parameters were measured: Plastic strength - the force of resistance of the body on which it is applied by a sharp tip; Structural strength - the resistance of the body to the deformation force applied by a rounded tip and Springiness - the capacity of the body to recover its initial form after the deformation force is removed. An OB - 05 penetrometer (Labor, Hungary) was used to conduct all the measurements. Before carrying out of the analysis the samples were left for 20 min at room temperature.

2.3.6. Microbiological status

The preparation and decimal dilutions of suspension were done according ISO 6887-2:2017. The microbiological status was presented by the Total viable count (TVC), Coliforms and Yeasts and Molds count (ISO4833-1:2013/ Amd 1:2022; ISO 4833-2:2013/ Amd 1:2022).

2.3.7. Light microscopy

The structure of cooked sausages cuts (2x2x1 cm) was prefixed using 10% formalin solution. A gradually increasing ethanol solutions (from 50% to absolute) were used for dehydration. After bleaching with acetone samples were transferred in xylene for one night and then embedded in paraffin. For the staining of the 5 μm thick slices was used haematoxylin-eosin (Barbut *et al.* 2005). The capturing of the images was done at x100 magnification using a light microscope (Olimpus BX41TF, Japan) equipped with digital camera (Olimpus SC30, Japan).

2.3.8. Data analysis

All results are expressed as mean ± standard error of means (SEM), with $n \geq 5$ for all analyses. The results are processed by methods of variation statistics with various software packages Microsoft Office 365 (SAS Institute Inc, 2018). The comparison in the values of the parameters is done by t-test at Student and ANOVA for all samples from one side and inside in the levels of the factors for statistically significant differences at the level of confidence $\alpha = 0.05$ ($p \leq 0.05$).

3.Results and discussions

3.1. Technological characteristics

The cross-cut surface color of the sausages is an important factor determining positive perception by consumers. One of the main stated disadvantages of plant or insect enriched hybrid meat products is the deterioration of sensory characteristics, for example their color (Talens *et al.,* 2022). The replacement of pork meat with mealworm flour (*T. molitor*) led to a decrease the color lightness (L*) of both batter and cooked sausages. After 3% substitution with mealworm flour (CM3), the decrease was 7% ($p<0.05$) in batters and 8.7% ($p \leq 0.05$) in

sausages, respectively (Table 3). Reduce in color lightness was observed in frankfurters made with the addition of mealworm flour (Choi *et al.*, 2017) and sausages with grasshopper flour (Cruz-López *et al.*, 2022). On the one hand, the darker color was expected, since the flour is darker in color. On the other hand, the color change may be due to enzymatic browning activated by heat treatment (Azzollini *et al.*, 2016) or a simple physical phenomenon explained by the decreased free water and increased pH, hence the decreased light scattering of the cross-cut surface (Pereira *et al.*, 2011).

The red color in the experimental cooked sausages increased linearly with percent meat replacement. In the meantime, no significant differences in the color yellowness (b*) was observed between all batter samples. On the 7^{-th} day of storage (0-4°C), the b* value was highest in CM3 (with 3% MF). The addition of mealworms (*T. molitor* larvae) increased the yellowness in frankfurters, too (Choi *et al.*, 2017). Previous studies found that the addition of cricket powder (*Acheta domesticus*) also affect b* values of formulated sausages (Ho *et al.*, 2022). From the point of view of the indicator red component (a*) the use of 1.5% mealworm meal is the most appropriate for the hybrid sausages processing without negative color changes. The data are consistent with the assumption made by Ho *et al.* (2022), namely that higher protein, fat and carbohydrate content and dietary fiber in insect flours influencing color changes.

The replacement of pork meat with mealworm did not affect pH of all examined batters (Table 3). After the applied heat treatment was established a significant increase in pH values in all cooked sausages ($p<0.05$). On the 1^{-st} day of storage (0-4°C) the lowest was pH in the control sausages C, and the highest - in CM3 (with 3% MF). The tendency was maintained on the 7^{-th} day of the experiment. The stabilization of pH in CM1, CM2 and CM3 during storage (0–4 °C) indicates suitable conditions for maintaining the cross-bind protein structure after heat treatment. A shift in pH in acidic reaction would disrupt the stability of the formed emulsion, associated with denaturation and disruption of cross-links in the protein matrix (Kim *et al.*, 2017). The pH can be used as an indicator of the batter stability in the production of sausages. The use of additives with a low pH carries the risk of decrease to the isoelectric point of the meat proteins, lead to low stability of the formed emulsion, poor texture and high cooking losses. Conversely, mealworm flour (*T. molitor)* is a suitable substitute in the production of hybrid meat products, due to its pH (Table 1) which favors the formation of a stable emulsion. Similar results were found by Ho *et al.* (2022) in the production of hybrid meat products with cricket powder (*Acheta domesticus*). The highest free water was found in the control batter C ($p<0.05$). The substitution of pork meat with mealworm flour led a decrease of free water in batter and cooked sausages (CM1, CM2 and CM3). Similar results were reported by Scholliers *et al.* (2020a) after partial replacement of meat by superworm (*Zophobas morio* larvae) in cooked sausages.

The appropriate pH of mealworm flour, together with the formed stable emulsion and good water binding ability both before and after cooking, confirmed the potential of suitable meat substitute in meat industry. Conversely, Wang *et al.* (2019) found a negative influence after partial replacement of meat with *Lentinula edodes* on cooking losses, used an indirect indicator of the emulsion stability.

Water activity (a_w) of all cooked sausages differ in no significant range ($p>0.05$) between 0.931 and 0.940 for CM3 and control, respectively. As well as a_w is considered as crucial indicator for microbial growth (Melgar-Lalanne *et al.*, 2019), the lack of difference after addition of mealworm flours up to 3% does not compromise the shelf life and safety of the hybrid meat sausages.

Table 3. Technological characteristics of batter and cooked sausages during storage (0–4 °C)

	Type sample/ Storage time		L*	a*	b*	pH	Free water (%)
C	Batter	-	66.66 ± 0.34^{d}	13.17 ±0.07^{d}	13.47 ±0.10^{a}	6.41 ±0.02^{a}	21.91 ±0.13^{d}
	Cooked sausages	Day 1	68.14 ±0.05^{d,y}	7.86 ±0.03^{a,x}	9.65 ±0.02^{c,y}	6.21 ±0.03^{a,x}	27.68 ±0.07^{d,x}
		Day 7	66.97 ±0.10^{c,x}	9.82 ±0.04^{b,y}	8.82 ±0.03^{b,x}	6.36 ±0.04^{a,y}	35.04 ±0.06^{c,y}
CM1	Batter	-	64.34 ±0.08^{c}	10.80 ±0.09^{c}	13.37 ±0.12^{a}	6.45 ±0.03^{a}	16.94 ±0.07^{b}
	Cooked sausages	Day 1	63.74 ±0.07^{c,x}	8.91 ±0.05^{b,x}	8.71 ±0.04^{a,x}	6.69 ±0.02^{b,y}	23.82 ±0.09^{c,x}
		Day 7	63.66 ±0.06^{b,x}	9.75 ±0.07^{b,y}	8.63 ±0.07^{a,x}	6.60 ±0.02^{b,x}	32.49 ±0.11^{b,y}
CM2	Batter	-	63.47 ±0.14^{b}	10.07 ±0.05^{b}	13.31 ±0.09^{a}	6.40 ±0.05^{a}	15.11 ±0.10^{a}
	Cooked sausages	Day 1	63.19 ±0.06^{b,y}	9.81 ±0.05^{d,y}	8.88 ±0.06^{a,y}	6.61 ±0.03^{b,x}	20.01 ±0.04^{a,x}
		Day 7	62.47 ±0.09^{a,x}	9.43 ±0.02^{a,x}	8.63 ±0.04^{a,x}	6.60 ±0.03^{b,x}	28.54 ±0.07^{a,y}
CM3	Batter	-	62.30 ±0.13^{a}	9.07 ±0.07^{a}	13.22 ±0.13^{a}	6.41 ±0.02^{a}	19.18 ±0.90^{c}
	Cooked sausages	Day 1	62.65 ±0.08^{a,x}	9.57 ±0.09^{c,x}	9.00 ±0.09^{b,x}	6.60 ±0.02^{b,x}	21.98 ±0.04^{b,x}
		Day 7	62.60 ±0.09^{a,x}	10.23 ±0.06^{c,y}	9.65 ±0.09^{c,y}	6.61 ±0.04^{b,x}	28.47 ±0.06^{a,y}

Note: ^{a,b,c,d} superscripts show significant difference ($p<0.05$) in means by columns (among the samples); ^{x,y} superscripts show significant difference ($p<0.05$) in means during storage.

Our results confirm the conclusion made by Ho *et al.* (2022), that meat replacement with mealworm flour causes minor changes in quality parameters but improves nutritional value and is environmentally friendly. Perhaps the origin of the flour and its technological characteristics such as pH, fat and protein content and solubility are the key to stability of the insect-based hybrid meat products.

3.2. Texture characteristics

The replacement of pork meat with mealworm up to 3% does not change the plastic strength (Table 4) of the batter ($p>0.05$). Obviously, the appropriate pH of the flour, its high protein content and low amount of water are suitable for stabilizing the native structure. However, after heat treatment, differences in the texture of the cooked sausages with pork meat substitution were found. Compared to control C, plastic strength was 3 times lower ($p<0.05$) in CM3 sausages (with 3% mealworm flour) and two times lower ($p<0.05$) in the cooked sausages with 2% mealworm flour (CM2). The 1% substitution of pork meat did not affect significantly ($p>0.05$) the plastic strength in the cooked sausages at the 1^{-st} day of storage (0-4°C). The opposite trend is observed in the structural strength (Table 4). In batter with 3% replaced pork meat (CM3) the structural strength increased twice ($p<0.05$). After 2% addition of *T. Molitor* flour (CM2) structural strength did not differ to control C ($p>0.05$). With an increase of MF, the structural strength of the insect-based hybrid cooked sausages decreases. Compared to the control, in CM3 the structural strength was 60% (Day 1) and 35% (Day 7) lower, respectively.

Table 4. Texture profile of batter and cooked sausages during storage (0–4 °)

	Type sample/ Storage time		Plastic strength (kg/cm^2)	Structural strength (kg/cm^2)	Springiness (%)
C	Batter	-	0.80 ± 0.19^{a}	5.90 ± 0.21^{a}	-
	Cooked sausages	Day 1	$16.80\pm0.42^{c,x}$	$232.70\pm20.50^{b,x}$	$86.0\pm10.0^{b,y}$
		Day 7	$26.60\pm0.39^{d,y}$	$314.10\pm4.50^{d,y}$	$44.0\pm2.0^{b,x}$
CM1	Batter	-	1.20 ± 0.19^{a}	$8.20\pm0.28^{a,b}$	-
	Cooked sausages	Day 1	$14.70\pm0.26^{c,x}$	$199.90\pm30.10^{a,b,x}$	$84.0\pm10.0^{b,x}$
		Day 7	$19.30\pm0.30^{c,x}$	$260.10\pm5.60^{c,y}$	$48.0\pm2.0^{b,y}$
CM2	Batter	-	1.50 ± 0.31^{a}	$7.00\pm0.24^{a,b}$	-
	Cooked sausages	Day 1	$8.50\pm0.37^{b,x}$	$158.00\pm21.40^{a,x}$	$77.0\pm3.0^{a,b,y}$
		Day 7	$11.90\pm0.42^{b,x}$	$212.00\pm8.10^{b,y}$	$38.0\pm6.0^{a,b,x}$
CM3	Batter	-	1.80 ± 0.34^{a}	10.20 ± 0.16^{b}	-
	Cooked sausages	Day 1	$5.50\pm0.27^{a,x}$	$142.80\pm26.00^{a,x}$	$62.0\pm11.0^{a,y}$
		Day 7	$8.40\pm0.25^{a,x}$	$231.50\pm9.10^{b,y}$	$29.0\pm7.0^{a,x}$

Note: ^{a,b,c,d} superscripts show significant difference ($p<0.05$) in means by columns (among the samples); ^{x,y} superscripts show significant difference ($p<0.05$) in means during storage.

With the increase of meat replacement with mealworm flour, the structural strength of the cooked sausages decreases. The gelation and emulsification process runs during cooling with formation of hydrogen bonds and improves the textural properties in final product (Scholliers *et al.,* 2020b). As a result, the textural properties are improved. But the mixtures of certain proteins, such as insect proteins, might be unable to aggregate in gel structure and therefore the gel strength decrease. Similar to our results were obtained after 5% meat replacing by superworm (*Zophobas morio* larvae) resulted in a drastic decrease in the textural properties (Scholliers *et al.,* 2020b).

In parallel with the structural strength, the springiness of the cooked sausages also decreases ($p<0.05$), and it was the lowest after meat substitution with 3% mealworm flour (CM3). Lower hardness in hybrid meat products (containing insect flour) were obtained by Choi *et al.,* (2017). The opposite effect was found by Kim *et al.,* (2016) and Kamani *et al.,* (2019). Probably the source of the protein flour - insects, plant proteins, milk proteins is a determinant of the texture of the hybrid product. A similar hypothesis was also expressed by Scholliers *et al.,* (2020b). They also add one more factor - the temperature during the cooking, as with the increase up to 90°C a partial improvement of textural properties in hybrid cooked sausages with *Zophobas morio* larvae was found.

3.3. Microbiological status

The replacement of pork meat with mealworm flour didn't increase the total viable count (TVC) (Table 5, Day 1). TVC in CM2 and CM3 were found lower compared to the control C and CM1 ($p<0.05$). In the same time lowest coliforms' count was found in cooked sausages with 2% MF (CM2) ($p<0.05$) and in CM3 they weren't even detected. At 1% substitution of pork meat with mealworm flour no significant difference as found compared to the control sausages No significant difference was found between the control C and CM1 and CM2 ($p>0.05$) in yeasts and molds growth, and minimal was the value in CM3 ($p<0.05$). On the 7^{-th} day of storage (0-4°C), minimal TVC growth was established in control C and CM1, followed by CM3 and CM2 ($p<0.05$). The lowest were found coliforms in control C ($p<0.05$). In the same time no significant difference between all experimental cooked sausages (CM1, CM2 and CM3) was established.

At the end of the studied period (Day 7, 0+4°C) lowest was growth of yeasts and molds in the control C and CM1.

Table 5. Microbiological status of the cooked sausages during storage (0–4 °C)

	Type sample/ Storage time		TVC (CFU/g)	Coliforms (CFU/g)	Yeasts & Molds (CFU/g)
C	Cooked sausages	Day 1	$5.29\pm0.18^{b,y}$	$5.12\pm0.21^{b,y}$	$4.32\pm0.28^{b,y}$
		Day 7	$3.59\pm0.33^{a,x}$	$2.30\pm0.37^{a,x}$	$2.75\pm0.62^{a,x}$
CM1	Cooked sausages	Day 1	$5.34\pm0.53^{b,y}$	$4.64\pm0.32^{b,x}$	$3.72\pm0.42^{b,y}$
		Day 7	$4.23\pm0.42^{a,b,x}$	$3.45\pm0.46^{b,x}$	$2.60\pm0.43^{a,x}$
CM2	Cooked sausages	Day 1	$4.06\pm0.37^{a,x}$	$3.18\pm0.24^{a,x}$	$3.15\pm0.39^{a,b,x}$
		Day 7	$4.47\pm0.41^{b,x}$	$3.45\pm0.38^{b,x}$	$3.20\pm0.61^{b,x}$
CM3	Cooked sausages	Day 1	$5.04\pm0.64^{a,b,x}$	N.F.	$3.00\pm0.21^{a,x}$
		Day 7	$4.73\pm0.32^{b,x}$	3.27 ± 0.55^{b}	$3.33\pm0.55^{b,x}$

Note: ^{a,b,c,d} superscripts show significant difference ($p<0.05$) in means by columns (among the samples); ^{x,y} superscripts show significant difference ($p<0.05$) in means during storage.

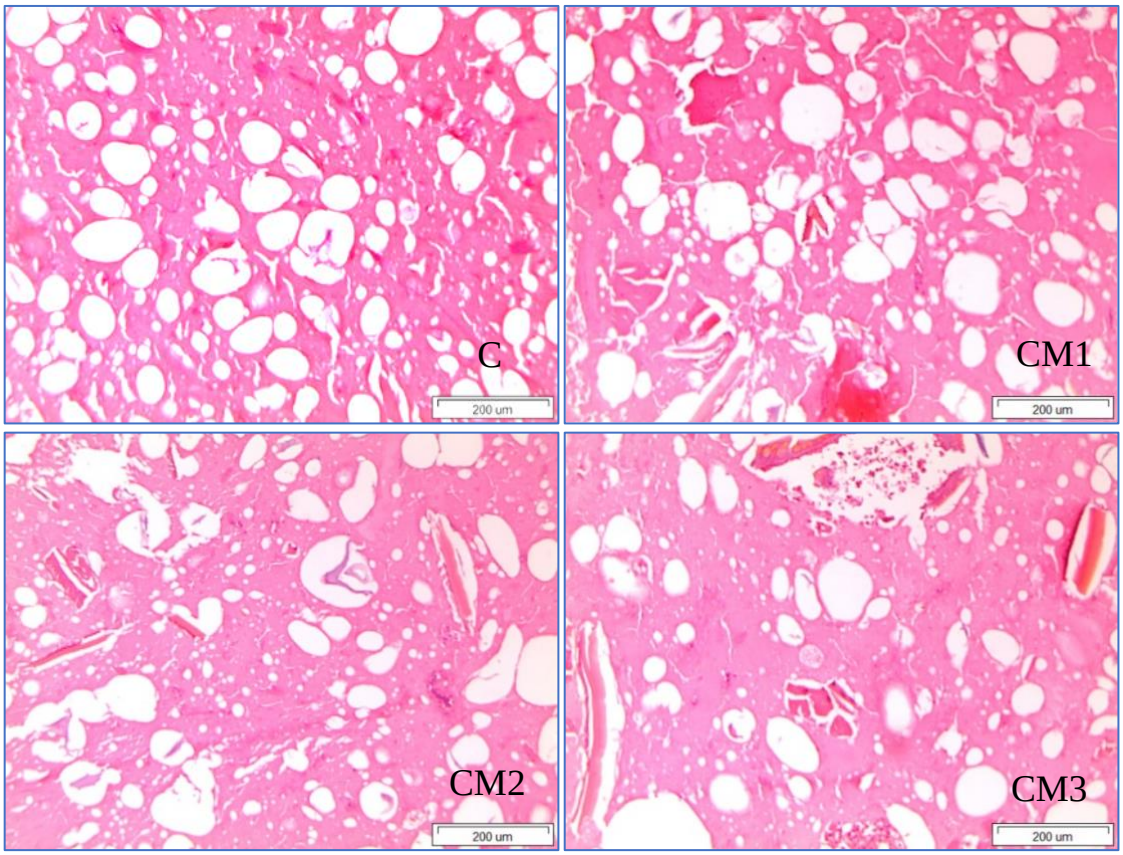

Figure 1. Light microscopy of cooked sausage at x100 time magnification

The obtained results confirm previous researches (Vandeweyer *et al.*, 2017) indicated that meat replacers, spices, salting materials like insect flour are a source of additional microbial contamination. However, the initial rate of microbial contamination was not increased by the meat replacement. The microbial growth during storage was similar in both control C and all experimental cooked sausages. According to the literature the chitin from the exoskeleton of insects exhibits antimicrobial properties (Shin *et al.*, 2019). The results obtained did not show a

significant antimicrobial effect after the meat substitution with mealworm flour.

3.4. Light microscopy

At Figure 1 are presented the microstructural images of the four investigated cooked sausages. Their matrix was made up of fat droplets wrapped in protein film, the water droplets are dispersed throughout the myofibrillar and connective tissue fragments. Control C had a characteristic uniform gel matrix. On the other hand, in all samples with MF the gel matrix was disturbed and there were clearly observable solid particles. A lager air bubbles and fat droplets were observed due to coalescence. Similar dose depended enlargement of the air bubbles due to the addition of insect flours was reported by Vlahova-Vangelova et al. (2022).

4. Conclusions

Overall color changes in the cooked sausages with 1.5% substitution of pork meat was minimal. Reduce in color lightness (L*) was observed by the darker flour color and due to enzymatic browning activated by heat treatment. The red component (a*) of the color increased after meat replacement with mealworm flour. The appropriate pH of mealworm flour (*T. molitor* larvae), together with the formed stable emulsions and good water binding ability both before and after cooking, confirmed the potential of suitable meat substitute in meat industry.

Meat replacement up to 3% mealworm flour decreases the free water in the experimental cooked sausages. The lack of difference in water activity after substitution of pork meat with mealworm flour up to 3% does not compromise the shelf life and safety of the produced meat products. The percent of meat replacement with mealworm flour in the cooked sausages affect their texture profile summarized by the decrease in structural and plastic strength and springiness of the experimental cooked sausages.

The expected microbial load of the mealworm flour affects TVC, coliforms count and yeasts & molds count in some sense established in the cooked sausages with 2 and 3% meat replacement. Yet the set limits for cooked meat products were not surpassed and their quality and safety were no compromised.

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CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

COMPARATIVE ANTIOXIDANT AND PHYTOCHEMICAL ACTIVITY OF RAW AND BOILED TUBER OF *DIOSCOREA BULBIFERA* COLLECTED FROM TRIBAL FOREST OF SUNDARGARH DISTRICT, ODISHA, INDIA

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https://doi.org/10.34302/crpjfst/2023.15.4.2

Article history:
Received: 18 September 2021
Accepted: 17 July 2023

Keywords:
Antioxidant;
Ascorbic acid;
Bioactive compound;
Flavonoid.

ABSTRACT

In the present study, we have investigated the differences in the composition of proximate minerals, vitamins bioactive compounds, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity between the raw and boiled tubers of *Dioscorea bulbifera.* The results showed that both the raw and boiled tubers have rich sources of carbohydrates (31.62% and 23.94%), proteins (3.48% and 2.25%), starch (8.6% and 11.67%), and free amino acids (1.45% and 0.59%); but have low-fat content (0.19% and 0.14%). Vitamin profiling of the tubers contained a substantial amount of ascorbic acid, vitamins B1, B2, B3, and B6. Further, the raw and boiled tuber of *Dioscorea bulbifera* had a very high amount of bioactive compounds like phenolics, flavonoid, diosgenin, tannin, and saponin. Phenolic and flavonoid content positively correlated with free radical scavenging activity of tuber and performed better scavenging activity compared to ascorbic acid and butylated hydroxytoluene (BHT). Thus, the tuber of *Dioscorea bulbifera* is a better food supplement to meet the calorie requirement of the tribal people and a rich source of antioxidants.

1.Introduction

Wild tubers *Dioscorea bulbifera,* known as air potato or yam, is primarily found in the tropical, subtropical, and temperate region of the world (Abara, 2011). *Dioscorea bulbifera* produces both underground and aerial tubers. However, underground tubers are very rich in starch hence mostly consumed. The tribal people used it as food as well as medicine (Kumar *et al*., 2013). The tubers are composed of a high amount of carbohydrates, fibers, and low fats and protein content with a good proportion of different amino acids, making them an excellent dietary source and could be consumed, boiled, steamed, baked, or fried (Osman, 1990). Due to lack of good nutritional information, the broad utilization of the yam is very limited; Studies of the nutritional composition of the locally available *Dioscorea bulbifera* is very important since it may help the tribal people to fulfill their dietary requirements. Besides excellent nutritional properties, *Dioscorea bulbifera* reported exhibiting antimicrobial, antioxidant, plasmid curing, analgesic, anti-inflammatory, antihyperglycemic, antihyperlipidemic, antidiabetic, antinociceptive, and antitumor activities (Ghosh *et al*., 2015). *Dioscorea bulbifera* is also reported to have good radical scavenging and singlet oxygen quenching ability hence used as potential herbal therapeutic agents for various diseases that occurred due to

oxidative stress (Ghosh *et al*., 2013). *Dioscorea bulbifera* (*D. bulbifera)* exhibits higher antioxidant capacities with lower IC_{50} values than the other species (Padhan *et al*., 2020). Detailed information on the profiling of tubers of *D. bulbifera* from the state of Odisha is very rare. In this regard, the present study is therefore aimed to evaluate the comparative phytochemical and antioxidant properties of the boiled and raw tuber of wild yam *D. bulbifera.*

2. Materials and methods

2.1. Collection and preparation of sample

The tubers specimen and the plant were collected from the forest of Sundargarh, Odisha, and identified at the Centre of Excellence in Natural Products and Therapeutics, Dept. of Biotechnology and Bioinformatics, Sambalpur University. Tubers were washed thoroughly, peeled, and sliced approximately 1-2 mm of thickness. Some of the sliced tubers were boiled and dried in a hot air oven at 80 ºC until a constant weight was obtained. In contrast, the other portion was taken fresh, without boiled, and dried in a hot air oven at 80 ºC until a constant weight was obtained. After drying, the samples were powdered and sieved through a 1mm sieve. Both raw and boiled tuber powder samples were preserved in an airtight glass bottle for further analysis.

2.2. Comparative Nutritional analysis

Nutritional analyses of both raw and boiled tuber were determined. Moisture, ash, and fat contents were determined using a hot air oven, muffle furnace and Soxhlet apparatus following the standard method described by Ranganna (2007). Total carbohydrate and starch content were estimated using the Anthrone reagent method (Thayumanavan and Sadasivam, 1984). Reducing sugar content of both the raw and boiled tuber samples was estimated by the Dinitrosalicyclic acid method (Sadasivam and Manickam, 2008). The protein content of both the raw and boiled tuber samples was estimated following the standard method of Lowery *et al*. (1951). The total free amino acid of the tuber samples was determined using Ninhydrin reagent (Sadasivam and Manickam, 2008). The amino acid profiling of the raw and boiled tuber samples was determined using 5.54 SP 5LAB solutions software after detection through Shimadzu LC-30 AD HPLC (Pal *et al*., 2016). Vitamins (B1, B2, B3, and B6) in tuber samples were analyzed in HPLC (Shimadzu HPLC and photodiode array detector. Supelcosil LC 17 DB column 250 mm×4.6 mm, 5µm; Sigma, USA) following the method of Perales *et al.* (2005). The mineral compositions were analyzed using an Inductively Coupled Atomic Adsorption Spectrometer (Perkin-Elmer Optical Emission Spectrometer, Optima 7000 DV) as per the methods of Kalra (1998). The ascorbic acid content was estimated as the standard method described by Sadasivam and Manickam (2008).

2.3. Comparative Bioactive compounds analysis

Total free phenolics and Total flavonoid content were determined in methanol, acetone, and water extract of tuber using modified Folin-Ciocalteau method (Ordon Ez *et al*., 2006) and the aluminum chloride colorimetric assay (Pallab *et al*., 2013), respectively. Tannin was estimated by following the methods of Schanderl (1970). The method of Obadoni and Ochuko (2001) was used for the determination of saponin content. Diosgenin was determined following the methods of Uematsu *et al*. (2000).

2.4. DPPH scavenging activity

Tuber extracts were concentrated to dryness under reduced pressures at 40 ºC using a rotary evaporator and dissolved in methanol to make a stock solution of 50 mg/ml and used for different antioxidant activities. The effect of the extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was determined following the standard method described by Liyana- Pathiranan and Shahidi (2005). The quantity of DPPH radical scavenged was calculated using the following equation:

DPPH radical scavenging activity (%) = [(AbsControl–AbsSample)]/(Abs Control)]x100….Eq^n… (1)

Abs Control = absorbance of DPPH radical+methanol; (2)

Abs Sample = absorbance of DPPH radical+sample extract/standard. (3)

Where Abs = Absorbency

2.5. Statistical Analysis

The results obtained were subjected to statistical analysis as mean and standard deviation (Zar, 1984). The mean values and standard deviations were calculated from the data obtained from three different experiments. The statistical difference at $p < 0.05$ was considered to be significant.

3.Results and discussions

3.1. Nutritional content

In the present study, a comparative analysis of nutritional composition between the raw and boiled tubers has been carried out, and the proximate content of the raw and boiled tubers is presented in Table-1. The moisture content of the raw tuber was found to be relatively low (74.89±0.54%) compared to the boiled tuber (80.48±1.18%). The ash content of the raw tuber was found to be high (2.57±0.04%) compared to the boiled tuber (1.66±0.34%). We found a very low amount of fats in both raw tubers (0.19±0.01%) and boiled tuber (0.14±0.012%), which are not significantly different. Total Carbohydrate (31.62±0.46%) and reducing sugar content (0.018±0.008%) of raw tuber was found to be relatively high compared to the boiled tuber (23.94±0.50% and 0.012±0.008%). In contrast, it was found that the boiled tuber contained an albeit high amount of starch (11.67±0.65%) compared to the raw tuber (8.6±0.54%). The protein content of the raw and boiled tuber was found to be 3.48±0.92% and 2.25±0.16%, respectively. The total free amino acid content was found to be slightly high in the raw tuber (1.45±0.05%) compared to the boiled tuber (0.59±0.13%). HPLC analysis of both the raw and boiled tubers for different amino acids revealed that out of nine essential amino acids, six amino acids (Histidine, methionine, lysine, phenylalanine, threonine, valine) are observed in the raw tuber and four amino acids (lysine, threonine, valine, and phenylalanine) in the boiled tuber (Table 1). Phenylalanine was present in the highest amount in both raw and boiled tuber, followed by valine. Histidine, methionine, and cysteine amino acids were found in minimum quantity in the raw tuber and were not detected in the boiled samples. The observations of the mineral compositions of the raw and boiled tubers are presented in Table 1. The sodium, potassium, phosphorus, iron, and calcium content of the raw tubers were observed in the range of 316±27.78, 677.33±21.38, 153.20±17.17, 6.16±0.89, 290±4.13 mg/100g dry mass, respectively. In contrast, the estimated value of the above mineral contents in boiled tuber were 119.36±16.25, 232.33±12.50, 60.43±1.72, 3.24±1.06, 180±2.28 mg/100g dry mass, respectively. The other essential elements such as magnesium, zinc, manganese, and copper were found to be 203±6.42, 0.45±0.95, 4.2±2.16, and 0.79±0.62 mg/100g dry mass, respectively in the raw tuber. In contrast, the boiled tuber contained a lower amount of magnesium, zinc, manganese, and copper elements (102±4.14, 0.18±0.83, 0.89±1.86, and 0.12±0.37 mg/100g dry mass, respectively).

Table 1. Nutritional composition analysis of raw and boiled tubers of *Dioscorea bulbifera*

Nutrient content	Raw tuber	Boiled tuber
Proximate		
Moisture (%)	74.89±0.54	80.48±1.18
Ash (%)	2.57±0.04	1.66±0.34
Total carbohydrate (%)	31.62±0.46	23.94±0.50
Starch (%)	8.6±0.54	11.67±0.65
Reducing sugar (%)	0.018±0.008	0.012±0.008
Fat (%)	0.19±0.01	0.14±0.012
Protein (%)	3.48±0.92	2.25±0.16
Free amino acid (%)	1.45±0.05	0.59±0.13
Amino acid quantity (mg/100g)		
Glutamic acid	14.27±0.51	10.24±0.35
Glutamine	8.95±0.11	7.6±0.29
Histidine	1.033±0.19	Nd
Arginine	3.9±0.23	3.34±0.15
Alanine	5.2±0.15	3.29±0.32
Serine	6.54±0.03	5.85±0.25
Tyrosine	4.18±0.17	3.58±0.24
Cysteine	0.37±0.13	Nd
Methionine	1.16±0.03	Nd
Proline	4.01±0.01	3.46±0.03
Glysine	5.5±0.03	6.3±0.08
Lysine	3.54±0.43	2.32±0.07
Threonine	4.42±0.27	2.05±0.05
Valine	5.19±0.11	4.43±0.03
Phenylalanine	5.29±0.35	4.83±0.21
Mineral element (mg/100g)		
Sodium	316±27.8	120±16.3
Potassium	677±21.4	232±12.5
Phosphorus	153±17.2	60.4±1.72
Iron	6.16±0.89	3.24±1.06
Calcium	290±4.13	180±2.28
Magnesium	203±6.42	102±4.14
Zinc	0.45±0.95	0.18±0.83
Manganese	4.2±2.16	0.89±1.86
Copper	0.79±0.62	0.12±0.37
Vitamin mg/100g		
Vitamin C	99.5±0.94	70.7±1.19
Vitamin B1	0.007±0.0008	0.005±0.0004
Vitamin B2	0.027±0.007	0.014±0.003
Vitamin B3	27.38±1.42	14.65±1.25
Vitamin B6	0.128±0.028	0.084±0.006

Not detected

The details of vitamin analysis of *Dioscorea bulbifera* are shown in Table 1. The ascorbic acid content of the raw tuber was found to be 99.5±0.94 mg/100g dry mass. In contrast, the ascorbic content of the boiled tuber was found to be 70.7±1.19 mg/100g dry mass. The content of vitamin B1, vitamin B2, vitamin B3, and vitamin B6 for the raw tuber was found to be 0.007±0.0004, 0.027±0.007, 27.38±1.42, and 0.128±0.028 mg/100g dry mass, respectively. In contrast, the boiled tuber's vitamin B1, vitamin B2, vitamin B3, and vitamin B6 content was found to be comparatively in lesser quantity than that of the raw tubers (Table 1)**.** Among all the contents analyzed, it has been observed that in comparison to the present finding, a higher amount of fat, starch, protein, potassium, calcium, zinc, and copper has been reported earlier by other researchers (Polycarp *et al.,* 2012). In contrast, carbohydrate and sodium contents reported earlier have been lower than the present findings (Sanful *et al.,* 2013). A very similar amount of ash, protein, phosphorus, manganese, magnesium, ascorbic, and vitamin contents has been observed compared to the earlier report (Okwu and Ndu, 2006). In comparison to the boiled tuber, raw tubers are found rich in nutrition (Table-1) which might be due to the leaching and degradation of most of the vitamins and mineral contents during boiling. In comparison to the previous reports, the variation observed in the nutritional content of the tuber in the current report might be due to the soil Physico-chemical properties, soil fertility, geographical and climatic condition, genetic variation, etc. (Bhandari and Kawabata, 2004). Overall the results revealed that the tuber of *Dioscorea bulbifera* are good sources of minerals and could be used as food supplements

3.2. Bioactive compounds

Bioactive compounds are secondary metabolites of the plant that have pharmacological or toxicological effects in humans and animals. Tannin is one of the phenolic compounds which gives an astringent and bitter taste. Tannins act as antidiarrheal, haemostatic and anti hemorrhoidal, anti-inflammatory, antiviral and antibacterial, antiseptic, antioxidant. Diosgenin content plants are grown for steroid preparation (Behera *et al*., 2010). Saponins are responsible for the reduction of cholesterol levels in animals along with other animals. (Desai *et al*., 2017). The analysis showed that raw tuber contained 160.2±0.84 mg/100g while boil tuber contained 12.5±0.11mg/100g of diosgenin**.** Tannin content was found to be 180.11±0.32 mg/100g and 12.09±0.12mg/100g for raw and boil tuber, respectively, in this present study**.** The saponin content of raw and boil tuber was found to be 150.34±0.67mg/100g and 21.26mg/100g, respectively. Ghosh et al. (2014) reported diosgenin exhibited potent inhibition against both porcine pancreatic alpha-amylase and alpha-glucosidase as well as against crude murine amylase and glucosidase and acts as lead candidate in managing Type II Diabetes Mellitus. Behera et al. (2010) reported 1383mg/100g of diosgenin for *Dioscorea bulbifera* tuber. Polycarp et al. (2012) reported 10.98mg/100g of tannin in *Dioscorea bulbifera*. Princewill-Ogbonna and Ibeji (2015) found 8.49- 14.03mg/100g of saponin content of three cultivars of *Dioscorea bulbifera*.

3.3. Antioxidant properties

Phenols and flavonoids are compounds having antioxidant activity hence can absorb and neutralize free radicals, quenching singlet and triplet oxygen, or decompose peroxides (Luis *et al.,* 2012). Among boiled and raw tuber, the latter one showed excellent radical scavenging activity, which is significantly higher than that of ascorbic acid and BHT (Butylated Hydroxytoluene). Methanolic extracts of raw and boiled tubers of *Dioscorea bulbifera* were used in the present study to determine the DPPH radical scavenging activity, and results were compared with standard ascorbic acid and BHT (Figure 1). The IC_{50} value (the concentration of the sample that reduced 50% of the absorbances of DPPH) of raw and boiled tuber, ascorbic acid, and BHT are included in Figure 2. Higher the IC_{50} value signifies less antioxidant activity and *vice-versa*. It was found that the methanolic

extract of the raw tuber has significantly higher antioxidant activity (IC_{50} value is 46.11 µg/ml) compared to the ascorbic acid (IC_{50} value is 92.86 µg/ml), BHT (IC_{50} value is 54.35 µg/ml) and boiled tuber extract (IC_{50} value is 455.37 µg/ml). Phenolic content of the raw tuber extracted with different solvents such as acetone, methanol, and water was found to be 95.92±11.78, 121.11±13.71, and 205.19±15.91 mg GAE/100g dry weight, respectively (Table 2). In contrast, the boiled tuber's acetone, methanol, and water extract contained 12.71 ± 5.28, 23.04 ±5.60, and 54.18± 10.40 mg GAE/100g dry weight, respectively of total phenols. Previously, Bhandari et al. (2003) have reported 166 mg GAE/100g fresh weight of total phenols for the acetone extract of *Dioscorea bulbifera* tuber. Total flavonoid content with the methanol, acetone, and water extract of raw tuber was 359.82±18.10, 232.10±34.22, and 387.71±9.96 mg quercetin equivalent/100 g dry weight (Table 2). In contrast, the methanol, acetone, and water extract of boiled tuber possessed a quite low flavonoid content viz. 75.91±12.63, 54.83±7.11, and 64.13±11.04 mg quercetin equivalent/100 g dry weight. Okwu and Ndu (2006) reported 8.04 mg quercetin equivalent /100g, and Adeosun et al. (2016) reported 5.36 mg quercetin equivalent/100g of flavonoid contents of *Dioscorea bulbifera* tuber on the dry weight basis. TPC (total phenol content) of tuber positively correlated with DPPH scavenging activity at the 0.01 level (R^2=0.9762) (Figure 3)**.** TFC (total flavonoid content) and DPPH also positively correlated at 0.01 level (R^2=0.9717) (Figure 4). The result of the analysis showed phenols are potent antioxidant and scavenging agents.

Overall, for treating radical-related pathological damage, the tuber of *Dioscorea bulbifera* can be used as a therapeutic agent. The current report also suggests that in *Dioscorea bulbifera*, polyphenol is essential component responsible for its antioxidant activities.

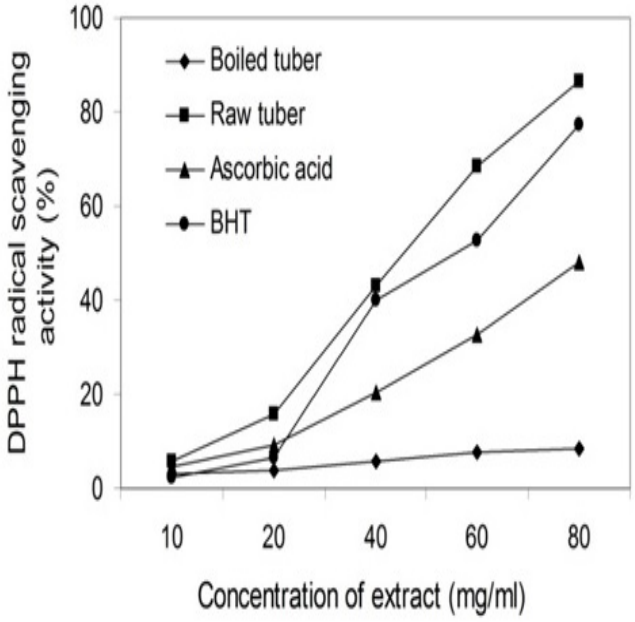

Figure 1. Comparison of DPPH radical scavenging activity of methanolic extract of the raw and boiled tuber of *Dioscorea bulbifera* with ascorbic acid and BHT.

Table 2. Phenols and flavonoid content of raw and boiled tubers of *Dioscorea bulbifera*

Polyphenol content	Raw tuber			Boiled tuber		
	Methanol extract	Acetone extract	Water extract	Methanol extract	Acetone extract	Water extract
Phenols (mg/100 g dry mass)	121.1±13.71	95.92±11.78	205.2±15.91	23.04±5.60	12.71±5.28	54.18±10.40
Flavonoid Mg/100 g dry mass) DPPH	359.8±18.10	232.1±34.22	387.7±9.96	75.91±12.63	54.83±7.11	64.13±11.04

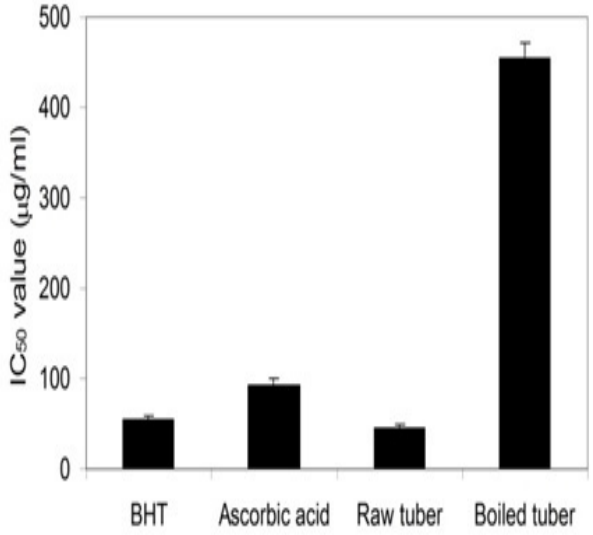

Figure 2. The IC_{50} value of raw and boiled tuber, ascorbic acid, and BHT

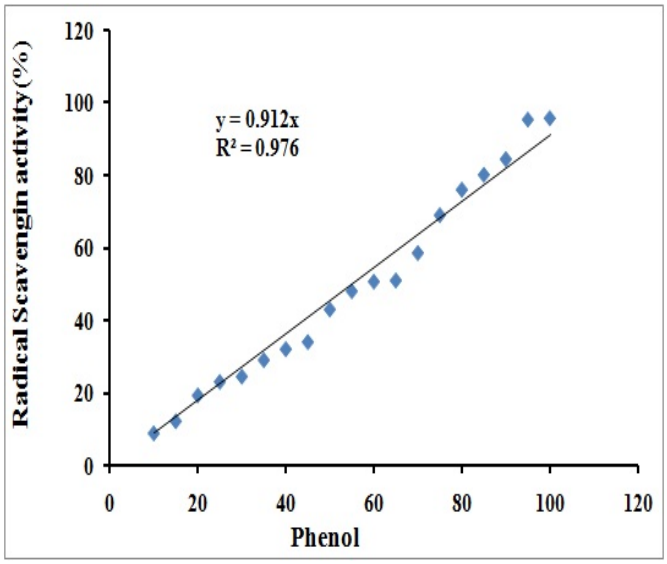

Figure 3. Correlation between TPC and DPPH

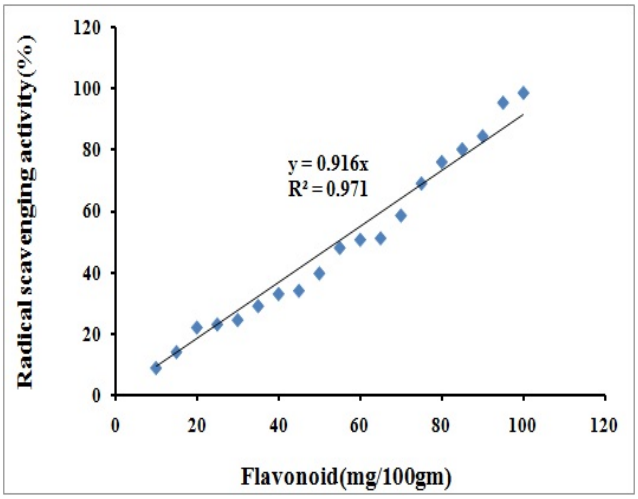

Figure 4. Correlation between TFC and DPPH

4. Conclusions

We have evaluated the nutritional composition and antioxidant activity of the tuber of *D. bulbifera*, both in raw and in boiled form. We found that the nutritional composition of the raw form is very rich than the boiled form of the tubers. The antioxidant activity of the raw tuber was found to be significantly very high compared to the ascorbic acid and BHT. The total phenolics and flavonoids are found to be significantly high in both raw and boiled tubers. The present study also emphasizes phytochemical analysis. Hence the tuber *D. bulbifera* could be a good candidate for functional foods.

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Acknowledgments

Financial support: OHEPEE, Govt. of Odisha through World Bank. Induar Sadhni acknowledges UGC, Govt. of India for RGNF Fellowship (201415-RGNF-2014-15-ST-ORI-70410).

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

HEAVY METALS AND THEIR ADVERSE EFFECTS: SOURCES, RISKS, AND STRATEGIES TO REDUCE ACCUMULATION IN TEA HERB - A SYSTEMATIC REVIEW

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https://doi.org/10.34302/crpjfst/2023.15.4.3

Article history:
Received: 6 April 2022
Accepted: 16 October 2023

Keywords:
Heavy Metal;
Tea Herb;
Ecological pollution;
Public health.

ABSTRACT

Heavy metals (HMs) such as arsenic, cadmium, chromium, lead, mercury, aluminum, iron, and barium can accumulate in tea herbs and pose health risks to consumers. This systematic review analyzes research from 2000-2022 on HM contamination in tea, associated health effects, and strategies to minimize exposure. Initial searches yielded 961 articles, with 157 selected for final review after the screening. HMs have no biological role; their toxicity depends on metal type, oxidation state, and solubility. Chronic ingestion of HM-contaminated tea can impair numerous organs and biochemical processes, potentially causing cancer, cardiovascular, neurological, reproductive, and developmental damage. Factors influencing HM levels in tea herbs include soil, air, and water contamination, proximity to pollution sources, genetic differences, brewing methods, and steeping time. Strategies to reduce HM uptake and toxicity include using cleaner irrigation water, avoiding acidic fertilizers, liming soil, and restricting tea cultivation near pollution sources. Processing methods and shorter brewing can also decrease HMs extracted into tea infusions. Ongoing research should further investigate phytoremediation and genetically engineering tea cultivars. Consuming contaminated tea poses cumulative risks, so sustainable agriculture and pollution control are vital to minimize HM exposure and protect public health. This review enhances understanding HM dynamics, toxicity, and mitigation strategies for producing safer tea.

1. Introduction

1.1. Tea Herb

The use of essential oils and extracts from medicinal plants has significantly increased recently due to growing awareness of the health benefits of herbs and the negative effects of synthetic chemicals. Medicinal herbs have been utilized in traditional medicine since ancient time (Aali *et al.*, 2017; Kazeminia *et al.*, 2017; Mahmoudi *et al.*, 2017).

Camellia sinensis is an evergreen shrub belonging to the *Theaceae* family. Its leaves are used to produce black tea, made by fermenting the leaves, and green tea, made by drying the leaves (Görür *et al.*, 2011). As the second most consumed beverage globally after water, tea contains bioactive polyphenols with medicinal

functions (Hayat *et al.*, 2015). Tea herb (TH) has different properties due to its various compounds (Zhang *et al.*, 2019). Flavonoids, antioxidants, and minerals in TH positively impact health. The polyphenol epigallocatechin-3-gallate has antioxidant effects (Bitu Pinto *et al.*, 2015). TH extract may benefit diabetes (Q.-Y. Fu *et al.*, 2017) and blood lipids levels (El-Bassossy *et al.*, 2016), and reduce risks of immune disorders, cardiovascular disease, stroke, and various cancers (Zhang et al., 2019). Thus, consuming black and green tea infusions is recommended. Table 1 outlines the medicinal compounds in TH.

Table 1. Effective medicinal compounds in tea herb

Ingredients	**Medicinal effect**
Polyphenols	Control of severe postprandial hypertension through inhibition of alpha-amylase production (Liu *et al.*, 2016), anti-inflammatory properties (Oz *et al.*, 2013), anti-cancer effects (Miyata *et al.*, 2019), immune-boosting, diuretic, and antimicrobial effects (Oz, Chen, *et al.*, 2005; Oz, McClain, *et al.*, 2005).
Epigallocatechin gallate	Antioxidants properties (Bitu Pinto et al., 2015), anti-inflammatory effects (Oz & Ebersole, 2010), cardioprotective effects in cardiovascular diseases (Widlansky *et al.*, 2007), insulin-sensitizing and anti-diabetic effects (Lin & Lin, 2008; Yamabe *et al.*, 2009), weight loss effects (Tabrez *et al.*, 2015), neuroprotective effects in Alzheimer's disease (Meng *et al.*, 2010), and anti-inflammatory effects in inflammatory bowel disease (Oz et al., 2013).
Catechin, chlorogenic acid, Caffeine, Theaflavin	Hypoglycemic effects through inhibition of α-glucosidase and α-amylase. Reduction of lipid metabolites and albuminuria (Liu et al., 2016).
Geraniol	Improvement of vascular function (El-Bassossy et al., 2016). Renoprotective effects in diabetic nephropathy (Yamabe et al., 2009).
Polysaccharides	Antioxidants properties (H. Chen *et al.*, 2009).
Catechins	Antioxidants properties (Liang *et al.*, 2008).
Gallic acid	Antioxidants properties (Kongpichitchoke *et al.*, 2016).
Caffeine	Weight loss effects (Zheng *et al.*, 2015).

TH is cultivated in around 45 countries spanning latitudes from 45°N to 34°S (Karak & Bhagat, 2010). Global tea production is estimated at 1.74 billion kg, with China as the largest producer and consumer, followed by India in production. Other producing countries include Sri Lanka, Kenya, Turkey, Indonesia, and Japan (Layomi Jayasinghe *et al.*, 2019).

As one of the most widely consumed beverages worldwide, approximately 98% of the global population considers tea their first hot drink, with around twenty billion cups consumed daily (Dufresne & Farnworth, 2001). Tea's popularity stems from its potential health benefits. However, heavy metals (HMs) in tea raise safety concerns, as they may adversely impact health. In modern times, HMs are hazardous substances. This review identifies the negative effects of HMs in tea and ways to minimize exposure, so consumers can be assured of a safe, uncontaminated product. Additionally, the methods discussed could inform policies aimed at reducing HMs contamination in the food chain.

1.2. Heavy Metals

There are 35 metals (Ms), of which 23 are classified as HMs. HMs include: Arsenic (symbol As), Aluminum (symbol Al), Antimony (symbol Sb from Latin: *stibium*), Barium (symbol Ba), Beryllium (symbol Be), Bismuth (symbol Bi), Chromium (symbol Cr), Cadmium (symbol Cd), Germanium (symbol Ge), Gallium (symbol Ga), Gold (symbol Au from Latin: *aurum*), Silver (symbol Ag from the Latin: *argentum*), Lead (symbol Pb from the Latin:

plumbum), Indium (symbol In), Lithium (symbol Li), Nickel (symbol Ni), Mercury (symbol Hg from the Latin: *hydrargyrum*), Platinum (symbol Pt), Strontium (symbol Sr), Tellurium (symbol Te), Titanium (symbol Ti), Thallium (symbol Tl), Tin (symbol Sn), Uranium (symbol U), and Vanadium (symbol V). HMs have relatively high density and electrical conductivity. HM levels in TH may vary by region (Khlifi & Hamza-Chaffai, 2010; Mahmoudi et al., 2017; F. Yang & Massey, 2019). Other Ms such as Copper (symbol Cu from Latin: *cuprum*), Zinc (symbol Zn), Iron (symbol Fe from Latin: *Ferrum*), Cobalt (symbol Co), Manganese (symbol Mn), Molybdenum (symbol Mo), Iodine, Bromine (symbol Br), and Selenium (symbol Se) are also present in TH. These are essential in trace amounts but can be toxic if intake exceeds thresholds (Mahmoudi et al., 2017; Nagajyoti *et al.*, 2010).

Recently, HM exposure has become an environmental and public health concern due to increased industrial and agricultural use. HMs are typically classified as non-essential (Fasae & Abolaji, 2022). Urbanization and technology have led to HM accumulation in soil and water, allowing entry into the food chain via plants and animals, including tea. This poses a health risk. It is critical to limit HM uptake and modulate plant response. Updating knowledge on HM dynamics in the soil-plant-environment is key (Thakur *et al.*, 2022). This review critically analyzes strategies to address HMs in tea and possible health risks. Understanding the risks of HM tea consumption and developing mitigation measures is imperative.

2. Materials and methods

The objective of this systematic review is to evaluate articles published from 2000-2022 that examine the adverse effects of HMs and potential solutions to reduce HM exposure. The search strategy involved keyword searches using terms such as "heavy metal," "side effects," "Cadmium," "Arsenic," "Mercury," "Chromium," "Lead," "Aluminum," "Iron," "Barium," "tea," "health effects," and "public health" in the Science Direct, PubMed, and Google Scholar databases. This review examined epidemiological, clinical, and experimental studies from different geographical areas that analyzed the toxic effects of various HMs on human health. It also looked at strategies proposed in these studies to reduce human exposure to harmful levels of HMs via contaminated food, water, air, and consumer products. The health impacts analyzed included effects on mortality, cancer, cardiovascular, neurological, kidney, bone, and developmental outcomes. The sources of exposure evaluated were drinking water contamination, polluted soil, industrial pollution, mining activities, herbal products, and metal cookware. By synthesizing key findings from the extensive literature on heavy metal (HM) toxicity and exposure reduction, this review aims to inform public health policies and interventions focused on protecting human health from the dangers of HM exposure.

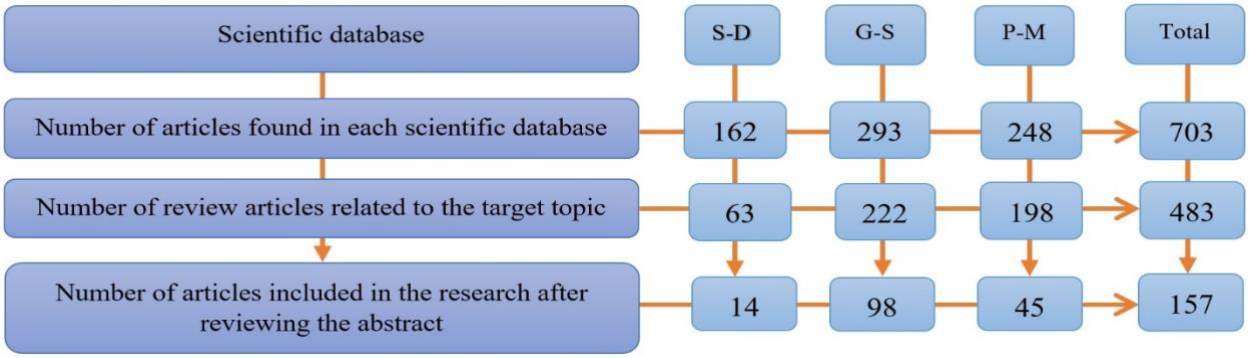

Figure 1. Procedures for selecting and entering studies into our research by searching scientific databases, including Science Direct (S-D), Google Scholar (G-S), and PubMed (P-M)

3. Results and discussions

This systematic review investigated the adverse health and environmental effects of several HMs, including As, Cd, Cr, Pb, Hg, Al, Fe, and Ba. It also aimed to identify effective solutions to reduce exposure. Initial searches yielded 961 potentially relevant articles. After screening titles and abstracts, 703 remained. Further screening for appropriateness eliminated 220 articles, leaving 483 for full-text review. To avoid duplicative findings and focus on current data, 326 additional articles were excluded. The final sample comprised 157 articles selected for analysis.

3.1. Tolerable daily intake of HMs

Health risk from HM contamination in foods can be assessed using the health risk index (HRI). This is calculated by dividing the estimated average daily intake by the tolerable daily intake. An HRI below 1 indicates food safety, while above 1 signals potential health risks (Apau *et al.*, 2014). The provisional tolerable daily intake (PTDI) and weekly intake (PTWI) for HMs were calculated using established methods (Tables 2 and 3).

Table 2. The maximum concentration of HMs for weekly and daily tolerable tolerance of raw black tea.

DDW[a] (unit) / HM	Ni	Co	Cr	Cd	Pb	Ar
PTDI (mg/kg×d)	0.51	0.39	0.18	0.018	0.16	0.006
PTWI (mg/kg×w)	3.60	2.73	1.30	0.13	1.12	0.042

[a]Dose daily and weekly

Table 3. The maximum concentration of HMs for weekly and daily tolerable tolerance of black tea infusion.

DDW[a] (unit) / HM	Ni	Co	Cr	Cd	Pb	Ar
PTDI (mg/kg×d)	0.13	0.073	0.06	0.009	0.002	0.001
PTWI (mg/kg×w)	0.91	0.51	0.42	0.064	0.016	0.009

[a]Dose daily and weekly

Two equations were used to evaluate the health risks from HM contamination in tea:
Equation 1: calculates the provisional tolerable daily intake of HM (mg/kg body weight/day). The PTDI is calculated for an average body weight of 65 kg.
Equation 2: calculates the target hazard quotient (THQ) to assess HM health risks in tea. THQ values <1 indicate low risks, values around 1 indicate chronic risks, and values >10 indicate high risks.

Measuring HM levels in tea is important for evaluating safety and potential health risks. Comparing HM types and concentrations to toxicity thresholds judges the overall safety status of tea samples.

Equation 1: $PTDI = \frac{P \times H}{W}$

P: as per capita consumption of HT, H: as HM concentration, W: as average body weight

Equation 2: $THQ = \frac{P \times H \times D}{L \times W}$

P: as per capita consumption of TH, H: as HM level, D: as duration of TH consumption, L: as average lifespan, W: as body average weight

3.2. Adverse effects and the entry routes of HMs

There are important links between food security, safety, pollution, and public health. In developed nations, major agriculture pollution sources include particle deposition on crops and using industrial effluents as fertilizers. In both developed and developing countries, irrigating with untreated wastewater (WW) causes contamination (Morais *et al.*, 2012).

Consuming contaminated vegetables poses greater health risks than contaminated fruits (Shaheen *et al.*, 2016). Main HM exposure routes are ingestion, inhalation, and skin contact. Increased environmental HM deposition can accumulate in food and drinks, entering the food chain and endangering safety and health (Zwicker *et al.*, 2010). HMs are absorbed and stored faster than excreted, potentially leading to poisoning, illness, or death (W.-Y. Han *et al.*, 2007).

HMs do not break down chemically or biologically, persisting in the environment (Soomro *et al.*, 2008). Even low concentrations have harmful effects due to persistence and bioaccumulation (Kaličanin & Velimirović, 2013). Prolonged exposure raises cancer risks including prostate, liver, lung, nose, laryngeal, and gastrointestinal (GI). HMs accumulate in tissues, potentially causing disorders in skeletal, nervous, cerebral, GI, dermal, renal, reproductive, circulatory systems, and immunodeficiency (Sofuoglu & Kavcar, 2008; Amouei *et al.*, 2012; Zhao *et al.*, 2014; El-Kady & Abdel-Wahhab, 2018).

3.2.1. Arsenic

As is the 20th most plentiful element on our planet and has metallurgical applications due to its semi-metallic properties (Singh *et al.*, 2007). As is present in minimal amounts in TH due to low water solubility. Predominant forms are arsenate III (As^{3+}) and arsenate V (As^{5+}) (Karak & Bhagat, 2010).

Humankind may be exposed to As through natural resources, industrial resources, or unwanted resources. Drinking water (DW) contamination occurs through minerals, chemicals, and pesticides. As exposure is a global issue, especially in Asia and South America where millions consume As-contaminated water for drinking and irrigation (Edition, 2011). The WHO set a 0.01 mg/L maximum for As in DW. Levels 10-100 times higher pose hazards (Hoque *et al.*, 2011).

High As doses via ingestion or inhalation can cause acute to severe poisoning, as shown in Figure 2 (Martin & Griswold, 2009). As impairs cellular respiration, enzymes, and mitosis as a protoplasmic toxin (Amouei et al., 2012). Recently, As exposure has become a key health concern due to associations with decreased male fertility (de Araújo Ramos *et al.*, 2017; Udagawa *et al.*, 2019) and reduced learning in children (Desai *et al.*, 2020; Hamadani *et al.*, 2011).

3.2.2. Cadmium

Cd is a toxic HM with high carcinogenicity (Oh *et al.*, 2015). Environmental Cd exposure is more likely in industrialized nations (J.-X. Han *et al.*, 2009). The WHO set a 0.003 mg/L maximum for Cd in DW (Joint *et al.*, 2007).

Cd is a byproduct of Zn production, first used in World War I to replace tin and as a pigment. Today it is used in batteries and alloys. Ingestion and inhalation are main exposure routes, with acute to severe effects (Figure 2). Cd persists in sediments and soil for years (Bernard, 2008; Mutlu *et al.*, 2012). Herbs uptake and transfer Cd to the food chain gradually (Olympio *et al.*, 2018). Cd accumulates in the body slowly, potentially impairing liver, kidneys, and bones (Boonprasert *et al.*, 2018; Tola *et al.*, 2007).

Cd and Zn have similar oxidation states. Cd can replace Zn in metallothionein, inhibiting Cd's free radical scavenging (Irfan *et al.*, 2013). Cd absorption is low in the gastrointestinal tract (GIT) but high in the lungs. Prenatal Cd increases risks of premature birth, low birth weight, and lower IQ (Henson & Chedrese, 2004; Kippler *et al.*, 2012). High Cd in children raises cancer likelihood and slows growth. Prolonged exposure also increases cardiovascular disease risks (Edition, 2011; Hoque et al., 2011).

Cd is more water-soluble than other Ms. Tobacco provides significant Cd exposure in smokers as it accumulates soil Cd. Non-smokers also ingest Cd through diet, although other absorption is unlikely (Mudgal *et al.*, 2010).

Cd exposure can lead to miscarriage and sexual issues like low semen quality, testicular/ovum defects (Udagawa et al., 2019). Cd rapidly accumulates in tissues, especially testes, strongly impacting reproduction (Cupertino *et al.*, 2017; De Franciscis *et al.*, 2015).

3.2.3. Chromium

Cr, the seventh most abundant element, exists in solid, liquid, and gaseous environmental forms (Monalisa & Kumar, 2013). Cr is widely used in industries like plating, metallurgy, wood protection, tanning, pigments, paints, and paper, contributing to environmental release (Ghani & Ghani, 2011). Industrial discharges and irrigating with

WW/Cr-contaminated water causes Cr transfer from soil to plants to food (Duan *et al.*, 2010).

Cr toxicity depends on the ion's charge. Cr^{3+} has low solubility, mobility, and toxicity. Cr^{6+} is highly water-soluble and toxic (Gardea-Torresdey *et al.*, 2004). Both harm health in excess (Gürkan *et al.*, 2017; Karaulov *et al.*, 2019). High Cr causes reduced root growth, chlorosis, necrosis, and inhibits germination in plants. It accumulates in tissues, causing organ damage and death (Ghani & Ghani, 2011). Compounds with Cr^{6+} like $ZnCrO_4$, $SrCrO_4$, $PbCrO_4$, and $CaCrO_4$, are highly poisonous and cancer-causing, damaging DNA and chromosomes (Matsumoto *et al.*, 2006). Cr^{3+} is an essential nutrient for glucose metabolism in trace amounts. However, Cr^{6+} absorbs faster in the GIT and lungs, increasing toxicity risks (Martin & Griswold, 2009).

The WHO set 0.2-1 μg/g and 0.05 mg/L maximums for Cr in vegetables and DW, respectively (Mahvi *et al.*, 2011; Narin *et al.*, 2004). In oxygen-rich environments, Cr^{3+} oxidizes to the highly toxic and water-soluble Cr^{6+} (Cervantes *et al.*, 2001).

3.2.4. Lead

Pb is a toxic, carcinogenic metal extensively used in industry, causing pollution and health issues globally (Amouei et al., 2012). Major Pb sources include bullet/casting production, dyeing, cable sheathing, battery manufacturing, brass alloys, and gasoline combustion (Pruvot *et al.*, 2006).

Pb limits in food/drink vary - Japan: 20 mg/kg, Canada/Australia/India: 10 mg/kg, Europe/China: 5 mg/kg (Joint et al., 2007). Pb is not biologically essential. High plant Pb, especially in shoots/leaves, creates reactive oxygen species damaging membranes and photosynthesis while suppressing growth (Najeeb *et al.*, 2017).

Pb disrupts nucleic acid metabolism (Achudume & Owoeye, 2010). Pb ions substitute for monovalent cations like Na^+ and divalent cations like Mg^{2+}, Ca^{2+}, Fe^{2+}, impairing protein folding, cell adhesion, apoptosis, transport, enzymes, and neurotransmitters (Flora *et al.*, 2008). Approximately 95% of absorbed Pb precipitates in bones as insoluble phosphate (Papanikolaou *et al.*, 2005).

Historically, Pb pipes contaminated water, causing poisoning (Brochin *et al.*, 2014). Pb toxicity primarily affects the GIT and nervous system (Markowitz, 2000). Fetuses and children are more vulnerable than adults (Sanders *et al.*, 2009). Childhood Pb exposure is linked to increased violence and criminality later in life (J. Sampson & S. Winter, 2018; Meyer & Rogers, 2018).

3.2.5. Mercury

Hg is a silvery, odorless liquid metal that evaporates into an invisible, toxic vapor when heated (C.-W. Chen *et al.*, 2012). The behavior of Hg in different forms is shown in Table 4.

Hg is widely applied in barometers, thermometers, lamps, and batteries. It is mainly available in three forms: metallic elements, inorganic compounds, and organic compounds, each with varying toxicity levels. These Hg forms occur in surface and groundwater, where they are absorbed by organisms and converted to methylmercury (MeHg), disrupting aquatic life. Human MeHg exposure is primarily through consumption of contaminated marine food (Trasande *et al.*, 2005).

Hg is present in beverages and foods at levels ranging from <1 to 50 μg/kg, often higher in seafood, especially fatty fish and fish liver (Reilly, 2006). The WHO sets the maximum permissible Hg level in DW at 0.002 mg/L (Joint & Additives, 2003).

Hg vapors can cause irritating breathing issues like bronchitis and asthma. Hg also damages tertiary and quaternary protein structures and inhibits cellular functions by binding to selenohydryl and sulfhydryl groups, which react with MeHg. Hg disrupts ribosomes, destroys the endoplasmic reticulum, and inhibits natural killer cell activity through effects on transcription and translation (Ynalvez *et al.*, 2016).

Table 4. The performance of Hg in different forms (Patrick, 2002).

Metal state	Hg	MeHg	Non-organic-Hg
Source	Fossil fuels, latex paints, incinerators, thermometers	Pesticides, fish, poultry	Oxidation of Hg, demethylation of MeHg by gut microflora
Absorption pathway	75-85% through inhalation	95-100% through GIT	7-15% through GIT, 2-3% through skin
Distribution areas	Throughout the body, crosses placental and blood-brain barriers	Throughout the body, crosses placental and blood-brain barriers	Kidney accumulation, cannot cross placental or blood-brain barriers
Excretion procedure	Feces, urine, saliva, sweat	10% urine, 90% feces/bile	Feces, urine, saliva, sweat
Causes of toxicity	Conversion to inorganic Hg	Demethylation to inorganic Hg, free radical formation, binds to enzyme/protein thiols	Binds to enzyme/protein thiols

3.2.6. Aluminium

Al, the third most abundant element on our planet, is dispersed in water, soil, and air. The extraction and processing of Al can increase its levels in the environment (Gupta *et al.*, 2013). Al has no biological function and is a toxic metal to humans. The main ways for humans to absorb Al are through inhalation, ingestion, and skin contact. Sources of Al exposure include DW, beverages, food, and Al-containing medications. Al is naturally present in many foods. Al and its compounds are poorly absorbed by the human body (Olaniran *et al.*, 2013).

Al toxicity increases with decreasing pH. Acid rain containing Al ions and increasing atmospheric acidification can have devastating effects on the environment, leading to deforestation, plant poisoning, crop failure, and destruction of aquatic organisms (Barabasz *et al.*, 2002).

Al intoxication results from an interaction between Al and the plasma membrane (Kochian *et al.*, 2005). The effect of Al on nerve cells is similar to that seen in alzheimer's disease. Replacement of Mg^{2+} and Fe^{2+} by Al^{3+} disrupts intercellular communication, cell growth, and gland secretory function (Vardar & Ünal, 2007).

Al poisoning can be exacerbated by living in environments with Al-containing dust, impaired kidney function, hemodialysis, and consuming Al-containing food, drinks or medications (Cannata Andia, 1996).

3.2.7. Iron

Iron, the second most abundant element on Earth, is vital for growth and survival of living organisms. Fe deficiency during growth and adulthood can affect the development and function of the nervous system (Baltussen *et al.*, 2004). Fe is an ideal transition metal for oxidation-reduction reactions because it can readily interconvert between ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions (Phippen *et al.*, 2008).

Lithuanians are exposed to high Fe levels in DW due to accumulation of Fe in Lithuanian groundwater above allowable limits (Grazuleviciene *et al.*, 2009). Environmental Fe deposits can cause significant damage by preventing fish from breathing properly (Clesceri *et al.*, 1998).

If Fe absorbed by the human body fails to bind to proteins, it generates high levels of harmful free radicals that can damage internal organs including the brain, liver, and heart. Unbound ferrous iron converts to ferric iron, disrupting oxidative phosphorylation, releasing hydrogen ions, and increasing metabolic acidity. Free Fe promotes lipid peroxidation, causing severe damage to microsomes, mitochondria, and other cellular components (Albretsen, 2006).

The clinical course of Fe toxicity is divided into four phases: (i) In the first phase (6 hours after Fe overdose), patients primarily exhibit GI symptoms such as diarrhea, vomiting, and GI bleeding. (ii) The second phase (6 to 24 hours) indicates a recovery stage. (iii) The third phase (12 to 96 hours) follows acute symptoms and is characterized by shock, hypotension, malaise, heart damage, liver necrosis, metabolic acidosis, and sometimes death. (iv) The fourth phase (2 to 6 weeks) involves GI ulcer formation. Excess Fe beyond the body's needs increases cancer risk (Nelson, 1992).

The formation of free radicals, including hydrogen peroxide and superoxide, occurs continuously during normal cellular processes. Superoxide releases Fe from ferritin. This free Fe reacts with hydrogen peroxide and superoxide to produce highly toxic hydroxyl radicals (Fine, 2000).

The adverse effects of HMs like lead (Kim *et al.*, 2016; Kuang *et al.*, 2020; Martin & Griswold, 2009; Pfadenhauer *et al.*, 2016;

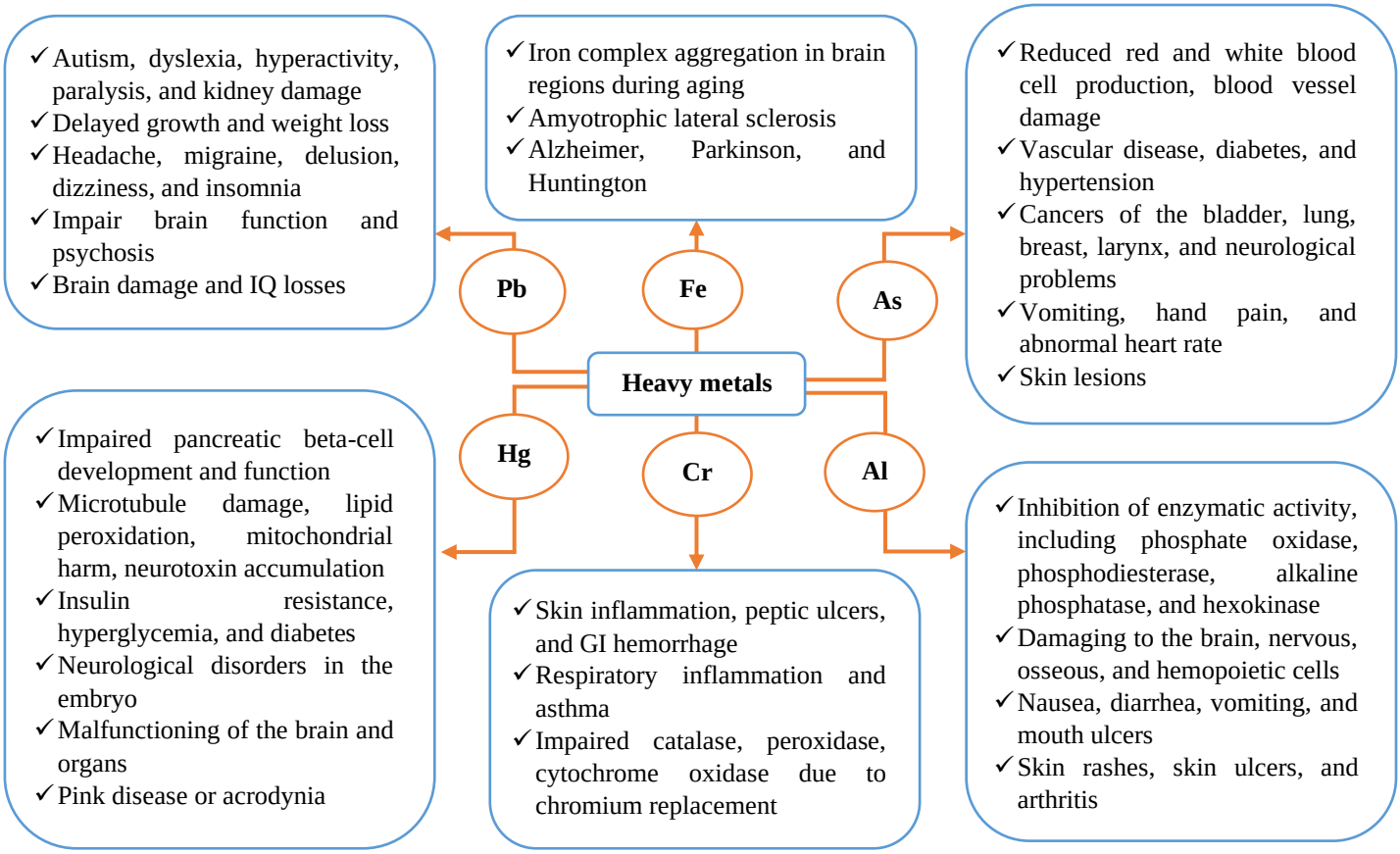

Figure 2. Adverse effects of exposure to HMs including Pb, Hg, Al, Fe, As, and Cr

Reuben *et al.*, 2017; Sanders et al., 2009; Zeng *et al.*, 2019), mercury (Haley, 2005; Morais et al., 2012; Patrick, 2002; Schumacher & Abbott, 2017; Wallin *et al.*, 2017; Qin *et al.*, 2021), aluminium (Barabasz et al., 2002; Krewski *et al.*, 2007), iron (Agrawal *et al.*, 2017; Ayton & Lei, 2014; Ghadery *et al.*, 2015; Li & Reichmann, 2016), arsenic (Roh *et al.*, 2018; Smith *et al.*, 2000; Román-Ochoa *et al.*, 2021; Q. Yang et al., 2022), chromium (Buters & Biedermann, 2017; Nath *et al.*, 2008; Rasoul *et al.*, 2017; Sofuoglu & Kavcar, 2008; Bjørklund *et al.*, 2022) are shown in Figure 2.

3.2.8. Barium

Ba is an unnecessary and rare element in food and DW (Schroeder & Kraemer, 1974). In the environment, Ba is usually found as Ba sulfate and Ba carbonate. Ba sulfate is used in plastics and paints due to its neutrality, low

toxicity, high density, and low solubility. Exposure to Ba occurs through ingestion of food or DW, or breathing Ba-contaminated air. Skin absorption of Ba is negligible. Ba bloodstream entry depends on solubility. Highly soluble Ba compounds like Ba nitrate and chloride can enter faster than insoluble sulfate and carbonate (Landsiedel *et al.*, 2014; F. Yang & Massey, 2019).

The body's reaction to Ba depends on its water solubility. Insoluble Ba compounds have minimal adverse effects. In contrast, soluble compounds are harmful, causing irregular heartbeat, paralysis or death, respiratory inflammation, vomiting, diarrhea, and cramps (Schwotzer *et al.*, 2017; F. Yang & Massey, 2019). Ba has low pulmonary toxicity (Olaniran et al., 2013). The cardiovascular system is a primary Ba toxicity target. Thus, consistent Ba monitoring in DW and food is critical (Afonso *et al.*, 2008). The WHO sets the maximum permissible Ba level in DW at 0.7 mg/L (Organization, 1990).

Ba poisoning occurs by disrupting potassium metabolism through two mechanisms: (i) Blocking potassium pumping channels. (ii) Increasing plasma membrane sodium permeability (F. Yang & Massey, 2019).

3.3. Presence of heavy metals in tea herb

HMs have serious adverse human health and environmental effects. Reducing HM levels in the environment and food chain is considered the most effective mitigation step. Thus, identifying factors causing HM accumulation in plants is a first step. Based on various papers, the following contribute to HM presence in TH: chemical fertilizer, improper waste, and fossil fuel use (Nazemi & Khosravi, 2011); HM presence in soil (Y. Yang *et al.*, 2018), water (Lokeshwari & Chandrappa, 2006), and air (Nabulo *et al.*, 2006); geographical location (Chabukdhara *et al.*, 2016; Saha *et al.*, 2015); planting and harvest time (Shekoohiyan *et al.*, 2012); tea brewing duration (Zazouli *et al.*, 2010); metal processing equipment in production (Seenivasan et al. 2008); HM amount and type (Gardea-Torresdey et al., 2004); and plant genetic diversity (Rattan *et al.*, 2005). These factors cause differences in herb HM concentrations and composition.

3.4. Strategies to control the value of heavy metals in tea herb

The accumulation of HMs in water and soil is one of the chief problems caused by inorganic contaminants. Their presence in high quantities in agricultural soils has substantially impacted food safety and human health. Among various physicochemical methods to reduce HMs in tea brews, the following have been most effective (Oladoye et al., 2022).

HM-contaminated water is a major environmental hazard. Using pre-treated WW effluent for irrigation can reduce HM levels (Cherfi *et al.*, 2015; Khan *et al.*, 2015). Various technologies remove HMs from WW, including solvent extraction, solid-phase extraction, gravity precipitation, electrodialysis, ion exchange, reverse osmosis, chemical precipitation, flotation, membrane separation, and adsorption (Bishnoi *et al.*, 2004; Dimoglo *et al.*, 2019). Ion exchange, adsorption, and membrane purification are among the most effective methods (F. Fu & Wang, 2011).

Plant roots play a vital role in HM uptake and transport (Antoniadis et al., 2017). HMs have low mobility in alkaline soils and are poorly absorbed by herbs. However, HM mobility increases in acidic soils, allowing root absorption and translocation to other plant parts. Soil acidification directly impacts HM transfer to TH (Mandiwana *et al.*, 2011; Moseti *et al.*, 2013). One study found liming acidic soil reduced Pb accumulation in TH (W.-Y. Han et al., 2007). Acid rain significantly lowers soil pH, so cultivating TH in industrial areas should be avoided or products experimented for HMs (Barabasz et al., 2002). Growing plants away from highways, mines, and industrial areas substantially reduces HM levels in products, as proximity to mines and roads increases Cu, Cd, Pb, Cr, and As in TH (Obiora *et al.*, 2016; Petit *et al.*, 2013; Rock *et al.*, 2017).

Longer brewing times directly increase HM concentration in tea (Shekoohiyan et al., 2012). Decreasing phosphorous fertilizer application also lowers HM levels in TH (Tola et al., 2007).

As transfer from leaves to tea depends on water temperature and steeping time. Higher temperatures reduce As extraction (Karak & Bhagat, 2010).

Tea from China and Japan, the leading exporters, should be consumed carefully due to their high environmental Cd contamination from industrialization (J.-X. Han et al., 2009). TH tannins form HM complexes, reducing their levels (Yuan *et al.*, 2007).

Cd concentrations in TH are low due to limited root-to-shoot translocation. TH roots also accumulate Cd, preventing transfer to upper plant organs (Gardea-Torresdey et al., 2004).

Phytoremediation uses plants to remove and accumulate HMs in harvestable sections. Strategies include phytoextraction, phytoevaporation, phytostabilization, rhizodegradation, and rhizofiltration. Using edible plants introduces HMs into the food chain, so ornamentals are better for phytoextraction to reduce soil HMs and prepare for food crops (Shao *et al.*, 2022; L. Wang *et al.*, 2022).

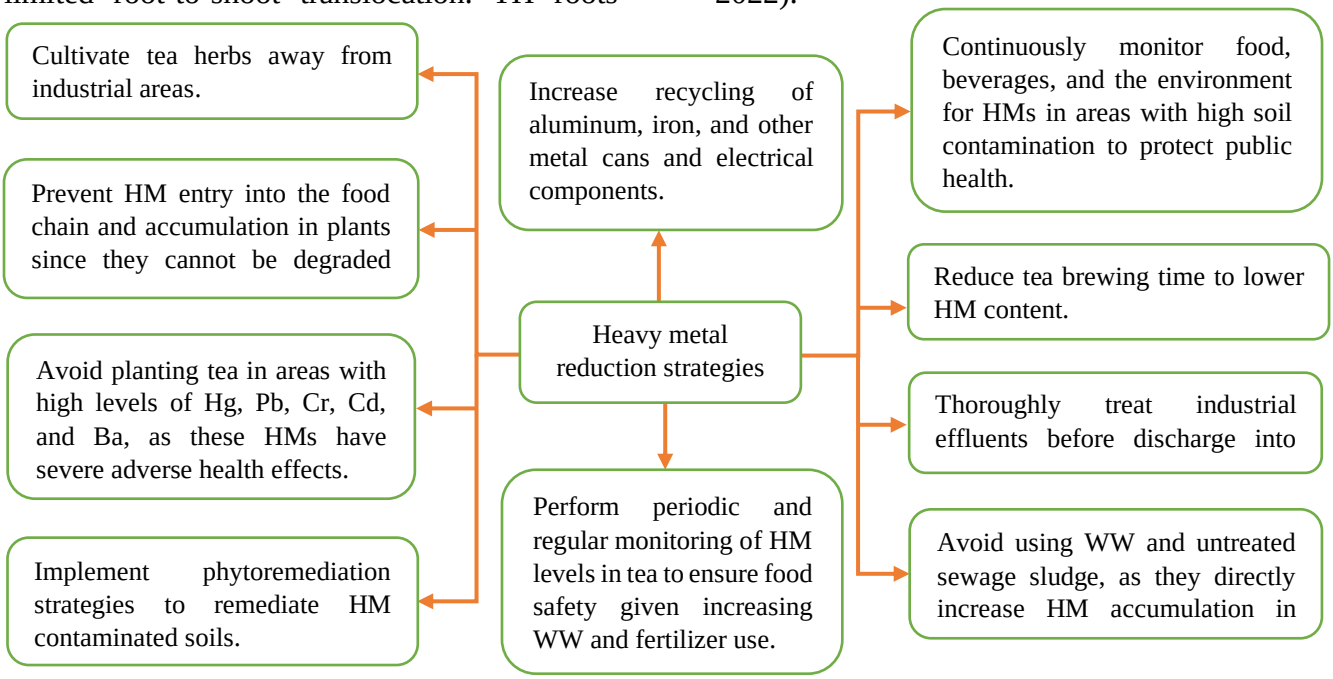

Figure 3. Main strategies to reduce exposure to HMs

4. Conclusions

The global concentration of HMs in soil and water has been increasing due to their stable, non-degradable nature. HM pollution and remediation remain major challenges for researchers. Consuming brewed tea containing HMs is a primary route of human exposure. HMs can also enter the body through contaminated air inhalation, skin contact, food ingestion, and drinking contaminated water. Once inside, HMs accumulate in fat and bone, then slowly release, potentially causing various illnesses.

Measures to reduce HM levels in the environment and TH include: recycling metal cans; properly treating industrial WW; avoiding fertilizers that acidify soil; avoiding planting TH in areas with high Hg, Pb, Cr, Cd, or Ba; growing TH in uncontaminated soil; and continuously monitoring HM levels in food, DW, and the environment.

In summary, HM contamination of TH is a complex issue involving many human and environmental factors. Careful monitoring, sustainable agricultural practices, and waste management can help mitigate risks. Ongoing research into optimal cultivation conditions and remediation strategies is important to support the safe production and consumption of TH.

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Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

The present article is taken from a research project with the number IR.QUMS.REC.1400.049. At this moment, we attempt to extend our gratitude to the Student Research Committee of Qazvin University of Medical Sciences, the Faculty of Health, and all the regarded persons, who assisted us in conducting this research.

Abbreviation

Tea Herb: TH, Heavy Metals: HMs, Heavy Metal: HM, Metals: Ms, Arsenic: As, Aluminum: Al, Chromium: Cr, Cadmium: Cd, Zinc: Zn, Lead: Pb, Mercury: Hg, Barium: Ba, Target hazard quotient: THQ, Health Risk Index: HRI, Provisional Tolerable Daily Intake: PTDI, Provisional Tolerable Weekly Intake (PTWI), World Health Organization: WHO, Drinking-Water: DW, Gastrointestinal: GI, Gastrointestinal Tract: GIT, Methylmercury: MeHg, Wastewater: WW

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

SINGLE AND PARALLEL DYE-BASED REAL-TIME PCR DETECTION OF FOODBORNE PATHOGENS *SALMONELLA ENTERICA* AND *CAMPYLOBACTER JEJUNI*

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https://doi.org/10.34302/crpjfst/2023.15.4.4

Article history:
Received: 9 September 2022
Accepted: 10 October 2023

Keywords:
Food safety;
Foodborne bacteria;
Real-time PCR;
Dye-based;
Artificial contamination.

ABSTRACT

Salmonella enterica and *Campylobacter jejuni* are some of the common foodborne pathogens causing gastrointestinal illnesses worldwide. The development of sensitive and specific detection methods is essential to ensure food safety. Dye-based real-time PCR assay using SYBR™ GreenER™ dye was developed for the detection of *Salmonella enterica* and *Campylobacter jejuni*. Designed primer sets specifically targeting the genes *ompF* and *omp50* in *Salmonella* and *Campylobacter*, respectively, were utilised in the study. The assay was able to detect *Salmonella* and *Campylobacter* at as low as 50 fg/µl and 10 fg/µl, respectively. Specificity analysis performed using 16 different bacterial strains to check for cross-reactivity with the respective bacteria found the assay to be specific to *Salmonella* and *Campylobacter*. The assay successfully detected *Salmonella enterica* in inoculated food at as low as 5 fg/reaction for some food samples. Meanwhile, the detection limit for *Campylobacter jejuni* in all inoculated food samples was 2000 fg/reaction. The coefficient variations (CV%) of the assays for both pathogens indicated that the assays were highly reproducible. Therefore, the developed real-time PCR assays for both *Salmonella enterica* and *Campylobacter jejuni* detections were specific and sensitive and can be used for rapid screening to detect these foodborne pathogens.

1.Introduction

Foodborne pathogens, such as bacteria, viruses and parasites, can cause gastrointestinal illnesses in humans. Factors such as globalization, population movement, and supply chain can introduce pathogens to different regions causing emerging infections. Approximately 600 million people, which is 1 in 10 people worldwide, have foodborne illnesses due to the consumption of contaminated food, and 420,000 people die from it annually (World Health Organization, 2022). Most foodborne illnesses are acute, with an infected person typically showing symptoms lasting four to seven days. The most common symptoms include fever, diarrhoea, headache, abdominal cramps, nausea, and vomiting. The disease is generally self-limiting, and most do not require medical treatment to recover. However, some foodborne diseases, such as gastroenteritis, may

lead to severe complications, thus, requiring medical attention, and untreated foodborne infections can lead to death, especially in immunocompromised patients and older adults. Hence, the disease can still burden public health in developed and developing countries.

The typical route for most foodborne illnesses in humans is through the consumption of contaminated food and water, also known as faecal-oral transmission. Other transmission routes include contact with an infected person or animals through zoonosis and via contaminated surfaces (North Dakota Department of Health, 2020). Fresh produce, such as fruits and vegetables, has a higher risk of contamination by foodborne pathogens due to the many access points for contamination from pre-harvesting, harvesting, packaging, and food preparation processes. Poor quality of irrigated water can easily transmit pathogens to fresh produce. Moreover, the transmission of foodborne pathogens can occur during the production, distribution, handling, and cooking of raw meat, such as poultry and beef (Bosch *et al.*, 2011).

Some of the most common foodborne pathogens include *Salmonella* and *Campylobacter* (North Dakota Department of Health, 2020). *Salmonella* is a Gram-negative and flagellated anaerobic bacterium. It causes gastroenteritis called salmonellosis, whereby the gastrointestinal tract is infected with *Salmonella* bacteria. Symptoms caused by *Salmonella* infection begin six hours to six days after the infection and can last up to four days to one week (Centers for Disease Control and Prevention, 2013). These symptoms include diarrhoea, fever, abdominal cramps, and nausea. *Salmonella enterica* (*S. enterica*) is the prominent cause of foodborne illness around the world (Silbergleit *et al.*, 2020). Approximately 93.8 million cases of *Salmonella* were reported every year worldwide, with 155,000 mortalities, of which 80.3 million cases were foodborne (Ao *et al.*, 2015; Majowicz *et al.*, 2010). Among those, 17 to 33 million cases are *Salmonella*-caused typhoid fever and diarrhoeal diseases, with 600,000 mortalities annually (Ranjbar *et al.*, 2014). The detection of *Salmonella* species is crucial, as the method must be able to detect the specific serovars, to prevent infection by the pathogen in food which could lead to illnesses and, if not controlled, an outbreak.

Campylobacter is Gram-negative, spiral-shaped, and can move in a corkscrew-like motion (Chon *et al.*, 2020; Frasao *et al.*, 2017). Gastroenteritis caused by *Campylobacter* bacteria is known as campylobacteriosis, which causes diarrhoea that can turn severe, fever, abdominal cramps and vomiting (Centers for Disease Control and Prevention, 2019; Same *et al.*, 2018). These symptoms typically begin two to five days after infection and can last up to one week (Centers for Disease Control and Prevention, 2019). *Campylobacter* infection is additionally a common antecedent of Guillain-Barré syndrome, an autoimmune disorder of the peripheral nervous system and is known to mainly cause acute flaccid paralysis (Centers for Disease Control and Prevention, 2022; Rees *et al.*, 1995; Sejvar *et al.*, 2011). *Campylobacter* infections that lead to Guillain-Barré syndrome have shown a slower recovery rate, degeneration of axons, and severe residual disability (Rees *et al.*, 1995). *Campylobacter* has also been reported as one of the etiological factors of Crohn's disease and ulcerative colitis in humans (Moore *et al.*, 2005). Moreover, other clinical manifestations of the infection include Bell's palsy, Miller Fisher syndrome, reactive arthritis and acute febrile disease. *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are the most common strains to cause campylobacteriosis in humans (Frasao *et al.*, 2017). The Centers for Disease Control and Prevention (CDC) has reported that *C. jejuni* is the second biggest cause of foodborne illnesses, with more than 1.5 million cases reported in the United States every year (Osaili and Alaboudi, 2016; Tack *et al.*, 2019). Approximately 9% of gastroenteritis was caused by *Campylobacter* spp., with 15% of the cases leading to hospitalisations (Scallan *et al.*, 2011).

The growing concern around outbreaks of foodborne illnesses demanded better control of food safety, including food handling, processing, storage and packaging. This highlighted the need to analyse food to ensure its safety and quality (Salihah *et al.*, 2016).

Molecular-based techniques such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) have made it easier for microbiologists to identify and detect foodborne pathogens (Saravanan *et al*., 2021). ELISA is a highly specific immunological approach for detecting foodborne pathogens (Saravanan *et al*., 2021); however, ELISA has low sensitivity, and cross-reaction between the antibodies and closely related antigens may occur, leading to false results (Law *et al*., 2015). PCR is a widely used method mediated by specific primers and requires thermostable DNA polymerase to amplify the target DNA sequence *in vitro.* Conventional PCR is a qualitative approach requiring gel electrophoresis to detect the presence of the PCR products (Kadri, 2019). Real-time PCR is the advanced variation of PCR whereby the target DNA sequence is amplified in real-time at the end of every amplification cycle. Among the molecular detection methods, real-time PCR is rapid and has higher sensitivity due to its ability to detect very low concentrations of the target sample. Thus, it has become the new "gold standard" for detecting and quantifying foodborne pathogens in food and microbial population studies with absolute and relative quantification (Malorny *et al*., 2008).

In comparison to conventional PCR, real-time PCR can be used as a one-step method without needing post-PCR analysis to determine the presence of the PCR products. Thus, real-time PCR is less labour-intensive and less prone to contamination due to the reduced number of steps. Unsurprisingly, the use of real-time PCR for *Salmonella* detection is favoured due to its accuracy, rapidity and sensitivity (Rodríguez-Lázaro *et al*., 2003) and has been utilised to detect and quantify *Salmonella* spp. from sheep faeces and tissue samples with 91% sensitivity and 100% specificity (Parker *et al*., 2020). Similarly, the detection of *Campylobacter* by real-time PCR has been reported to produce rapid and sensitive results compared to conventional culture methods (Sails *et al*., 2003; Vencia *et al*., 2014; Yang *et al*., 2003). Methods employing biosensors have facilitated the speedy detection of foodborne pathogens. However, the biosensor's practicality is dependent on the samples' condition, and the non-interaction of the target with the bioreceptor may contribute to inaccurate data and findings (Saravanan *et al*., 2021). Being broadly applied for pathogen detection, real-time PCR has been chosen as the detection method in this study. Furthermore, real-time PCR is relatively more straightforward than other detection methods, and it serves a central role in DNA amplification, making it a crucial application in pathogen detection.

The purpose of this study was to develop rapid and sensitive real-time PCR assay methods for the detection of the two most common foodborne pathogens found in Brunei Darussalam, *S. enterica* and *C. jejuni*, using novel designed primers that specifically identify targeted genes of the pathogens. The majority of the developed *Salmonella* PCR assays amplify virulence genes like invasion gene (*inv*), fimbriae Y protein gene (*fimY*), and type-1 fimbrial protein subunit A gene (*fimA*) (Azinheiro *et al*., 2020; Wang *et al*., 2018; Zhai *et al*., 2014). Target genes for *Campylobacter* PCR assays include 16S ribosomal RNA (*16S* rRNA), hippuricase (*hipO*), flagellin (*flaA*), and elongation factor G (*fusA*) (Hong *et al*., 2007; Perelle *et al*., 2004; Reis *et al*., 2018). To the best of found knowledge, only a number of studies have investigated the use of outer membrane protein F gene (*ompF*) and 50kDa outer membrane protein gene (*omp50*), which are the target genes in this study, for the detection of *S. enterica* and *C. jejuni*, respectively. The assays were developed in such a way that both assays employed the same temperature protocol; hence, the detection of the two bacterial species could be conducted simultaneously in a single PCR run or it can be performed separately. Assessment of assays' performance demonstrated better sensitivity and applicability for *Salmonella* and *Campylobacter* detection in foods.

2. Materials and methods

2.1. Bacterial strains and bacterial culture

The genomic DNA (gDNA) of *S. enterica*, *C. jejuni* and 15 other bacterial strains were

obtained from American Type Culture Collection (ATCC, USA) and are listed in Table 1. The strains were stored at -80 °C and aliquoted to tubes to be used as working stock and kept at -30 °C. All strains were tested with gel electrophoresis to check the quality of the gDNA using 0.8% agarose gel and electrophoresed at 90 V for 45 min. Quick-load 2-log DNA ladder (New Englands Biolabs, USA) was used to determine the molecular weight of the gDNA. NanoPhotometer™ P-Class (Implen, Germany) was used to measure the concentration and purity of the gDNA of all bacterial strains. To prepare artificially contaminated food samples, *S. enterica* subsp. enterica serovar Paratyphi ATCC strain number 9150D-5 bacterial cultures in blood agar (BA) plates and *C. jejuni* AS-84-79 ATCC strain number 33292D-5 bacterial cultures in Campylobacter Selective agar (CAMPY) plates were received from Microbiology Lab, Department of Laboratory Services, Ministry of Health, Brunei. The bacteria colony was cultured in buffered peptone water (BPW) and incubated at 37 °C for 24 hours.

Table 1. List of bacterial strains used in this study

Bacterial Strains	ATCC strain number
Salmonella enterica subsp. enterica serovar Paratyphi	ATCC 9150D-5
Campylobacter jejuni AS-84-79	ATCC 33292D-5
Streptococcus pyogenes group A	ATCC 19615D-5
Yersinia enterocolitica subsp. enterocolitica	ATCC 27739D-5
Escherichia coli H10407	ATCC 35401D-5
Bacillus subtilis 168	ATCC 23857D-5
Shigella flexneri type 2 24570	ATCC 29903D-5
Clostridium perfringens	ATCC 13124D-5
Aeromonas hydrophila CDC 359-60	ATCC 7966D-5
Plesiomonas shigelloides	ATCC 51903D
Mycobacteria avium K-10	ATCC BAA-968D-5
Cronobacter sakazakii 2001-10-01	ATCC BAA-894D-5
Escherichia coli FDA Seattle 1946	ATCC 25922D-5
Staphylococcus epidermidis	ATCC 12228D-5
Pseudomonas aeruginosa Boston 41501	ATCC 27853D-5
Legionella pneumophila Philadelphia-1	ATCC 33152D-5
Bacillus subtilis subsp. spizizenii	ATCC 6633D-5

Table 2. List of primer pairs designed for the detection of *S. enterica* and *C. jejuni*

Bacteria species	Target gene	Primer Sequences (5' – 3')	Location	Amplicon length (bp)
S. enterica	*ompF*	F: CAACGACCGGCGATAGTAAA R: ATTCCCACTGACCGAAACC	137 – 157 223 – 242	105
C. jejuni	*omp50*	F: GTAGGCGGACGCTATGATTT R: GTTGATACTTGGACGGCTCATA	1143 – 1163 1220 – 1242	99

2.2. Primer design of *S. enterica* and *C. jejuni*

The *ompF* and *omp50* genes in *S. enterica* (Accession number CP035301.1) and *C. jejuni* (Accession number AJ582064.1), respectively, were targeted, and primer pairs were designed accordingly (Tatavarthy and Cannons, 2010; Dedieu *et al*., 2004). DNA sequences of the target genes were derived from Standard Nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The

primer pairs were designed using PrimerQuest Tool at Integrated DNA Technologies (IDT PTE, Singapore) website : (https://www.idtdna.com/Primerquest/Home/Index). *In silico* PCR amplification was performed to confirm the specificity and compatibility of the designed primers for *Salmonella* and *Campylobacter* detection : (http://insilico.ehu.es/PCR/).

Designed primer sets for *S. enterica* and *C. jejuni* presented in Table 2 were purchased from SBS Genetech Co., Ltd. (Beijing, China) and Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China), respectively. Lyophilised primers were then suspended in Tris-EDTA (TE) buffer (10 mM, pH 8, 1 mM) at their respective amounts.

2.3. Real-time PCR protocol

For each real-time PCR reaction, a total volume of 20 µl containing 10 µl of 2× SYBR™ Select Master Mix (Applied Biosystems, Thermo Fisher Scientific), 0.5 µl of 10 µM forward and reverse primers, 5 µl nuclease-free water and 4 µl DNA template was set up. The real-time PCR assay was performed on Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, USA), with the following thermal cycling conditions: 50 °C for 5 min, followed by 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The melt curve was set after amplification at 95 °C for 5 s, 60 °C for 1 min, followed by 95 °C for 30 s and finally 60 °C for 15 s. All real-time PCR runs include nuclease-free Ultrapure MilliQ water as negative control and *S. enterica* subsp. enterica serovar Paratyphi ATCC 9150D-5 and *C. jejuni* AS-84-79 ATCC 33292D-5 as the positive control for *S. enterica* and *C. jejuni* detection, respectively.

2.4. Specificity analysis

The specificity analysis of the real-time PCR assay was performed against 1×10^2 pg/µl of the bacterial strains (Table 1). The PCR products were validated and confirmed by electrophoresis in 2% agarose gel stained with 1.5 µl FloroSafe DNA stain (1st BASE, Singapore). The gel was electrophoresed in TBE buffer at 90 V for 45 to 50 min and subsequently visualised under UV light to obtain the gel image.

2.5. Sensitivity analysis

The gDNA of *S. enterica* subsp. enterica serovar Paratyphi ATCC 9150D-5 and *C. jejuni* AS-84-79 were diluted to 10-fold serial dilutions from 1×10^6 fg/µl to 1 fg/µl with TE buffer. Serial dilutions were amplified in triplicates in an assay following the real-time PCR protocol for subsequent determination of the limit of detection (LOD). Data obtained from the amplification plot was subsequently used to plot the standard curve, which will be used to further validate the real-time PCR assay.

2.6. Validation of real-time PCR assay

Standard curves were plotted, and the R^2 value and PCR efficiency were determined to validate the real-time PCR assays. The standard curves were plotted using the 10-fold serial dilutions of respective gDNA strains, *S. enterica* and *C. jejuni*, ranging from 1×10^6 fg/µl to 1 fg/µl. PCR efficiency (E) was calculated using the following equation:

$$E = \left(10^{(-1/gradient)} - 1\right) \times 100\% \quad (1)$$

where the gradient is the slope of the curve.

To determine the reproducibility of the assay, the intra- and inter-assay coefficient variations (CV%) were calculated by dividing the analysed sample's standard deviation by its average.

2.7. Preparation of artificially contaminated food samples

Bacterial cell cultures of *S. enterica* subsp. enterica serovar Paratyphi on BA agar plate and *C. jejuni* on CAMPY agar plate were obtained from Microbiology Laboratory, Department of Laboratory Services, Ministry of Health, Brunei. The serial dilutions of *Salmonella* and *Campylobacter* culture were performed by pre-enrichment to obtain the 10-fold serial dilution suspensions, which were then incubated at 37 °C for 24 h. The concentration range of serial dilutions was made 1 to 1×10^6 cells/ml in which one cell of *S. enterica* and *C. jejuni* was equivalent to approximately 5 fg and 2 fg of DNA, respectively. Food samples were diced and autoclaved at 121 ℃ for 15 min. 22.5 ml

BPW was added to 2.5g of the autoclaved food sample to yield a dilution of 1:10 (w/v), and 500 µl of the diluted culture was inoculated to the sample. After incubation for 24 h at 37 °C, 1 ml of the sample was heat-treated at 100 °C for 10 min, followed by DNA extraction using Purelink™ Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, USA).

2.8. Detection of *S. enterica* and *C. jejuni* in artificially contaminated food

Following DNA extraction, 4 µl of the extracted DNA was used as the template for the real-time PCR assay. Autoclaved sterile food samples without artificially inoculated bacterial culture and nuclease-free Ultrapure MilliQ water were used as negative controls. The detection of pathogens in artificially contaminated food was performed in triplicates using the real-time PCR protocol.

3. Results and discussions

3.1. Specificity analysis and primer designing

Primer sets that target the *ompF* and *omp50* genes of *Salmonella* and *Campylobacter*, respectively, were confirmed to be homologous to *S. enterica* and *C. jejuni* via *in silico* PCR amplification. As shown in Table 3 and the amplification plot in Figure 1, *in vitro* analysis of the primer sets specificity revealed no cross-

Table 3. Specificity analysis of *S. enterica* and *C. jejuni*

Bacterial strains	**Cross-reactivity analysis[a]**	**Cross-reactivity analysis[b]**
Salmonella enterica subsp. enterica serovar Paratyphi	+	-
Campylobacter jejuni AS-84-79	-	+
Streptococcus pyogenes Group A	-	-
Yersinia enterocolitica subsp. enterocolitica	-	-
Escherichia coli H10407	-	-
Bacillus subtilis 168	-	-
Shigella flexneri type 2 24570	-	-
Clostridium perfringens	-	-
Aeromonas hydrophila CDC 359-60	-	-
Plesiomonas shigelloides	-	-
Mycobacterium avium K-10	-	-
Enterobacter sakazakii 2001-10-01	-	-
Escherichia coli FDA Seattle 1946	-	-
Staphylococcus epidermidis	-	-
Pseudomonas aeruginosa Boston 41501	-	-
Legionella pneumophila philadelphia-1	-	-
Bacillus subtilis subsp. Spizizenii	-	-

[a] – Cross-reactivity for *S. enterica* detection, [b] – Cross-reactivity for *C. jejuni* detection, + showing positive amplification, - showing negative amplification

reactivity with non-target bacterial species. The specificity assay was performed in triplicates and further confirmed using 2% agarose gel electrophoresis, which shows positive bands for the target bacterial strains and no bands for the non-target strains. By allowing substrates to cross the membrane, *ompF* contributes to *Salmonella*'s physiology (Abd El Tawab *et al*., 2016; Elkenany *et al*., 2019). Studies on the *ompF* gene found that the gene is not identified in other prevalent foodborne pathogens such as *Vibrio* spp., *Listeria* spp., *Staphylococcus* spp., *Bacillus* spp., *Shigella* spp., *Escherichia coli*, and *Campylobacter* spp. (Abd El Tawab *et al*., 2016; Tatavarthy and Cannons, 2010). Similarly, the *omp50* gene was discovered to be

Campylobacter-specific (Dedieu *et al*., 2004; Xia *et al*., 2013). As the genes are specific to the targeted pathogen species, one might argue that the specificity of the P2ompF and Pomp50 assays to detect *S. enterica* and *C. jejuni*, respectively, is expected to be high.

3.2. Sensitivity analysis and limit of detection

Sensitivity analysis using different concentrations of the pathogens' gDNA from 1×10^6 fg/µl to 1 fg/µl was performed in triplicate, with three replicate measurements taken for each serial dilution (Table 4). LOD is

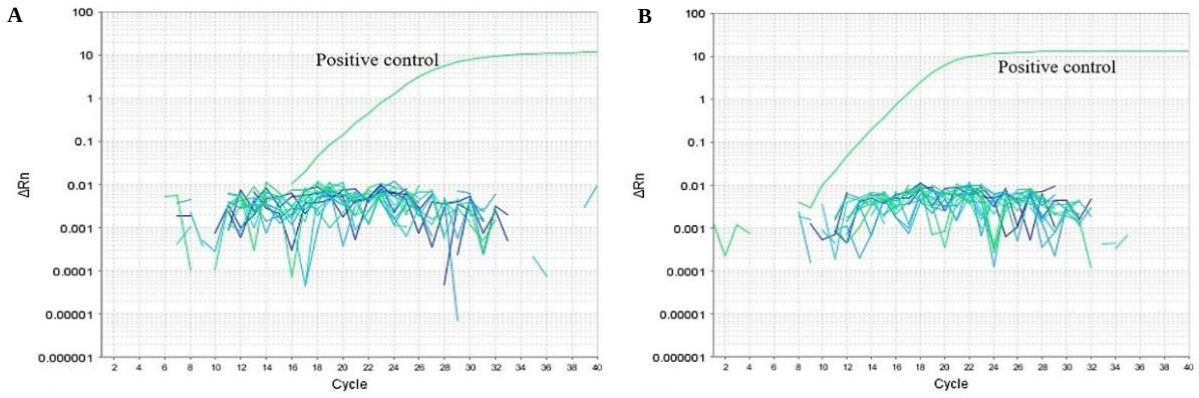

Figure 1. Amplification plot for specificity analysis. A – P2ompF assay. B – Pomp50 assay

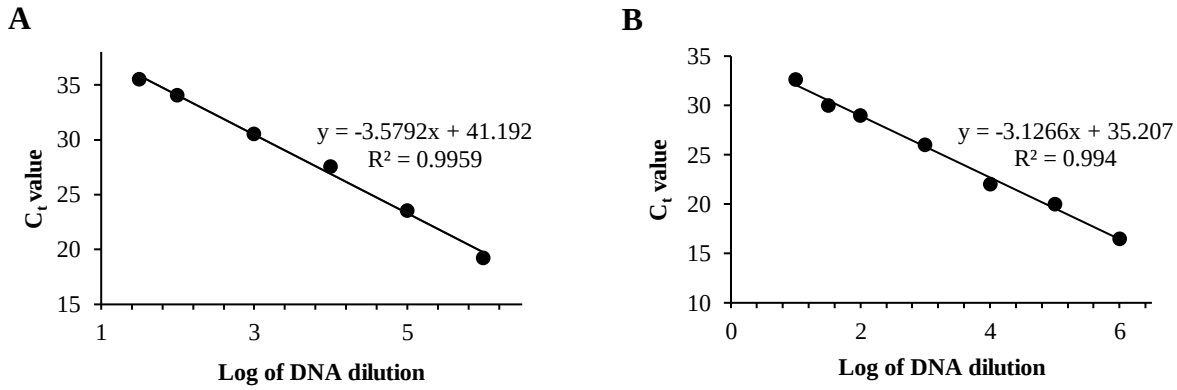

Figure 2. Standard curves of the real-time PCR assays. A – P2ompF assay. B – Pomp50 assay

the lowest analyte concentration at which positive amplification can be obtained with at least a 95% confidence level (US FDA, 2020). The LOD of the P2ompF assay for *S. enterica* was 50 fg/µl. Compared to previous research that targets the virulence genes, the limit of the P2ompF assay is lower. Azinheiro *et al*. (2020), Wang *et al*. (2018), and Zhai *et al*. (2014), reported a limit of 1.6 pg/µl, 10^{-3} ng/µl, and 1.87 pg/µl *Salmonella* gDNA, respectively. The Pomp50 primer set used in this study successfully detected as little as 10 fg/µl of *C. jejuni*, whereas the primers utilised by Reis *et al*. (2018) to target the *fusA* gene of *C. jejuni* achieved an analytical sensitivity of 200 fg.

3.3. Validation of real-time PCR assay

The performance of the assay was validated by evaluating the amplification plot and the standard curve (Figure 2). The standard curve was generated by plotting the 10-fold serial dilutions of the gDNA of each bacterial strain, *S. enterica* and *C. jejuni*. The PCR assay for *S. enterica* detection achieved a PCR efficiency of 91% and an R^2 of 0.9959 (Table 5). The intra- and inter-assay coefficient variations (CV%) were calculated as 0.784 ± 0.194 and 0.988 ±

0.243, respectively. On the other hand, the PCR assay for *C. jejuni* showed an efficiency of 104% and an R^2 of 0.994 with intra- and inter-assay coefficient variations of 0.639 ± 0.139 and 0.658 ± 0.139, respectively. The assays' efficiency and R^2 value were within the recommended range of E = 90 to 110% and $R^2 > 0.99$ (Johnson *et al*., 2013), demonstrating that the designed primer sets were of high quality. Furthermore, as the intra- and inter-assay coefficient variations (CV%) were less than 1%, the developed PCR assays were indicated to be highly reproducible and repeatable (Pfaffl, 2004).

Table 4. Sensitivity analysis of *Salmonella* and *Campylobacter* real-time PCR assay, ratio of positive amplification, copy number and LOD

Bacteria	Assay	Concentration[a] [fg/µl]	Ratio of positive amplification[b]	Copy number[c]	LOD[d] [fg/µl]
S. enterica	P2ompF	1×10^6	6/6	4.41×10^5	50
		1×10^5	9/9		
		1×10^4	9/9		
		1×10^3	9/9		
		1×10^2	9/9		
		10	4/9		
		1	0/9		
C. jejuni	Pomp50	1×10^6	6/6	9.36×10^4	10
		1×10^5	9/9		
		1×10^4	9/9		
		1×10^3	9/9		
		1×10^2	9/9		
		10	9/9		
		1	4/9		

[a] – serial dilutions of respective *S. enterica* and *C. jejuni* in fg/µl, [b] – ratio of positive amplification per 3 individual reactions, [c] – copy number per bacterial cell, [d] – limit of detection of assay

Table 5. PCR efficiency, R^2 value and intra- and inter-assay coefficient variations (CV%) for P2ompF and Pomp50 assays

Bacteria	Assay	Concentration[a] [fg/µl]	R^2 value	PCR Efficiency (E)	Mean CV% ± SD[b]	
					Intra-assay	Inter-assay
S. enterica	P2ompF	1×10^6	0.9959	91%	0.784 ± 0.194	0.988 ± 0.243
		1×10^5				
		1×10^4				
		1×10^3				
		1×10^2				
		10				
		1				
C. jejuni	Pomp50	1×10^6	0.994	104 %	0.639 ± 0.139	0.658 ± 0.139
		1×10^5				
		1×10^4				
		1×10^3				
		1×10^2				
		10				
		1				

[a] – serial dilutions of *S. enterica* and *C. jejuni* in fg/µl, [b] – standard deviation

Table 6. The LOD of *Salmonella enterica* in artificially contaminated food samples

Food sample	Concentration[a] [fg/reaction]	Ratio of positive amplification[b]	LOD[c] [fg/reaction]
Lamb	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Fish	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Chicken	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Beef	5×10^6	3/3	5
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	3/3	
Pork	5×10^6	3/3	50
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	3/3	
	5	0/3	
Processed Fish	5×10^6	3/3	500
	5×10^5	3/3	
	5×10^4	3/3	
	5×10^3	3/3	
	5×10^2	3/3	
	50	1/3	
	5	0/3	

[a] – approximate quantity of gDNA of *S. enterica* subsp. Enterica serovar Paratyphi ATCC 9150D-5 in fg/reaction, [b] – ratio of positive amplification per 3 individual reactions, [c] – limit of detection of the assay

Table 7. The LOD of *Campylobacter jejuni* in artificially contaminated food samples

Food sample	Concentration[a] [fg/reaction]	Ratio of positive amplification[b]	LOD[c] [fg/reaction]
Lamb	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	
Fish	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	0/3	
	20	0/3	
	2	0/3	
Chicken	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	
Beef	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	
Pork	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	0/3	
	20	0/3	
	2	0/3	
Processed Fish	2×10^6	3/3	2000
	2×10^5	3/3	
	2×10^4	3/3	
	2×10^3	3/3	
	2×10^2	1/3	
	20	0/3	
	2	0/3	

[a] – approximate quantity of gDNA of *C. jejuni* AS-84-79 ATCC 33292D-5 in fg/reaction, [b] – ratio of positive amplification per 3 individual reactions, [c] – limit of detection of assay

3.4. Detection of *S. enterica* and *C. jejuni* in artificially contaminated food

To determine the practicality of the real-time PCR assay to detect *S. enterica* and *C. jejuni* in foods, food samples artificially inoculated with the bacteria were prepared and analysed using the proposed techniques. Detection of *Salmonella* and *Campylobacter* in artificially contaminated lamb, fish, chicken, beef, pork, and processed fish was performed with 4 µl of extracted DNA using respective primer sets. Autoclaved sterile food samples without artificially inoculated bacterial culture and nuclease-free Ultrapure MilliQ water were used as negative controls in which no amplification was observed for both samples. The real-time PCR assay for *S. enterica* reached a detection limit of as low as 5 fg/reaction for lamb, fish, chicken, and beef. However, the LOD for pork and processed fish was 50 fg/reaction and 500 fg/reaction, respectively (Table 6). Meanwhile, the real-time PCR assay for *Campylobacter* detected as low as 2000 fg/reaction in all the inoculated food samples (Table 7).

Taking into account that 5 fg of *S. enterica* gDNA corresponds to one cell, the P2ompF assay could detect 1 cell/ml of *S. enterica* in lamb, fish, chicken, and beef. In contrast, a minimum of 10 cells/ml and 100 cells/ml of *S. enterica* was successfully detected in pork and processed fish, respectively. Considering that one cell of *C. jejuni* is equivalent to approximately 2 fg gDNA, the Pomp50 assay detected 1000 cells/ml of *C. jejuni* in all the inoculated food samples. The detection limits in foods achieved by the assays are comparable to published works. A real-time PCR assay developed by Alves *et al*. (2016) detected 1 CFU/ml of *Salmonella* spp. and *Campylobacter* spp. in spiked chicken meat. Toplak *et al*. (2012) were able to detect 10 to 10000 CFU/ml of *C. jejuni* in spiked tap water and chicken juice. A study on *Salmonella* has reported a limit of detection of 8.5 CFU/ml in inoculated poultry meat (Siala *et al*., 2017). The differences in the detection limits may be attributed to the varying food matrices and inhibitory features of the food type under investigation (Siala *et al*., 2017). According to the findings of this study, the novel primer sets could contribute to enhancing the sensitivity of real-time PCR approaches for the detection of *Salmonella* and *Campylobacter* spp. in food samples.

4. Conclusions

The proposed SYBR™ GreenER™ dye-based real-time PCR assays successfully detected *S. enterica* and *C. jejuni* with 100% specificity and high sensitivity, reproducibility and repeatability. The primer sets target the *ompF* and *omp50* genes, which are known to be inclusive for *Salmonella* and *Campylobacter*, respectively. Application of the assays to various artificially contaminated food samples showed the potential and usefulness of the assay to be implemented in food microbiology laboratories and for food monitoring.

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Acknowledgment

The authors acknowledge the financial support provided by Universiti Brunei Darussalam and the Ministry of Education of Brunei Darussalam.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

PHYSICOCHEMICAL QUALITY AND STORAGE STABILITY OF RETAIL CAKES AVAILABLE IN TANGAIL CITY, BANGLADESH

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https://doi.org/10.34302/crpjfst/2023.15.4.5

Article history:
Received: 19 September 2022
Accepted: 15 September 2023

Keywords:
Cake;
Physicochemical analysis;
Textural and microbial quality;
Storage stability.

ABSTRACT

The study was carried out to assess the physicochemical and storage stability of seven different retail cake samples available in Tangail city, Bangladesh. The proximate composition of the collected cake samples was found significantly different ($p<0.05$) and was found within the acceptable range of the Bangladesh Standard and Testing Institution (BSTI) standards though most of the cake samples (except S7) had moisture content near to the BSTI borderline range for moisture (~25%). Volume and specific volume of the cake samples ranged from 12.00 to 29.99 cm^3 and 1.64 to 2.93 ml/g respectively. The color measurement of all samples were analyzed for L*, a* and b* values, hue angle, saturation index and whiteness index. Textural properties of the cake samples were analyzed for 7 days of storage where hardness of all samples were increased significantly ($p<0.05$) and springiness was decreased with storage time. Regarding the storage stability most the cake samples (except S7 containing lowest moisture) showed higher total viable count (TVC) (1×10^4 cfu/g to 2.71×10^6 cfu/g) and total fungal count (TFC) (0 cfu/g to 1.4×10^5 cfu/g) than the WHO acceptable range for consumption just after the 2nd day and 6th day of storage respectively.

1. Introduction

From the advancement of modern civilization and urbanization, different types of ready to eat foods such as confectionery and bakery goods have been consumed widely since the food habit of people has also been changed with time (Bandara *et al*., 2021). Among those cakes is one of the most delicious and liked most baked products. It is usually made up of a number of ingredients, principally flour, sugar, egg, baking powder and others (Conforti, 2006). Good quality cake should be fluffy, spongy, smooth, and should satisfy the consumers (Malav *et al.,* 2015; Jiang *et al.,* 2017). The quality and quantity of ingredients along with processing methods and baking conditions influence the wholesomeness and overall eating quality of prepared cake (Salehi F and Aghajanzadeh, 2020). The quality of cake can be compromised by faulty production (higher moisture content), improper handling, packaging and sanitization which eventually lead to microbial spoilage (fungi, bacteria etc.) (Saranraj and Geetha, 2012). To maintain good quality of cake, the Bangladesh Standard and Testing Institution (BSTI) has provided a number of specifications (e.g., moisture level, fiber content etc.) for finished cake (BSTI,

2006). Proper food processing and storage techniques can provide benefits to both commercial producers as well as consumers with a prolonged shelf life of food products (Ilbery and Maye, 2005). A wide range of processed bakery products are available in both local and supermarkets in Bangladesh with a production record of 3,472.000 Metric Ton in July, 2021 (Al-Fuad *et al.,* 2018; CEIC, 2021). A large quantity of these bakery products are being produced by numerous unlicensed and semi-authorized local manufacturers at smaller scale which are hardly scrutinized by government food regulatory authorities. As many of these local bakeries are manufacturing bakery products in unhygienic conditions with low grade raw materials even they don't have BSTI certification which may eventually endanger public health to some extent (Al-Fuad *et al.,* 2018). Now it has become a public health safety concern for bakery products (Islam *et al.,* 2013).Therefore, this study was conducted to analyze the physicochemical quality parameters as well as the storage stability regarding textural (hardness and springiness) and microbial count of different locally produced and marketed cake in Tangail region, Bangladesh. In addition, the study also drew a comparative analysis of the above specifications with standards developed by the BSTI.

2. Materials and methods

2.1. Materials

2.1.1. Study design and Sample collection

It was a laboratory-based analytical study which was based on assessment of physicochemical, microbial stability and shelf life of cake samples. Seven different locally produced cake samples (Table 1) were purchased from the different local retail shops of Tangail city (24.264423°N 89.918140°E) located in the central region of Bangladesh (Fig. 1).

Among those S7 cake samples was a countrywide recognized brand and largely consumed by the population of the study area. All samples were kept at ambient room temperature until analysis. The analysis was conducted in the Department of Food Technology and Nutritional Science (Microbiology Lab), Mawlana Bhashani Science and Technology University, Tangail, Bangladesh.

Table 1. Sample coding and identification

Sr.	Name of the cake samples	Sample ID
01.	Asha special plain cake	S1
02.	Jamuna cake	S2
03.	Master cake	S3
04.	Unknown brand	S4
05.	Tripti plain cake	S5
06.	Bondhon cake	S6
07.	Fuang anytime cake	S7

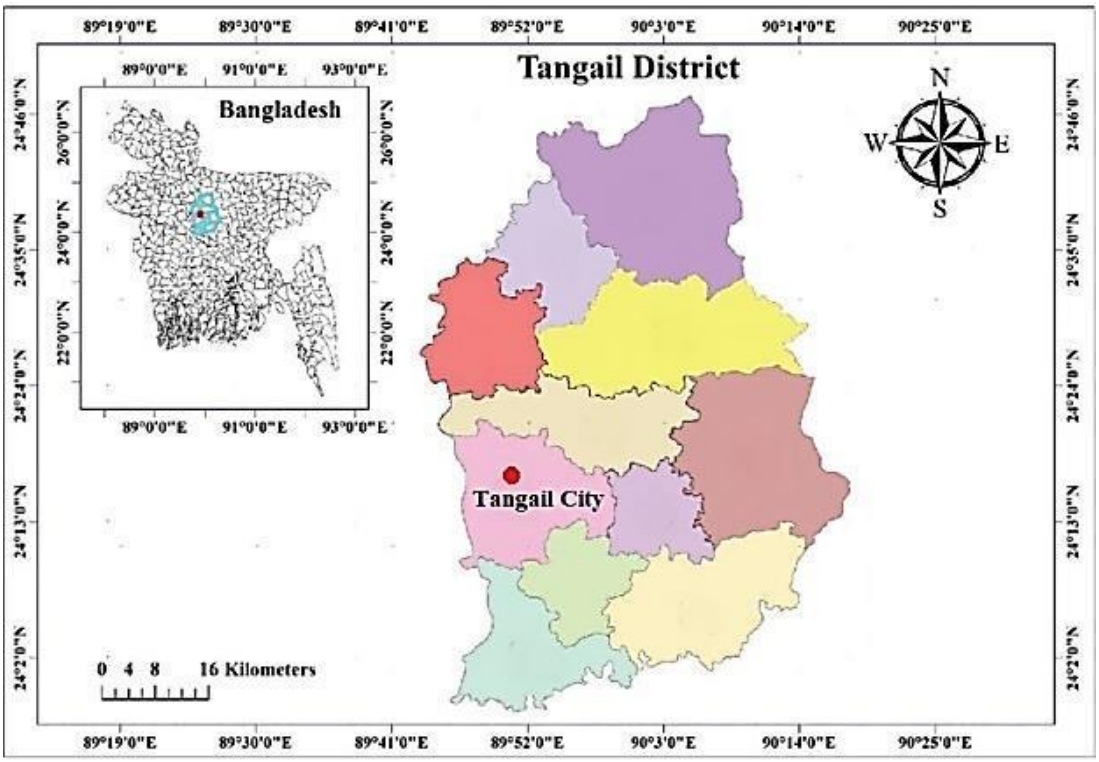

Figure 1. Map showing the study area of Tangail district, Bangladesh

2.2. Analysis of proximate composition

All the cake samples were analyzed for the moisture, ash, crude fat, crude protein and crude fiber content according to the approved methods of AOAC (2005). The utilizable carbohydrate content was determined by using the following equation (Edeogu, 2007).

% Carbohydrate= 100- {Moisture (%) + Protein (%) + Fat (%) + Ash (%) + Crude fiber (%)}

2.3. Color measurement

Color measurements were evaluated by measuring the color values (L*, a*, b*) through Hunter color scale of various cake samples (Kim *et al.*, 2011). The L, a*, b* values of the cake sample were measured by reflection using a Chromameter (CR-400; Konica-Minolta, Tokyo, Japan). Where L defines lightness, a* denotes the red/green value, and b* defines the yellow/blue value. The instrument was calibrated with standard white tiles (Y=85.1, X=0.3171, y=0.3234) prior to measurement. Other color parameters such as hue angle (h*), saturation index (SI) and whiteness index (WI) were also calculated according to the formula described by (Pathare *et al.*, 2012) At least three measurements were taken separately from three different locations on the sample case and the mean, including the standard deviation was analyzed.

2.4. Volume and specific volume of cake

Mass of cake samples were measured by using an analytical balance (Model: ALE-223, India), volumes were measured with displacement of mustard seeds as described by Begum *et al.* (2011) The specific volumes of breads were calculated according to AACC (2000) method based on the volume/mass ratio.

2.5. Texture profile analysis (TPA)

The texture profile of collected cake samples were determined by using a texture analyzer (Imada FRTS Series, Japan) adopting the method described by Feili *et al.*, (2013). A cake sample with a height of 50 mm were submitted to two cycles of compression and compressed up to 20% of the original height using a 20-mm cylindrical probe, at a speed of 1.0 mm/s, a trigger force of 5 g, and a recovery period

between compressions of 15s. The parameters measured were hardness and springiness.

2.6. Storage-stability

The microbial stability of the collected cake samples were analyzed according to the U.S. Bacteriological Analytical Manual (BAM, 1980). The total bacterial and fungal counts were analyzed every day for 7 days in a row using serial dilutions method. Nutrient agar and potato dextrose agar medium were used as media for bacterial and fungal analysis respectively.

2.6.1. Bacterial and fungal count

1 g of cake sample was homogenized in 10 ml distilled water. 0.85% NaCl solution was used to make serial dilutions. After reaching the desired concentration, 0.1 ml of the solution was spread on the surface of the Petri dish containing nutrient agar and potato dextrose medium for analyzing bacterial and fungi count respectively. Prepared nutrient agar and potato dextrose media were incubated at 37^{o}C and 25^{o}C for 7 days and all plates were examined every day for recording the bacterial and fungal growth respectively. The colonies were counted by using the Stuart scientific colony counter (Stuart: SC6 PLUS, UK). After counting the colonies, they are multiplied by the dilution factor to analyze the number of CFU/g in the cake sample (Bennet, 1984; Upasen and Wattanachai, 2018).

2.7. Statistical analysis

Data analysis was performed using Statistical Package for the Social Sciences (SPSS version 20.0 SPSS Inc. Chicago, Illinois, USA). Analysis of variance (ANOVA) was carried out for the determination of significant differences ($p < 0.05$) between the means. Microsoft Excel version 10.0 was used for graphic illustration.

3. Results and discussions

3.1. Composition of cake

The chemical composition of the cake samples is detailed in Table 2. The moisture content of the samples ranged from 16.05-24.81% with significantly highest ($p<0.05$) in S2 and lowest in S7 samples. These results are in agreement with the findings of Begum *et al.* (2016). The reported higher range of moisture content indicated that the cake samples might capture the atmospheric moisture because of improper packaging after baking or due to the hygroscopic properties of wheat flour (Bhat and Bath, 2013). The reported results of moisture content complied with the acceptable moisture range (14-25%) of Bangladesh Standard and Testing Institution (BSTI, 2006) but increased amount of moisture content may faster the spoilage of cake samples.

Table 2. Proximate composition of cake samples

Samples	Moisture (%)	Ash (%)	Fat (%)	Protein (%)	Fiber (%)	Carbo-hydrate (%)	Calorie (Kcal/100g of dry matter)
S1	23.23±0.01^{b}	1.0±0.001^{a}	12.67±0.12^{ab}	6.98±0.16^{b}	0.1±0.00^{c}	56.12±0.42^{d}	366.43±0.77^{c}
S2	24.81±0.02^{a}	0.77±0.00^{c}	10.83±0.04^{b}	6.02±0.1^{d}	0.1±0.00^{c}	57.57±0.23^{cd}	351.85±0.16^{e}
S3	22.37±0.01^{bc}	1.09±0.001^{a}	10.47±0.07^{c}	7.0±0.17^{b}	0.2±0.01^{bc}	59.36±0.36^{b}	359.68±0.42^{d}
S4	19.15±0.01^{d}	0.90±0.003^{b}	15.93±0.12^{a}	6.71±0.11^{c}	0.2±0.02^{bc}	57.32±0.35^{cd}	399.48±0.76^{a}
S5	20.37±0.03^{c}	0.66±0.003^{d}	12.63±0.09^{ab}	7.72±0.06^{a}	0.3±0.01^{b}	58.72±0.26^{c}	378.51±0.46^{b}
S6	22.34±0.02^{bc}	0.76±0.003^{c}	10.63±0.12^{c}	6.21±0.07^{cd}	0.2±0.00^{bc}	60.06±0.31^{b}	360.74±0.70^{d}
S7	16.05±0.01^{e}	0.73±0.002^{c}	9.23±0.06^{d}	6.22±0.04^{cd}	0.4±0.03^{a}	67.77±0.16^{a}	379.03±0.35^{b}

Values are means ± SD of triplicate. Values with the different superscript in the same column are significantly different ($p<0.05$)

The index of mineral constituents of any food can be calculated from the ash content of that food. The ash content of the cake samples was recorded in the range of 0.66-1.09% with the highest value in S3 and lowest value in S5. Though the plain cakes contained an inferior amount of ash content, these findings are complied with the acceptable range of BSTI standards (BSTI, 2006). The fat content of the cake samples ranges from 9.23-15.93% where S4 cake samples recorded significantly higher ($p<0.05$) fat content than other samples and S7 had the lowest fat content. Thus, the highest fat content of S4 sample contributed to the highest calorie value (399.48 Kcal/100g) also. Protein content of the cake samples was found 6.02-7.72%. Lower protein content of plain cakes may be due to the usage of soft wheat flour for cake preparation which can impart softness to the final product (Al- Dmoor, 2013) or may be usage of smaller egg portions or no egg for final cake preparation. However baking operations could also denature the protein content of the raw materials used for cake production as it was studied that different unit operations including roasting, frying and baking could unfavorably minimize the food protein content (Swaminathan, 1986). Among different nutrient content the lowest value was recorded for fiber content of the cake samples. The inferior amount of fiber (<1.0%) was reported in the analysis with the highest content (0.4%) in S7. These might be due to the fact that the soluble fibers from the cake samples may be possibly leached out though the oils and stayed on the packaging materials of the cakes (Begum *et al.,* 2016). The carbohydrate content of the cake samples was in the range of 56.12-67.77% with the highest content in S7 and lowest in S1. Carbohydrate content of S7 cake samples were found significantly higher ($p<0.05$) than other samples. Final calorie values of all the cake samples ranged from 351.85 to 399.48 Kcal/100 g indicating the samples having highest fat content would contribute to the highest calorie value. On the basis of nutritional analysis regarding moisture, ash, fat, protein, carbohydrate and total calories of the seven different plain cakes revealed that some of them are good in quality whereas numbers of them are lacking in good quality. Thus it became very difficult to make any declaration that which one is best, rather the samples exhibited their superiority in different nutrient parameters over one another.

3.2. Physical properties

3.2.1 Weight, volume and specific volume of cake samples

Consumers desire high volume cakes; thus, volume is one of the most important physical qualities of cakes (Lebesi and Tzia, 2011). It is a significant quality indicator which correlates with the dough and textural properties of cake (Pomeranz, 1980). According to Table 3 among the cake samples, S5 was analyzed with highest volume and specific volume which is a clear indicator of entrapping more air or absorbing oil and may result in development of higher cake volume. Another fact is that higher cake volume would also result from lower batter density which is very common with sponge cake (Go´mez *et al.,* 2008). Whereas S1 cake samples found to have lowest values of volume and specific volume. It may be due to lower batter quality, lower capacity of entrapping larger air volumes, improper gelatinization of starch and losses of gas by different processing operations due to baking (Begum *et al.,* 2016). Low batter quality of S1 samples might be due to the low carbohydrate and fiber content of S1 cake samples (Table 2.) as lower amount of fiber and carbohydrate can lower the batter quality which results in lower volume (Lebesi and Tzia, 2012).

Table 3. Physical properties of cake samples

Samples	Sample weight (g)	Volume (cm^3)	Specific volume (ml/g)	Crumb Color		
				L*	a*	b*
S1	7.34	12.00	1.64	72.42 ± 0.75^{c}	-0.84 ± 0.07^{a}	24.19 ± 0.18^{d}
S2	6.52	14.00	2.15	77.11 ± 0.21^{a}	-1.79 ± 0.17^{b}	22.99 ± 0.30^{e}
S3	7.81	22.00	2.82	77.38 ± 0.25^{a}	-1.95 ± 0.07^{b}	22.90 ± 0.18^{e}
S4	9.62	24.96	2.60	76.14 ± 0.22^{b}	-1.78 ± 0.13^{b}	26.20 ± 0.28^{b}
S5	10.24	29.99	2.93	76.03 ± 0.59^{b}	-4.92 ± 0.03^{c}	35.94 ± 0.36^{a}
S6	10.77	17.99	1.67	76.93 ± 0.22^{a}	-1.21 ± 0.08^{b}	19.39 ± 0.05^{f}
S7	6.24	15.00	2.41	77.42 ± 0.30^{a}	-1.72 ± 0.05^{b}	25.21 ± 0.12^{c}

Color values are means ± SD of triplicate. Values with the different superscript in the column are significantly different ($p<0.05$)

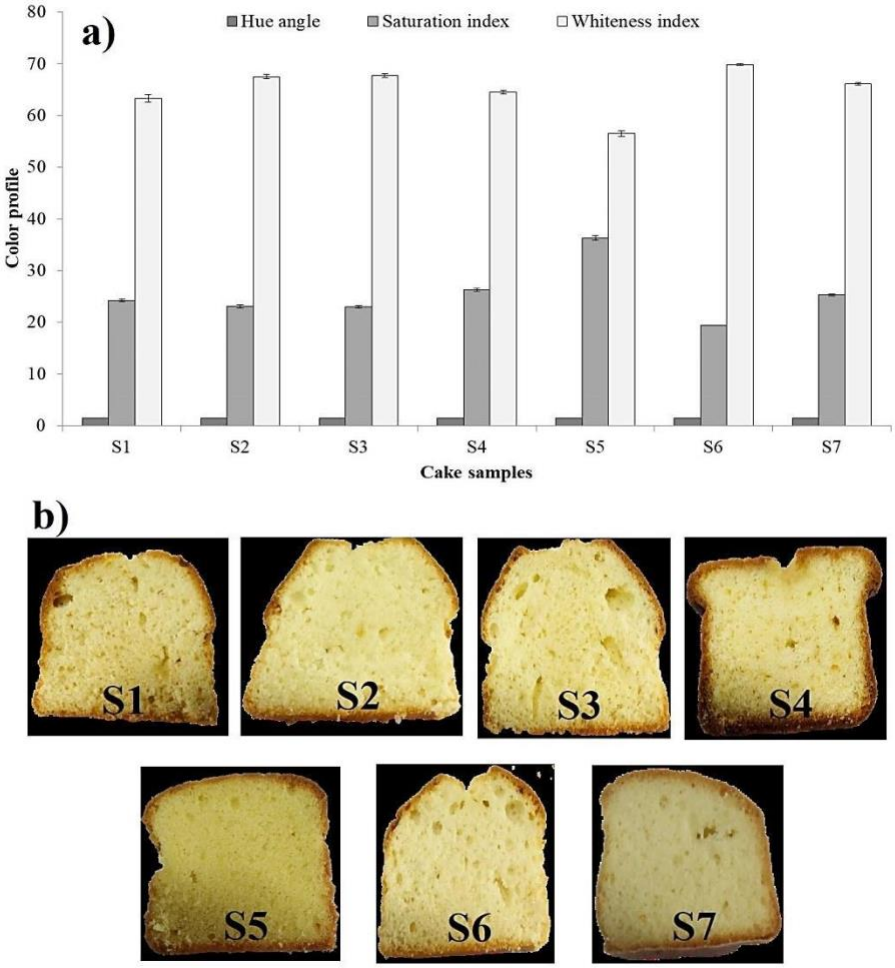

Figure 2. a) Color profiles of cake sample b) photographs of sliced cake samples

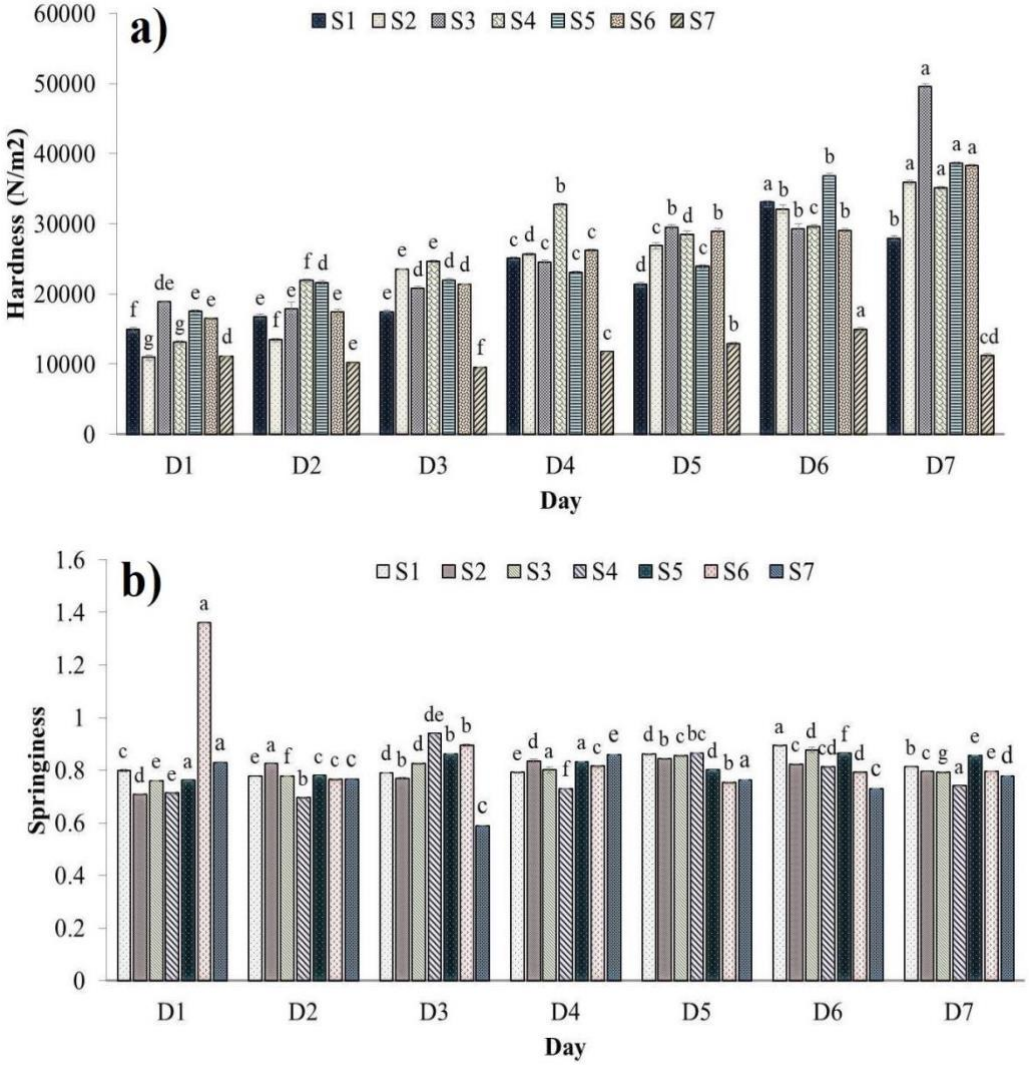

Figure 3. Textural stability of cake; a) Hardness, b) Springiness

3.2.2. Color indices of cake samples

Appearance is considered one of the primary criteria that a consumer usually considered during buying food (Kashim and Kashim, 2015). Among many other indices color has always been a key parameter in food choices, food preference and acceptability, and even effect taste thresholds, pleasantness and perception (Rico *et al.*, 2007).

Table 3 and Fig. 2 jointly showed the color profile of the cake crumb regarding L*, a*, b* value (Table 3) as well as hue angle, saturation index and whiteness index (Fig. 2). It reveals that highest lightness was observe for S7 cake sample whereas darker texture was observed in S1 sample with lowest L* value. No significant difference ($p>0.05$) was observed among the L* values of cake samples (except S1 sample). S5 cake sample was tend to be more yellowish in appearance than other cake samples with highest a* and b* value. The hue angle is another parameter frequently used to characterize color in food products (Barreiro *et al.*, 1997).

The lowest hue angle may also contributed to this yellowish appearance of S5 sample (Pathare *et al.*, 2012) whereas S1 sample was analyzed with highest hue angle and maximum b* value. Another parameter is the saturation

index (SI) or chroma that indicates color saturation and is proportional to its intensity (Barreiro *et al.,* 1997). S5 cake samples showed higher color saturation than other samples. Whiteness index mathematically combines lightness and yellow–blue into a single term (Rodriguez-Aguilera *et al.,* 2011). Overall whiteness of the S6 cake samples showed higher value than other samples indicating the higher extent of discoloration during the baking process (Hsu *et al.,* 2003) with simultaneous lowest color saturation.

3.2.3. Textural properties of cake samples

The quality of bakery items degrades over time due to starch retrogradation, which leads to amylose recrystallization resulting in increased hardness (Hussain *et al.,* 2022). One of the key elements in improving the stiffness of the cake is the moisture migration from the crumbs to the surface (Huang *et al.,* 2020). Thus, in this study the cake quality was evaluated after the first and seven days of storage.

Fig. 3 showed the changes in textural properties of cake samples for seven days storage. It revealed that the hardness of the maximum cake samples (except S1 and S7) was gradually increased with storage time. Highest hardness was recorded in the 7^{th} day of storage and it was significantly different ($p<0.05$) from the initial values. This change was due to the loss of capability of the cake to entrap air that led to the loss of moisture (Schiraldi *et al.,* 1996). However, the increasing hardness of S1 and S7 was not found consistent. Bread-crumb hardness is generally lower in samples with increased moisture content (Hussain *et al.,* 2022). Thus cake samples having higher moisture percentage in the crumbs after 7 days of storage, less hardness might be predicted. Surprisingly, there was no evident link between the moisture content of the cake and its hardness. According to the springiness statistics, the S4 cake samples had the highest springiness values after 7 days of storage. In contrast, the S3 cake sample had the least-springy cake crumb after 7 days. Overall, the springiness of the cake was not affected as much as its hardness when the storage time was changed. Moreover, according to Fig. 3 the seven days of storage exhibited a significant increase in the hardness ($p<0.05$) whereas the springiness decreased.

3.3. Microbial stability of cake samples

The local bakery products which either were wrapped individually and then sealed manually by candle lights or were over-packed in large open or partially sealed poly-packs with more than few pieces together – that increases the chance of those local products to come in contact with airborne microbes, insects, dusts, and hot & humid environment of the vendors outside. This along with the poor post baking handling and storage conditions in the vendors themselves may be one of the major reasons for high microbial count observed in the local bread and cakes.

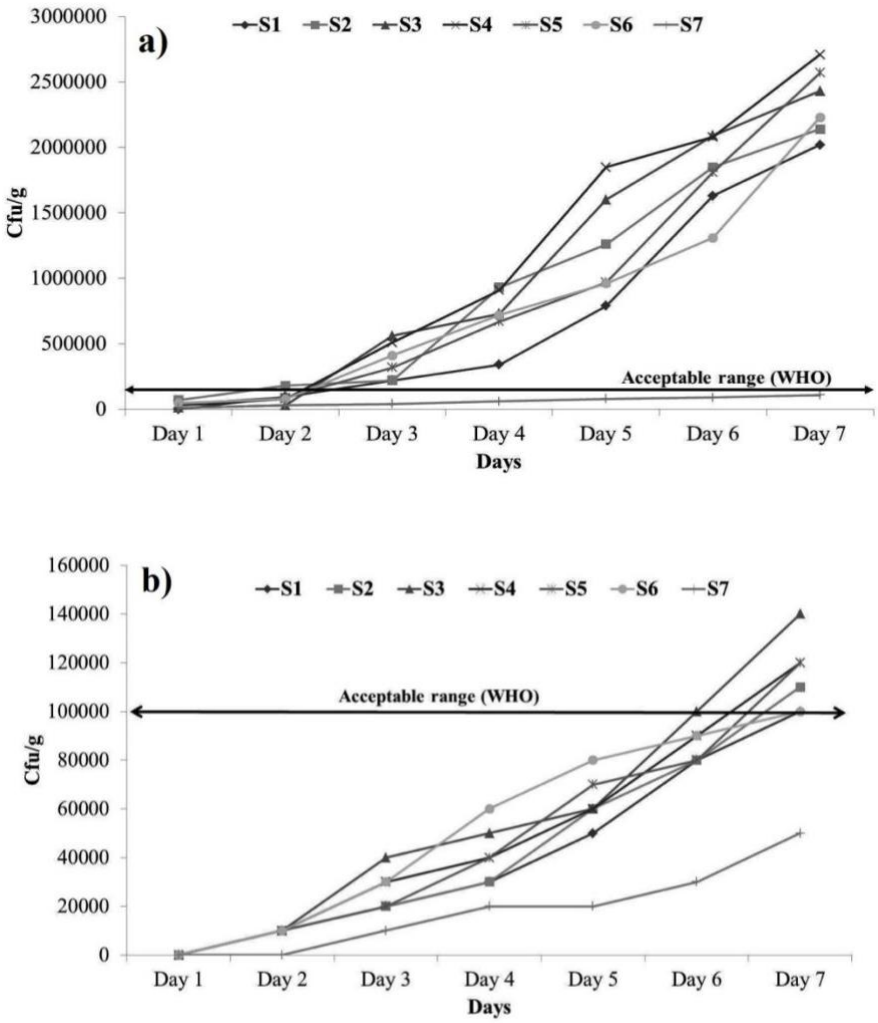

Figure 4. Microbial stability of cake samples; a) Total viable count (TVC), b) Total Fungal count (TFC)

The microbial stability as well as shelf life of bakery products like cake and bread largely depends on packaging materials, technology used, packaging condition and importantly post baking handling and storage conditions (Al-Fuad *et al.*, 2018). The cake samples were stored for seven days where the bacterial and fungal count were analyzed and recorded to assess their shelf.

Fig. 4. (a) revealed the total viable count of different cake samples from day 1 to day 7 at every 24 hours interval. Most of those cake samples contained higher TVC content than the acceptable range of TVC ($2.0x10^5$ cfu/g) suggested by WHO (WHO, 1994), just after the 2nd day of storage. The findings are well in agreement with the TVC values of cake samples observed by Talukder *et al.* (2017). The exception was recorded for the S7 cake sample which was found to contain TVC within the WHO acceptable range even till the last day of analysis. The more stability of S7 cake samples might be due to the low moisture content (Table 2) than other samples. Moreover the packaging quality of this cake sample was comparatively better than others which might contribute to maintaining better microbial resistance. The other cake samples that did not comply with the WHO bacteriological standard just after 2nd days of storage might be manufactured or stored in unhygienic condition or packaged inappropriately. Fig. 4. (b) also showed the total

fungal content (TFC) of the cake samples. At the initial period of analysis no fungal growth was observed for any cake samples. Except for the S7 sample the fungi started to grow just after the very first day. But the growth was limited and within the acceptable range ($1.0x10^5$ cfu/g) of WHO standards (WHO, 1994). At the end of the storage all the cake samples except S7 were reached to the maximum or beyond the limit of TFC suggested by WHO (1994). The cake samples of the current study were found considerably more fungal resistant than the analysis of Talukder *et al.* (2017) where most of the samples started to cross the acceptable range of TFC just after 3 days of storage. Considering both TVC and TFC it was clear and lucid that bacterial contamination may be more prominent in cake than fungal contamination and among the cake sample S7 showed superior microbial stability both in the case of bacterial and fungal contamination than other cake samples.

4. Conclusions

Although the locally produced cake samples showed minimum quality standards for consumption regarding different proximate parameters according to BSTI standards, for manufacturer, care must be taken in reducing moisture content and improving packaging quality to ensure better self-life and storage stability. As most of the cake samples had lower microbial and textural stability during seven days of storage, the storage condition in retail shops also needed to be improved for maintaining better wholesomeness and palatability.

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CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

FUNCTIONAL RICOTTA CHEESE WITH *DUNALIELLA SALINA* ALGAE EMULSION

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https://doi.org/10.34302/crpjfst/2023.15.4.6

Article history:
Received: 5 November 2023
Accepted: 10 December 2023

Keywords:
Antioxidant capacity;
Dunaliella salina;
Emulsion;
Microalgae;
Ricotta cheese.

ABSTRACT

Microalgae are a remarkable source of essential biomolecules with several uses that may be exploited for commercial applications. The objective of the present work was to prepare a novel Ricotta cheese supplemented with *Dunaliella salina* algae emulsion formula containing mint essential oil (mint EO) as a functional food ingredient. Total phenolic compounds (TP), total flavonoids (TF), DPPH antioxidant capacity, and identification of phenolic and flavonoids compounds of algae were determined. Moreover, the chemical, physical, rheological, microbiological, organoleptic characteristics, and chemical score of the resulted Ricotta cheese were investigated. The results showed that TP, and TF contents in *D. salina* algae crude extract are 8.48 (mg GAE/g DW), and 5.93 (mg QE/g DW), respectively. Also, *D. salina* has high levels of phenolic acids (e.g., gallic acid, chlorogenic acid, caffeic acid, and rosmarinic acid); as well as high flavonoid content (e.g., catechin, naringenin, quercetin, rutin, and hesperetin). The DPPH procedure showed that *D. salina* algae antioxidant activity is 42.19±0.39 mg/ml as IC_{50} value. The amino acids profile revealed that *D. salina* algae contains 17 amino acids with the highest value of threonine, as well as total amino acid (TAA) is 949.69, which contains 424.2 essential amino acids (EAA) with a 0.447 TAA/EAA ratio. However, *D. salina* algae/Mint EO emulsion was prepared using a non-ionic surfactant (Tween 80). Ricotta cheese supplemented with an algae emulsion formula 0.25%(I), 0.5%(II), and 1.00%(III) showed gradual adequate free radical scavenging ability as the algae level increased. The incorporation of *D. salina* algae emulsion into Ricotta cheese processing led to an increase in their protein, fat, and mineral levels compared to control cheese. Also, all color attributes were decreased for algae Ricotta cheese in comparison to the control sample. Texture profile of cheese showed that algae Ricotta cheese had lower hardness, gumminess, and chewiness than control cheese with opposite trends for other textural parameters; while no significant ($p \leq 0.05$) changes in adhesiveness, cohesiveness and springiness parameters were recorded after 21 days of storage at 5 °C. The total bacterial counts were significantly ($p \leq 0.05$) lower in the algae cheese samples than the control. The yeast and mold count in control cheese appeared after 14 days, whereas it didn't appear in II and III algae Ricotta cheese until day 21 of storage. However, the addition of an algae emulsion formula improved all sensorial attributes of Ricotta cheese during storage at 5 °C for 21 days, especially at the level of 0.5% (II), and the same level of algae emulsion led to an increase in most amino acid content, and the chemical score of amino acids (e.g., methionine + cysteine, histidine, and isoleucine) compared to control cheese.

1.Introduction

The global consumption of cheese is increasing continuously and is predicted to rise by ~13.5% between 2016 and 2025 (OECD/FAO, 2016). However, the cheese industry produces approximately ~145 million tons of whey annually which represents an environmental issue (Ganju and Gogate, 2017). Recent research has focused on remanufacturing cheese whey, a well-known unconventional protein source, into different food products to address the societal dilemma of food and protein scarcity to sustain human population growth. Ricotta cheese, which is primarily made from cheese whey, is a creamy, soft, un-ripened cheese with a high moisture content. It also has important nutritional, health, and functional features. Traditionally prepared by heat-induced coagulation of whey protein and adding acetic, citric, or lactic acid to the heated whey to reach a pH of ~5. The flavor of fresh ricotta cheese is nutty, mild, and pleasant flavor (Camerini *et al*., 2016; Rubel *et al*., 2019; Nzekoue *et al.*, 2021; Hesarinejad *et al*., 2021). The whey proteins in Ricotta cheese have a high biological value because they contain a large amount content of sulfur-containing amino acids (Smithers, 2008). Fresh ricotta is used extensively around the world due to its nutritional advantages. The increasing awareness among consumers regarding the significance of diet for health and overall well-being has led food scientists and cheese manufacturers to focus on enhancing the quality of their current products or creating novel and innovative ones. (Lamichhane *et al*., 2018). Many natural components, such as dietary fiber, natural antioxidants, vitamins, etc., can be used to boost Ricotta cheese into a functional product. (Siyar *et al*., 2022). Consumers are often looking for dairy products with new flavors in addition to their high nutritional value. Many consumers continued to enjoy exploring new flavors and combinations with recognizable and approachable formats and flavors during the COVID-19 pandemic, such as key lime-flavored yoghurt or barbecue flavored unripe firm cheese. (Falcão *et al*., 2023). The addition of essential oils with their characteristic flavor and microalgae with their potent health benefits to a traditional product like cheese conforms to this trend. Microalgae are a remarkable source of essential biomolecules with several uses that may be exploited for commercial applications. Thus, the use of microalgal biomass resources presents a resource-efficient and ecological way to utilize algal metabolism to synthesize and accumulate a wide range of molecules of industrial interest and high value-added products including proteins, lipids, vitamins, carotenoids, pigments, and other bioactive compounds (Becker, 2007; De Jesus Raposo *et al*., 2013; Sui and Vlaeminck, 2019).

Dunaliella salina (Dunaliellaceae), one of the few species of green microalga that may naturally flourish in very salty media. It synthesizes extreme amounts of carotenoids, especially β-carotene and zeaxanthin beside lutein and astaxanthin (El-Baz *et al*., 2020a; El-Baz and Ali, 2023). In addition to carotenoids, *D. salina* produce remarkable amounts of polyphenolic compounds, including caffeic acid, vanillic acid, gallic acid, coumarin, and vanillin (Simon *et al*., 2015). Different carotenoids, in particular the lipid-soluble orange pigment β-carotene, are utilized as coloring constituents in food and feed thanks to their strong antioxidant and free radical scavenging efficiency (Cakmak *et al*., 2014). *D. salina* had remarkable anti-inflammatory, hepatoprotective, and antioxidant therapeutic benefits (El-Baz *et al*., 2020a) and counteractive agent in liver fibrosis (El-Baz *et al*., 2019, 2020b). Different epidemiological findings advised that daily consumption of enough amounts of natural antioxidants like phenols and carotenoids could potentially lower the likelihood of cardiovascular complications and inhibit the growth of different cancer cells (Sheu *et al*., 2008; Singh *et al.*, 2016; Gaafar *et al*., 2020). Nonetheless, the US Food and Drug Administration (FDA) has designated Dunaliella as a form of microalgae with Generally Regarded as Safe (GRAS) classification. It is primarily utilized in various applications, producing proteins with excellent quality and digestibility. It is primarily utilized in various uses, producing proteins with

excellent quality and digestibility (Amaya *et al*., 2014).

According to our hypothesis, using cheese whey to make Ricotta cheese will assist to reduce the environmental problem of excessive cheese whey waste, while incorporating new functional microalgae *Dunaliella salina* into such cheese will satisfy customer desire for highly nutritive and therapeutic dairy product. As a result, the main goal of this research is to develop a novel functional Ricotta cheese enriched with *Dunaliella salina* microalgae emulsion formula which includes mint essential oil (mint EO). The chemical composition and antioxidant characteristics of Ricotta cheese and *Dunaliella salina* microalgae were determined. Additionally, the physical, rheological, microbiological, organoleptic characteristics, and chemical score of the developed Ricotta cheese were examined.

2.Materials and methods

2.1. Materials

The *D. salina* pulverized biomass utilized in this investigation was secured from the National Research Center in Cairo, Egypt by the Algal Biotechnology Group at the New Central Laboratories Network. Mint essential oil (Mint EO) was obtained from the production and marketing of medicinal plants and their extracts unit, National Research Centre, Egypt. Sweet whey was got from the dairy plant at the Faculty of Agriculture, Cairo University, Giza, Egypt. Commercial fine grade salt (Sodium-chloride, NaCl) was obtained from the Saudi-Egyptian company for salt and minerals (SECOASALT), 10th of Ramadan, Egypt. Citric acid was obtained from SRL-INDIA. Gallic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Tween 80, standard amino acids, and Folin-Ciocalteau phenol reagent were purchased from Sigma Aldrich (St. Louis, Missouri, USA). All other chemicals were used in analytical grade.

2.2.Methods

2.2.1.Preparation of D. salina extract

Two grams of pulverized *D. salina* were sonicated (5 sec. on, 50 sec. off, for 30 min) in 40 ml of a water/methanol/formic acid (28:70:2, v/v/v) mixture (Martini *et al*., 2019), using a Prop Ultrasonic (Vibra Cell (VCX750), USA) with 750 Watts power and 20 KHz frequency. The mixture was centrifuged (5000 xg, 20 min, 4 °C). The resultant supernatant was completed to 50 ml with the same solvent solution.

2.2.2.Total phenolic content (TP) assessment

Using the Folin Ciocalteu reagent test and our previously reported procedures, the TP of the crude extract was assessed (Ali *et al*., 2018). In a 25 ml volumetric flask, the extract (1 ml), distilled water (9 ml), and Folin reagent (1 ml) were mixed well. Then 7 % Na2CO3 (10 ml) was added to the mixture after 5 min. The mixture was completed to 25 ml with distilled water, mixed well, and incubated at room temperature for 90 min. A spectrophotometer (Unicum UV 300) was used to measure the absorbance at 750 nm against the blank (all reagents without the sample). The sample's TP was reported as mg Gallic acid equivalents (GAE)/g DW. Every sample was assessed three times.

2.2.3. Total flavonoid content (TF) assessment

The aluminum chloride method was used for the determination of total flavonoid (TF) content in the crude extract following our former published methods (Ali *et al*., 2018). In a volumetric flask, the extract (1 ml), distilled water (4 ml), and 5 % NaNO2 (0.3 ml) were mixed well. After 5 min, 10 % AlCl3 (0.3 ml) was added. Following an additional six minutes, 2 milliliters of 1M NaOH were added, and then the mixture was completed with distilled water to a final volume of 10 ml. A spectrophotometer (Unicum UV 300) was used to measure the absorbance at 510 nm against the blank (all reagents without the sample). The sample's TF was reported as mg quercetin equivalents (QE)/g DW. Every sample was assessed three times.

2.2.4. Identifying phenolic ingredients

For fifteen minutes, a 10 mg sample of *D. salina* extract was vortexed in 2 mL of HPLC spectral grade methanol. A 0.2µm Millipore membrane filter was used to filter this extract, and its volume was set at 2 mL, and 5 µl of it was injected into an HPLC (Agilent Technologies 1260 series, Germany) with an auto-sampling injector. To separate phenolic

ingredients, C8 column (ZORBAX Eclipse Plus, 4.6 mm x 250 mm i.d., 5 µm) was utilized. The temperature in the column was kept constant at 40°C. At a flow rate of 0.9 ml/min, the mobile phase was composed of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). The following mobile phases were programmed in a linear gradient: 0 min (82% A); 0-1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A); 22-24 min (82% A). At 280 nm, the multi-wavelength detector was observed (Goupy *et al.*, 1999). The relative retention durations of phenolic compounds were compared to those of the reference mixture chromatogram. The peak area measurements were used to compute the concentration of each phenolic component (µg/g DW).

2.2.5. Determination of antioxidant activity

DPPH• radical scavenging test

After preparing the DPPH• (0.1 mM) in methyl alcohol, 1 ml of this solution was added to 100 mg/ml of Ricotta cheese or 2 ml of *D. salina* extract at concentrations ranging from 1 to 7 mg/ml. After giving the mixture a good shake, it was left to remain at room temperature in the dark for half an hour. The absorbance was then measured at 515 nm (Gaafar *et al.*, 2020). The control sample comprised all reagents except the extract. Using the following formula, the ability to scavenge the DPPH• radical was determined:

DPPH• scavenging effect (%) = (Ac- As / Ac) × 100

Where Ac is the absorbance of the control sample and As is the absorbance of the tested sample. The results were represented as IC50 (the extract's concentration (in mg/ml) that scavenges 50% of the DPPH• radical).

2.2.6. Amino acids profile

The amino acids profile of *D. salina* algae powder and Ricotta cheese samples were determined using HPLC-Pico-Tag method according to Cohen *et al.*, (1989). The Pico-Tag method was developed commercially by Waters Associates which was an integrated technique for amino acids analysis. Phenylisothiocyanate (PITC, or Edman's reagent) was used for pre-column derivatization, while reversed-phase gradient elution high-performance liquid chromatography (HPLC) separates the phenylthiocarbamyl (PTC) derivatives, which were detected by their UV absorbance. The Liquid chromatography apparatus was equipped with 600 E Multisolvent Delivery System and the following gradient of Pico-Tag solvent A and B (Waters Eluent A and B) at 38 °C, flow rate 1 ml/min. Twenty microliter of sample was injected and loaded on amino acids column Pico-Tag amino acids (150 x 3.9 mm, stainless steel) using linear gradient elution. Detection of the PTC derivatives is by ultraviolet absorption measurements using a fixed wavelength 254 nm (2489 UV/Vis Detector).

2.2.7. Preparation of algae emulsion formula

The algae emulsion formula was prepared by mixing 1.5% *D. salina* algae powder, 1.5% Mint EO, and 0.1% Tween 80 with distilled water using high speed homogenizer (Ingenieurbüro CAT, M. Zipperer GmbH) for 15 min at 25000 rpm/min. The mixture was sonicated using ultrasonicator (ULTRASONIC Get 750) micro tip probe of 400 watt with amplitude of 40% for 30 min. During sonication, the temperature of algae emulsion formula was maintained at 25°C by cooling in an ice bath. The resulted emulsion was stored in sterile containers at 5 °C until further analysis.

2.2.8. Characterization of algae emulsion formula

The algae emulsion formula was assessed by Transmission Electron Microscopy (TEM, JEM-1230, JEOL) to evaluate the structure and surface morphology of the prepared emulsion. Also, the particle size of the resulted algae emulsion was performed and expressed as intensity particle size distribution and average particle size at 20 °C using a particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK) using dynamic light scattering technique.

2.2.9. Preparation of Ricotta cheese

Sweet whey collected after the production of Mozzarella cheese from cow milk, was heated up to 85 – 90°C, thereafter, citric acid (25 ml/L) was added followed by the addition of 1 % NaCl to the mixture and a gentle stirring was provided. Following coagulation, the cheese curd was formed and left in the whey for 10-15 min and

then scooped in a mold lined with muslin filter to separate the whey and allowed to attain complete drainage in 24 hr. The curd was divided into four parts. The first part was considered as a control Ricotta cheese without any additives. *D. salina* algae/Mint EO emulsion was added at the ratio of 0.25, 0.5 and 1.0% (w/w) to second, third, and fourth portion and mixed well to prepare (I), (II), and (III) Ricotta cheese with algae emulsion formula, respectively. Three replicates were done for each treatment. All Ricotta cheeses were packaged in a plastic container (100 g) and stored in refrigerator at 5±1°C for 21 day. The samples were analyzed fresh and after 7, 14, and 21 days of cold storage.

2.2.10. Cheese chemical analysis

Moisture, fat, total protein, ash, and titratable acidity were analyzed according to the method described in AOAC (2012). The pH value of cheese was measured electrometrically using Lab. pH meter with a plastic electrode, JENWAY 3510 digital pH meter. Minerals content was estimated as described by Hankinson (1975) using atomic absorption spectrophotometer No. 3300 (PerkinElmer, US instrument Division Norwalk, CT, USA).

2.2.11. Cheese texture profile analysis

Texture profile analysis was determined using the (TMSPro testing machine) equipped with (250 lbf) load cell and linked to a computer programmed with ProTM texture analysis software. Texture of cheese samples was evaluated in triplicate for each batch of cheese at room temperature (±2ºC). Cheese samples were analyzed through a storage period; they were measured in a 25-ml cup, and two-bite compression experiments with a flat cylinder probe (25 mm diameter) were applied to each sample. All texture profile analysis (TPA) measurements are performed with two cycles of uniaxial compression tests which generated a plot of force (g) vs. time (s). Compression and release of the samples and the force exerted on the probe was automatically registered. The data obtained from the force decompression curve was used to calculate the maximum and residual force, while the data got from the TPA curve was used for the calculation of textural parameters. The parameters simulating involved hardness (the peak force through the first compression cycle), adhesiveness (the negative force area of the first compression cycle), cohesiveness (the ratio of the positive force area through the second compression cycle to that during the first compression cycle) and springiness (the length to which the sample recovers in height during the time that terminates between the end of the first compression cycle and the start of the second compression cycle).

2.2.12. Color development of cheese

Color parameters of Ricotta cheese during storage for 21 days were performed using Hunter colorimeter (model D2s A-2, Hunter Assoc. Lab., Virginia, USA) tri-stimulus values of the color namely l*, a* and b* as described by Hunter and Harold (1987).

2.2.13. Cheese microbiological analysis

The total bacterial count was enumerated using plate count agar (Oxoid), incubated at 37 °C for 48 h as a method recorded by Laird *et al*., (2004). Coliform was counted using violet red bile agar (VRBA) at pH 7.0-7.2, 37˚C for 48 hrs as described in Hitchins (1992). Yeast and mold were detected according to IDF (1990).

2.2.14. Organoleptic evaluation of cheese

Sensorial attributes of Ricotta cheese for 21 days of storage were evaluated as described by Pappas *et al*., (1996). Cheese samples were evaluated by means of regular panelists from the staff of the Dairy Sciences and Technology Research Department (Food Technology Research Institute, Giza, Egypt), with a maximum score of 50 points for flavor, body and texture (40 points) and cheese appearance (10 points).

2.2.15. Chemical score of cheese

The chemical score, protein efficiency ratio and biological value of Ricotta cheese samples were calculated based on their amino acid content according to Bhanu *et al*., (1991), as follows:

Chemical score = (mg of amino acid in 1g test protein/ mg of amino acid in 1g reference protein) × 100

2.2.16. Statistical analysis

The results average values were analyzed by SAS software (SAS Institute, Cary, North

Carolina, USA) using ANOVA procedure for analysis of variance. The results were expressed as mean ± standard error and the differences between means were tested for significance using Duncan's multiple range at $p \leq 0.05$.

3.Results and discussion

3.1. Algae phenolic and flavonoid contents

The result showed that the total phenolic and total flavonoid contents of *D. salina* crude extract are 8.48±0.11 (mg GAE/g DW) and 5.93±0.40 (mg QE/g DW), respectively (Table 1). The total phenolics of the *D. salina* extract in the current research is consistent with the content (8.78±1.49 mg GAE g^{-1} DW) of *D. salina* aqueous extract and higher than the content (1.30±0.37 mg GAE g^{-1} DW) of *D. salina* methanol extract as reported by Andriopoulos *et al.*, (2022). Other studies reported various total phenolic contents in different *D. salina* extracts, including 4.672 mg GAE g^{-1} methanol extract (Widowati *et al.*, 2017), 53.27±7.7 mg GAE g^{-1} hexane extract and 56.45±4.50 mg GAE g^{-1} dichloromethane extract (Cakmak *et al.*, 2014), and 19.3±0.70 mg GAE g^{-1} ethanol extract (Maadane *et al.*, 2015).

Table 1 shows that the *D. salina* crude extract contains high concentrations (µg/g DW) of phenolic acids, including gallic acid (47.82), chlorogenic acid (46.84), caffeic acid (25.05), and rosmarinic acid (12.42), in addition to high flavonoid concentrations (µg/g DW), catechin (22.58), naringenin (14.88), quercetin (14.25), rutin (11.10), and hesperetin (9.80). Our findings are in accord with Simon *et al.*, (2015), who identified many phenolic components in the HPLC-MS/MS chemical profile of *D. salina* ethyl acetate extract, including caffeic acid, gallic acid, vanillic acid, coumarin, and vanillin. The concentration of total phenolic content and phenolic individuals in *D. salina* microalgae varies in this study compared to other studies because of differences in algal growth conditions, extraction solvent selection, and condition of extraction processes (Khawli *et al.*, 2021).

Table 1. Phenolic and flavonoid compounds profile of *D. salina* microalgae powder.

Compounds	***D. salina* algae content (µg/g DW)**
Phenolic compounds	
Gallic acid	47.82
Chlorogenic acid	46.84
Caffeic acid	25.05
Rosmarinic acid	12.42
Cinnamic acid	8.21
Ferulic acid	3.95
Syringic acid	3.06
Flavonoids	
Catechin	22.58
Naringenin	14.88
Quercetin	14.25
Rutin	11.10
Hesperetin	9.80
Kaempferol	7.06
Daidzein	6.05
Other phenolics	
Vanillin	4.33
Methyl gallate	1.94
Total phenolic contents (TP)	8.48±0.11 (mg GAE/g DW)
Total flavonoid contents (TF)	5.93±0.40 (mg QE/g DW)

3.2.Antioxidant activity of algae

The antioxidant efficiency of *D. salina* algal crude extract increased with concentration using the DPPH technique (Fig. 1a), with adequate IC_{50} value of 42.19±0.39 mg/ml. The powerful free radical scavenging capacity of *D. salina* extract is strongly linked to its quantities of various phenolic acids and flavonoids, these findings are in accord with former conclusions that demonstrated the high antioxidant ability of *D. salina* different extracts and attributed it to their contents of phenolic elements (Cakmak *et al*., 2014; Maadane *et al*., 2015; Widowati *et al*., 2017; Andriopoulos *et al*., 2022). The antioxidant effects of *D. salina* microalgae in this research could also be attributed to its high quantities of carotenoids, the powerful natural antioxidant pigments, in recent study (El-Baz and Ali, 2023) found that *D. salina* microalgae contain total carotenoids (0.0039-1.39 mg/g DW), β-carotene (0.0037-0.0565 mg/g DW), lutein (0.0004-0.1596 mg/g DW), zeaxanthin (0.0002-0.0341 mg/g DW), and astaxanthin (0.0003-0.0231 mg/g DW) depending on the extraction solvent. The free radical scavenging ability of the Ricotta cheese significantly ($p \leq 0.05$) enhanced as the quantities of the supplemented algae emulsion formula (*D. salina* algae/Mint EO) increased, revealing DPPH scavenging effects of 12.21, 43.36, and 43.99 % for I (0.25%), II (0.5%), and III (1%), respectively, compared to 5.71% of the control cheese (Fig. 1b). The numerous identified phenolic compounds in *D. salina* microalgae, including rosmarinic acid, gallic acid, caffeic acid, chlorogenic acid, rutin, catechin, naringenin, hesperetin, and quercetin are principally in charge of the elevated antioxidant capacity of Ricotta cheese fortified with algae emulsion formula (*D. salina* algae/Mint EO) compared to the control cheese in this study. These outcomes align with those of Falco *et al.*, (2023), who showed that adding *Chlorella vulgaris* microalga increased the antioxidant capacity of cream and quark cheese significantly in contrast to the cheese used as a control. Tohamy *et al*., (2018) revealed that spreadable cheese treated with *C. vulgaris* had stronger antioxidant activity than control cheese. The current findings suggested *D. salina* microalgae as a natural, safe, and potent source of antioxidants to lessen oxidative cellular damage and stop oxidative deterioration in manufactured dairy products like Ricotta cheese. Antioxidants, such as phenolic compounds and carotenoids found in *D. salina* microalgae, work through a variety of mechanisms to prevent chain initiation, chelate transition metal ion catalysts, degrade peroxidases, stop further hydrogen absorption, and act as radical scavengers (Valko *et al*., 2006; Andriopoulos *et al*., 2022).

3.3. Algae amino acids profile

The amino acids analysis of *D. salina* microalgae found that it contained 949.69 mg of total amino acids (TAA) and 424.2 mg of essential amino acids (EAA), with a ratio of 0.447 between TAA and EAA. The amino acid profile of *D. salina* microalgae contains 17 essential amino acids (Fig. 2), that are important for human growth and health including threonine (117.98 mg/g protein), lysine (112.88 mg/g protein), histidine (60.06 mg/g protein), valine (56.96 mg/g protein), phenylalanine (53.17 mg/g protein), methionine (40.16 mg/g protein), leucine (19.93 mg/g protein), and isoleucine (18.64 mg/g protein). Similar findings were recorded for the amino acids profile of *Chlorella vulgaris* microalgae (Mohamed *et al.*, 2013).

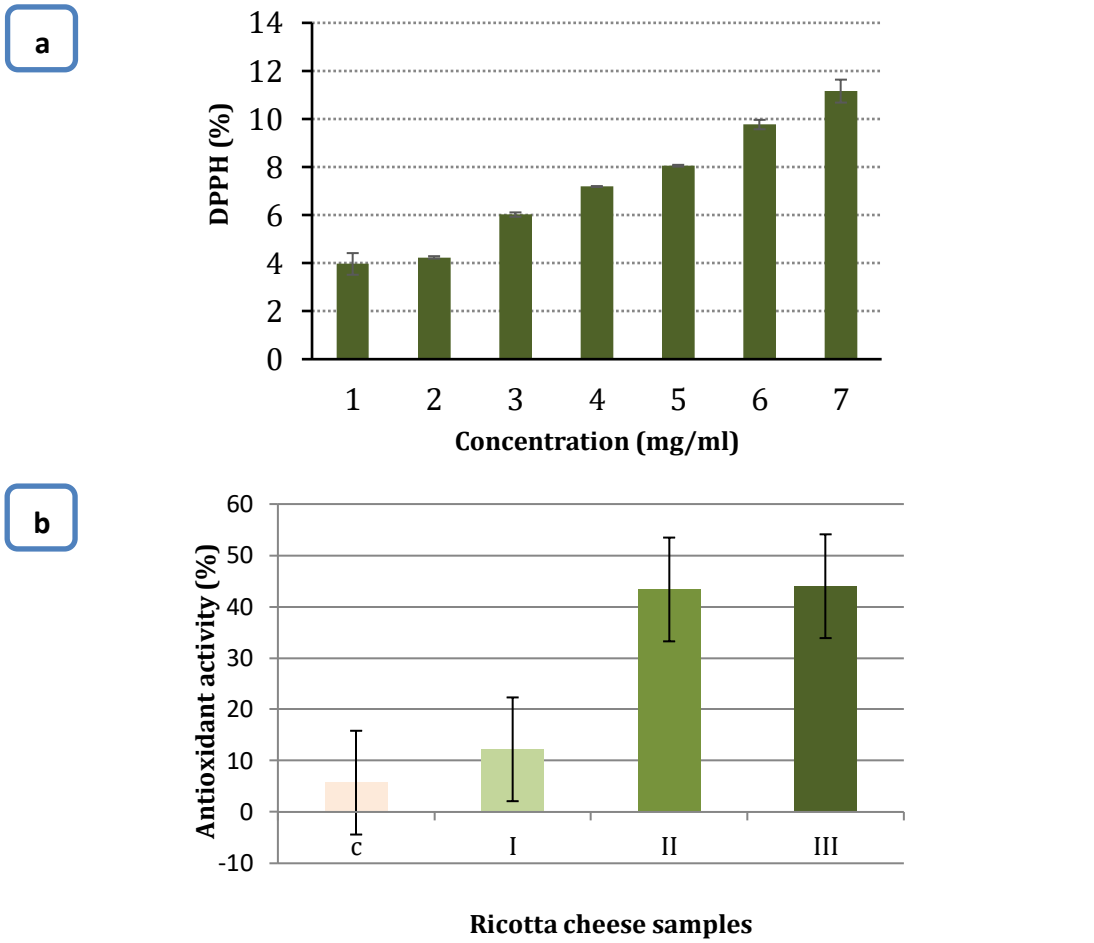


Figure 1. Antioxidant activity of *D. salina* algae and Ricotta cheese.
(a) DPPH• radical scavenging assay of *D. salina* algae crude extract, **(b)** Antioxidant activity of Ricotta cheese.
C: Ricotta cheese without algae emulsion, **I**: Ricotta cheese supplemented with 0.25% algae emulsion, **II**: Ricotta cheese supplemented with 0.5% algae emulsion, **III**: Ricotta cheese supplemented with 1% algae emulsion.

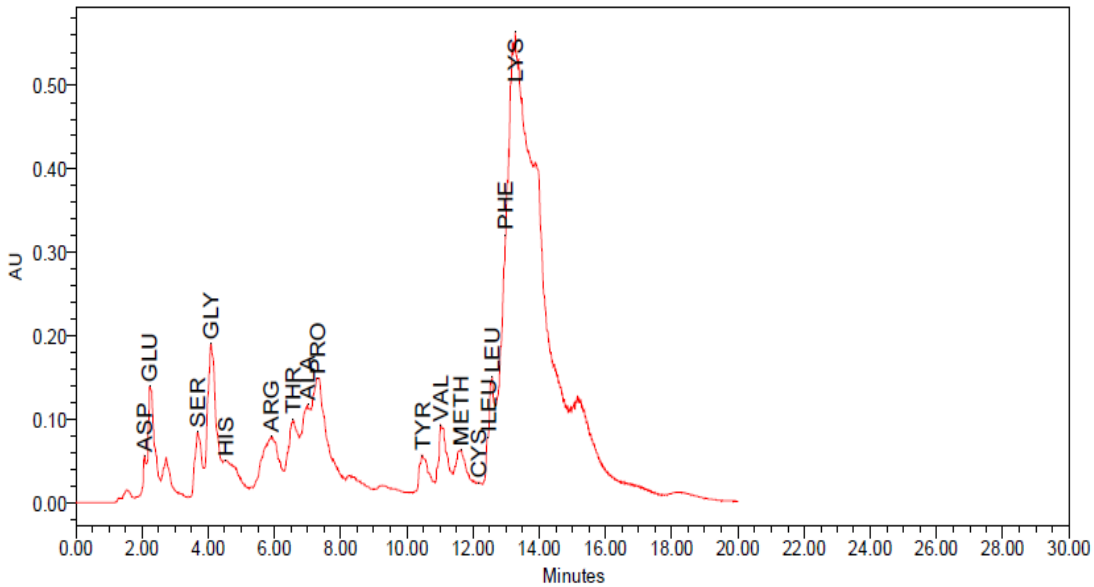


Figure 2. Amino acids profile of *D. salina* algae powder.

3.4. Characteristics of algae emulsion

The algae emulsion contains *D. salina* algae powder, and mint essential oil was prepared with non-ionic surfactant (Tween 80) using ultrasonication technique. The morphology of algae emulsion formula using TEM shows that it was mostly sphere-shaped, or oval in shape and very smooth surface as shown in Fig. 3 (A1, A2) at low and high magnifications. Also, the dynamic light scattering (DLS) result of particle size analyzer of the prepared algae emulsion is shown in Fig. 3 (B) which displays the average diameter is about 116 nm which similar with the average diameter of algae/cinnamon oil/epirubicin (117.2 nm) (Alkhatib *et al.*, 2020). In particular, the molecular geometry of a surfactant is known to play a major role in determining the formation and stability of emulsions and nanoemulsions (Israelachvili, 2011). It means the presence of double bonds in the nonpolar chains of Tween 80 as non-ionic surfactants improves the formation of *D. salina* algae/mint EO emulsion. Similar effects in other studies which have been reported that the surfactant type also had an appreciable effect on the droplet diameter mean, which Tween 80 as a food-grade nonionic surfactants had the smallest droplets (Wang *et al.*, 2009; Chang *et al.*, 2013).

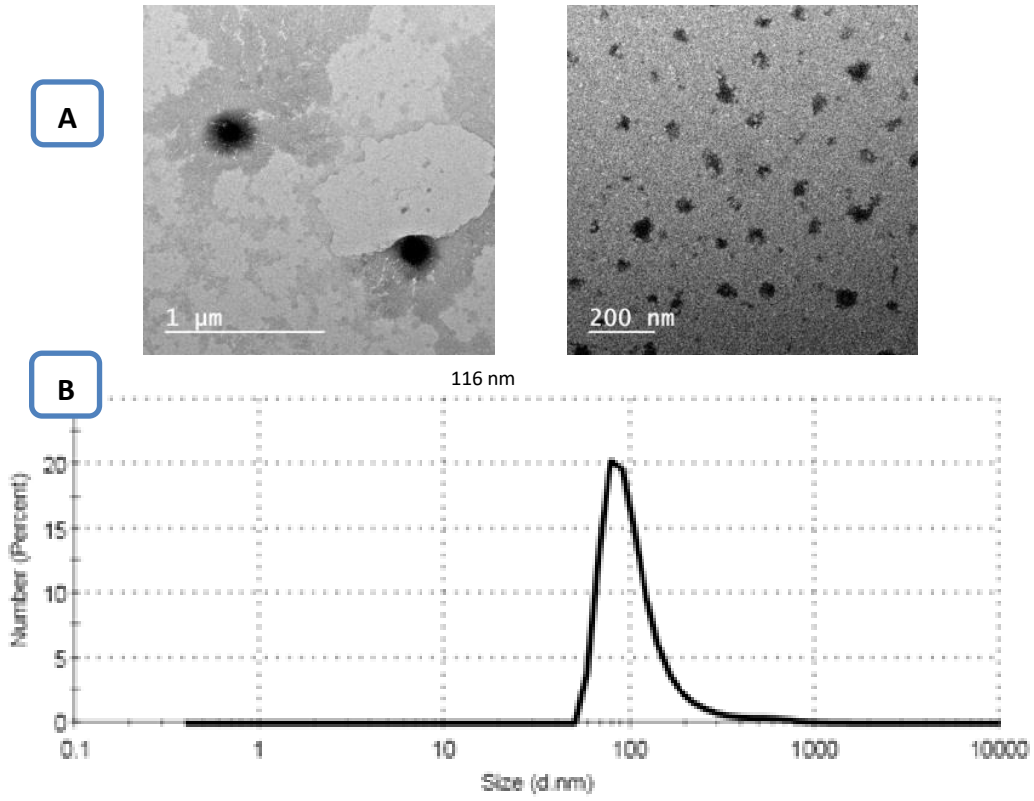


Figure 3. TEM images different magnifications (A), mean diameter and relative particle size (B) of the prepared algae emulsion formula.

3.5. Chemical changes of algae Ricotta cheese

The results obtained in this study for moisture, protein, fat, and ash, are shown in Table 2. The moisture content of Ricotta cheese ranged from 73.29 to 75.11%. Consequently, Ricotta cheese is categorized as a high moisture cheese as its moisture content exceeds 55% (Mangione *et al.*, 2023). Treatments III showed the lowest moisture value and differed significantly ($p \leq 0.05$) from control. Throughout the storage period, all cheese samples showed a decrease in moisture, most likely as a result of moisture evaporation from the cheese surfaces

(Pérez-Soto *et al.*, 2021). The current results of moisture content are consistent with those reported by Niro *et al.*, (2013), and Miele *et al.*, (2021).

The protein content of Ricotta cheese ranged from 17.59 to19.04%. The incorporation of the algae emulsion into the Ricotta cheese formulation causes significant changes ($p \leq 0.05$) in the protein content of cheeses. The fat content of Ricotta cheese ranged from (0.40-0.85%) for control and III treatments, respectively. The ash content significantly different ($p \leq 0.05$) between treatments; ash content of 1.78% was reported for Ricotta with 1% algae emulsion. However, the moisture loss of all samples during the storage period is most likely the cause of the apparent gradual increase in fat, protein, and ash content in the cheeses.

3.6. Acidity and pH changes of algae Ricotta cheese

The pH and titratable acidity (TA) values of the Ricotta cheeses that contained different concentrations of algae emulsion during storage period are shown in Table 2. The titratable acidity and pH values of the cheese samples were in the ranges of 0.47–1.06% and 5.02–5.39, respectively. The pH of cheese samples decreased and TA significantly increased ($p \leq 0.05$) in all samples throughout the storage period. According to Dermiki *et al.*, (2008), the production of organic acid, mainly by lactic acid producing bacteria, increased during storage, which is likely what caused the increase in TA. The slight increase in the total number of bacteria in the cheese samples from algae emulsion in the first three weeks could explain the constant TA values.

Table 2. Chemical changes of algae Ricotta cheese during storage at 5 °C for 21 days.

Treatment	Storage period (Day)	% (wt/wt)				Titratable acidity (%)	pH
		Moisture	Protein	Fat	Ash		
Control	**Fresh**	75.11±0.03^{aA}	17.59±0.10^{iA}	0.40±0.01^{dA}	0.70±0.02^{gB}	0.53±0.02^{dD}	5.39±0.05^{ghA}
	7	74.93±0.46^{abAB}	17.61±0.04^{hiA}	0.40±0.01^{dA}	0.75±0.02^{fgAB}	0.64±0.02^{cC}	5.29±0.01^{hiAB}
	14	74.60±0.16^{abcAB}	17.87±0.09^{ghiA}	0.43±0.02^{dA}	0.80±0.02^{fgAB}	0.84±0.02^{hiB}	5.23±0.07^{iB}
	21	74.32±0.04^{bcdeB}	17.95±0.03^{fghA}	0.44±0.01^{dA}	0.87±0.04^{fA}	1.05±0.02^{aA}	5.02±0.01^{jC}
I	**Fresh**	74.57±0.17^{abcA}	18.08±0.14^{efgA}	0.60±0.01^{cA}	1.56±0.01^{eC}	0.35±0.02^{hiBC}	5.60±0.08^{cdeA}
	7	74.49±0.56^{abcdA}	18.11±0.19^{efgA}	0.62±0.01^{cA}	1.58±0.02^{deBC}	0.39±0.02^{ghB}	5.52±0.08^{efAB}
	14	74.35±0.08^{bcdeA}	18.25±0.17^{efA}	0.64±0.02^{cA}	1.61±0.08^{cdeABC}	0.46±0.02^{bA}	5.49±0.01^{efgAB}
	21	74.01±0.03^{cdefA}	18.33±0.06^{eA}	0.65±0.02^{cA}	1.73±0.03^{bcA}	0.47±0.01^{efA}	5.42±0.02^{fgB}
II	**Fresh**	73.99±0.27^{cdefA}	18.69±0.03^{dA}	0.72±0.02^{bA}	1.71±0.02^{bcdA}	0.34±0.02^{ghB}	5.76±0.03^{bA}
	7	73.85±0.07^{defgA}	18.73±0.10^{dA}	0.74±0.01^{bA}	1.74±0.02^{bcA}	0.37±0.03^{fghAB}	5.67±0.01^{bcAB}
	14	73.70±0.11^{efghA}	18.79±0.03^{cdA}	0.74±0.01^{bA}	1.79±0.06^{abA}	0.38±0.01^{fghAB}	5.58±0.03^{cdBC}
	21	73.51±0.07^{fghiA}	18.90±0.04^{bcdA}	0.76±0.01^{bA}	1.85±0.03^{abA}	0.42±0.02^{efA}	5.50±0.02^{defC}
III	**Fresh**	73.29±0.10^{ghiA}	19.04±0.10^{abcdA}	0.85±0.02^{aA}	1.78±0.02^{abA}	0.32±0.02^{iC}	5.88±0.05^{aA}
	7	73.24±0.08^{ghiA}	19.12±0.19^{abcA}	0.86±0.01^{aA}	1.80±0.12^{abA}	0.35±0.03^{hiBC}	5.78±0.02^{abA}
	14	73.10±0.09^{hiA}	19.19±0.11^{abA}	0.87±0.01^{aA}	1.85±0.06^{abA}	0.39±0.02^{ghAB}	5.61±0.01^{cdeBC}
	21	72.96±0.02^{iA}	19.32±0.18^{aA}	0.89±0.04^{aA}	1.92±0.04^{abA}	0.42±0.02^{fgA}	5.59±0.03^{deC}

All parameters are represented as mean of triplicates ± standard error. Different lowercase letters in the same row differ significantly between treatments at $p \leq 0.05$. Different capital letters in the same column differ significantly at $p \leq 0.05$ over the storage period. **Control:** Ricotta cheese without algae emulsion, **I:** Ricotta cheese supplemented with 0.25% algae emulsion, **II:** Ricotta cheese supplemented with 0.5% algae emulsion, **III:** Ricotta cheese supplemented with 1% algae emulsion.

3.7. Minerals content of algae Ricotta cheese

Data presented in Table 3 showed that the content of minerals (mg/100 g) in Ricotta cheese increased in linear proportion to the concentration of *D. salina* microalgae that was incorporated in the algal Ricotta cheese. Along with having a high protein content, algal emulsion ricotta cheese (1%), had the greatest micronutrient concentration, with 398, 143.63, 118.75, 22.35, and 0.85 mg/100g of calcium, phosphor, potassium, magnesium, and iron, respectively. These findings are consistent with those of Osman *et al*. (2011), Michalak and Chojnacka (2014), and Oluwamukomi and Adeyemi (2015). In the current investigation, fortification of *D. salina* microalgae in the ricotta cheese resulted in ricotta cheese enriched with appropriate concentrations of minerals including magnesium, zinc, copper, and iron, which are necessary for immune support (Weyh *et al*., 2022). Because dairy products are often low in iron (Gaucheron, 2000), the fortified ricotta cheese used in this study with *D. salina* microalgae had a high iron content, which is a significant advantage for consumers, particularly those who consume vegetarian or plant-based diets. Hence, in accordance with Regulation (EU) No. 1169/2011, *D. salina* microalgae-enriched Ricotta cheese can use the nutritional demand of 'source of iron'. Falcão *et al*., (2023) also revealed that the addition of 4 % of *C. vulgaris* into cream cheese can use the nutritional demand of a source of iron compared to control cheese.

Table 3. Minerals content of algae Ricotta cheese.

Mineral	Control	I	II	III
Ca	121±0.58^{d}	225±0.42^{c}	297±0.72^{b}	398±0.78^{a}
Na	415.38±0.06^{d}	442.86±0.61^{c}	495.04±0.03^{b}	569.69±0.04^{a}
K	114±0.29^{d}	115.04±0.07^{c}	116.38±0.12^{b}	118.75±0.03^{a}
Zn	0.030±0.003^{b}	0.032±0.001^{b}	0.035±0.002^{ab}	0.040±0.002^{a}
Fe	0.14±0.01^{d}	0.30±0.04^{c}	0.58±0.02^{b}	0.85±0.02^{a}
Cu	0.001±0.02^{a}	0.0013±0.01^{b}	0.0015±0.0001^{d}	0.061±0.01^{c}
Mn	0.025±0.003^{c}	0.050±0.01^{bc}	0.085±0.003^{b}	0.250±0.03^{a}
P	142.11±0.31^{c}	142.49±0.06^{bc}	142.87±0.06^{b}	143.63±0.07^{a}
Mg	13.02±0.06^{c}	21.08±0.04^{b}	21.96±0.26^{a}	22.35±0.12^{a}

All parameters are represented as mean of triplicates ± standard error. Different lowercase letters in the same row differ significantly between treatments at $p \leq 0.05$. Different capital letters in the same column differ significantly at $p \leq 0.05$ over the storage period. **Control**: Ricotta cheese without algae emulsion, **I**: Ricotta cheese supplemented with 0.25% algae emulsion, **II**: Ricotta cheese supplemented with 0.5% algae emulsion, **III**: Ricotta cheese supplemented with 1% algae emulsion.

3.8. Color development of algae Ricotta cheese

The enrichment of Ricotta cheese with *D. salina* algae emulsion led to innovative green color which mainly due to the presence of varied pigments in the microalga biomass including chlorophyll and carotenoids (El-Baz and Ali, 2023). The addition of algae emulsion formula during Ricotta cheese processing showed significant ($p \leq 0.05$) darkening of the cheese treatments, where it can be seen reduction in all color tonalities (Fig. 4). It could be mainly due the color of *D. salina* algae is green while the control Ricotta cheese is creamy color. The

obtained results are in the line with Falcão *et al.*, (2023) who demonstrated that the incorporation of *C. vulgaris* microalga results in a significant ($p \leq 0.05$) decrease in the L* color attributes for both cream and quark cheese samples compared to control cheese.

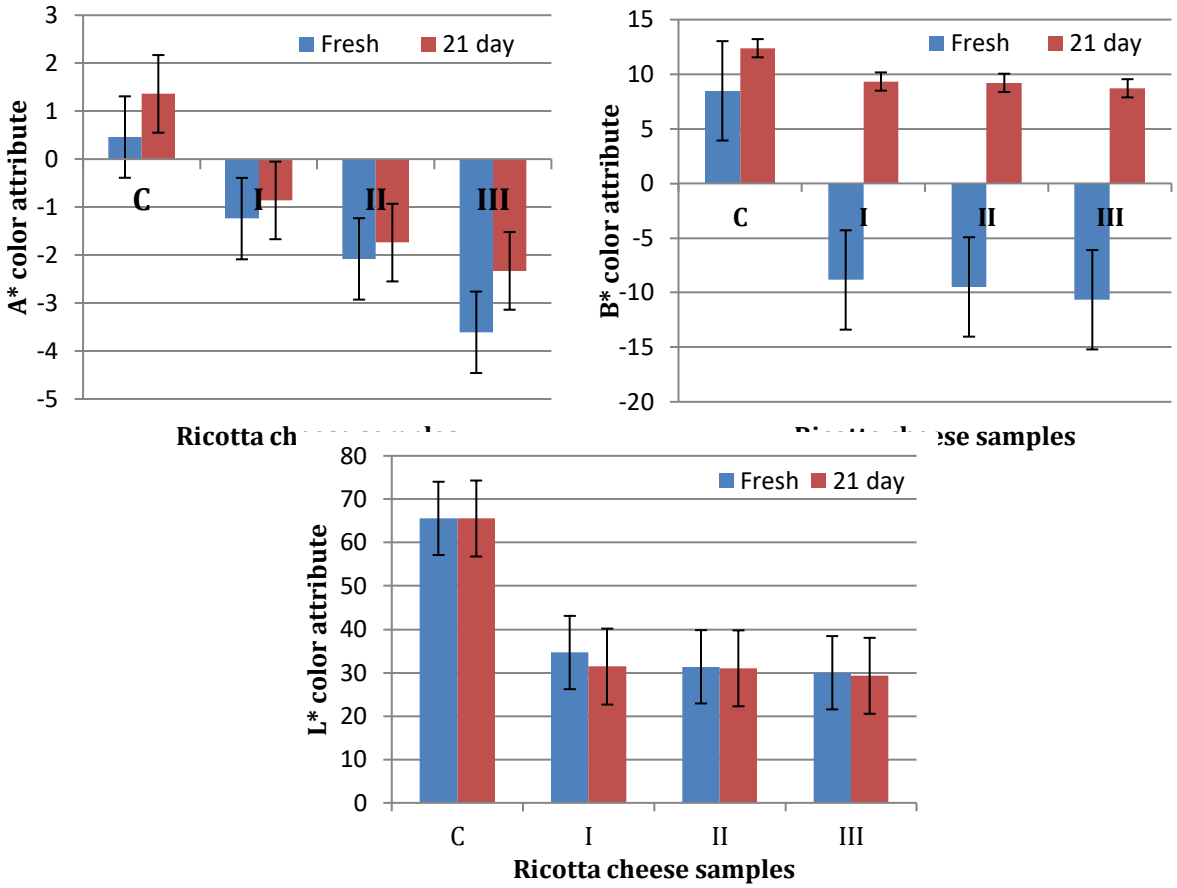


Figure 4. Color development of algae Ricotta cheese during storage at 5 °C for 21 days.
l* value represents darkness from black (0) to white (100); **a*** value represents color ranging from red (+) to green (−); **b*** value represents yellow (+) to blue (-).
C: Ricotta cheese without algae emulsion, **I**: Ricotta cheese supplemented with 0.25% algae emulsion, **II**: Ricotta cheese supplemented with 0.5% algae emulsion, **III**: Ricotta cheese supplemented with 1% algae emulsion.

3.9. Texture profile analysis of algae Ricotta cheese

The texture profile of Ricotta cheese is characterized as a viscoelastic food (Fox *et al.*, 2000) not pasty and friable with fragile characteristics (Tunick *et al.*, 2012). Texture profile analysis (TPA) analysis of Ricotta cheese at different ratios of algae emulsion was carried out on fresh samples and after 21 days of storage at 5 °C (Table 4). Hardness is the force required to attain a specific deformation. Hardness values of fresh samples ranged from 10.3 to 16.8 N. It could be seen that the control cheese sample had significantly higher hardness than the other varieties that contained different percentages of algae emulsion. This result agrees with Bryant *et al.*, (1995); Araque *et al.*, (2017); Azarashkan *et al.*, (2022) who reported that fat content affected cheese firmness significantly. As the cold storage period progressed, the hardness values of the cheeses significantly increased at the end of storage ($p \leq 0.05$) a similar finding was reported by Araque *et al.*, (2017). Adhesiveness is the work required to overcome attraction between food and other surfaces. Because the fat content affected the adhesiveness, therefore significant change was observed in the adhesiveness of algae-based Ricotta cheese

treatments. Algae-based Ricotta cheese samples showed greater adhesiveness than the control sample at the end of the cold storage period. Cohesiveness is the mechanical textural attribute relating to the degree to which a substance can be deformed before it breaks. The cohesiveness value without the addition of algae emulsion was 0.45. The lower value of cohesiveness in Ricotta cheese can be attributed to the low fat/protein ratio (Pizzillo *et al*., 2005). The addition of algae emulsion increasing cheese cohesiveness which means that the force exerted by the internal cheese bonds was stronger. The ability of the deformed cheese to gradually return to its original position following the removal of force is measured by its springiness. The higher values of springiness of II and III treatments show that the deformability of Ricotta cheese remained unchanged (Ferrandini *et al*., 2011). No significant differences in springiness ($p \leq 0.05$) observed in cheese samples; but only among control and III treatments. There was a slight decrease in springiness, which could be related to the proteolytic breakdown of the protein matrix. Chewiness is a secondary cheese textural characteristic. and derivative of hardness which means the overall work required for the double compression. Zheng *et al.*, (2016) found that chewiness values were highest for low-fat cheese samples and lowest for high-fat or high-moisture cheese samples. Additionally, it was noted that restructured Ricotta cheese made with mozzarella cheese whey including a combination of gelatin and guar gum had a higher chewiness value (Hesarinejad *et al*., 2021). In our study, the value of chewiness decreased with increasing the percentage of algae emulsion (4.75mj). There were significant differences ($p \leq 0.05$) in gumminess between the cheese treatments. The control recorded the highest value of guminness (9.07 N). At the end of the storage period, this secondary attribute increased and exhibited similar behavior with primary attribute of hardness. Our TPA results are in agreement with literature (Tunick *et al*., 2012; Hesarinejad *et al*., 2021) who described that Ricotta cheese texture is very soft consistency, compressible, and not too cohesive. In terms of textural parameters, adhesiveness, cohesiveness, and springiness, which are essential for preserving product acceptance over its shelf life, there were no significant changes observed after 21 days of refrigerated storage and the texture profile was generally stable.

Table 4. Texture profile changes of algae Ricotta cheese during storage at 5 °C for 21 days.

Parameter	Storage period (day)	Ricotta cheese treatments			
		Control	I	II	III
Hardness (N)	**Fresh**	16.80 ± 0.04^{bB}	14.30 ± 0.06^{dB}	11.70 ± 0.11^{fB}	10.30 ± 0.13^{hB}
	21	17.20 ± 0.02^{aA}	16.10 ± 0.15^{cA}	13.20 ± 0.10^{eA}	11.28 ± 0.09^{gA}
Adhesiveness (mj)	**Fresh**	0.80 ± 0.01^{fA}	0.87 ± 0.02^{efB}	1.15 ± 0.04^{dB}	1.51 ± 0.02^{bB}
	21	0.83 ± 0.006^{efA}	0.94 ± 0.06^{eA}	1.34 ± 0.07^{cA}	1.84 ± 0.03^{aA}
Cohesiveness (Ratio)	**Fresh**	0.54 ± 0.03^{dA}	0.61 ± 0.02^{cdA}	0.69 ± 0.02^{abcA}	0.71 ± 0.04^{abA}
	21	0.59 ± 0.03^{dA}	0.68 ± 0.03^{bcA}	0.74 ± 0.02^{abA}	0.77 ± 0.03^{aA}
Springiness (mm)	**Fresh**	0.65 ± 0.03^{bA}	0.65 ± 0.03^{bA}	0.83 ± 0.03^{abA}	0.86 ± 0.07^{aA}
	21	0.66 ± 0.03^{bA}	0.66 ± 0.03^{bA}	0.85 ± 0.08^{aA}	0.89 ± 0.05^{aA}
Gumminess (N)	**Fresh**	9.07 ± 0.09^{cB}	8.72 ± 0.02^{cB}	8.07 ± 0.22^{dB}	7.31 ± 0.06^{eB}
	21	10.15 ± 0.04^{bA}	10.95 ± 0.36^{aA}	9.77 ± 0.32^{bA}	8.69 ± 0.15^{eA}
Chewiness (mj)	**Fresh**	7.78 ± 0.09^{bB}	7.24 ± 0.14^{cB}	6.05 ± 0.02^{dB}	4.75 ± 0.12^{eB}
	21	9.03 ± 0.21^{aA}	9.31 ± 0.17^{aA}	7.43 ± 0.12^{bcA}	5.74 ± 0.06^{dA}

All parameters are represented as mean of triplicates ± standard error. Different lowercase letters in the same row differ significantly between treatments at $p \leq 0.05$. Different capital letters in the same column differ significantly at $p \leq 0.05$ over the storage period. **Control**: Ricotta cheese without algae emulsion, **I**: Ricotta cheese supplemented with 0.25% algae emulsion, **II**: Ricotta cheese supplemented with 0.5% algae emulsion, **III**: Ricotta cheese supplemented with 1% algae emulsion.

3.10. Organoleptic attributes of algae Ricotta cheese

In 2020, green colored food varieties a major pattern as consumers looked for items that reconnected them with nature which such variety is frequently connected with nutritional and healthy products (Smail, 2021; Falcão *et al*., 2023). In the present investigation, the addition of algae emulsion formula during Ricotta cheese processing improved all sensorial attributes of cheese including flavor, appearance and texture during the storage period for 21 days at 5 °C. The Ricotta cheese with algae emulsion formula (0.5 %) had the highest flavor (Fig. 5A), and appearance (Fig. 5B) scores among other treatments; while at the higher level of algae emulsion such sensorial attributes were decreased. The mint essential oil content of algae emulsion improved the acceptability of cheese flavor and appearance especially for both *D. salina* algae color is green and its taste could be unaccepted, while the higher level of algae emulsion increased the green color level in the resulted cheese which decreased their overall acceptability. Also, Fig. 5C shows that the body and texture of Ricotta cheese was increased with the algae emulsion increased. Falcão *et al*., (2023) also reported a promising sensory testing result in cream cheese supplemented with *C. vulgaris* compared to control cheese.

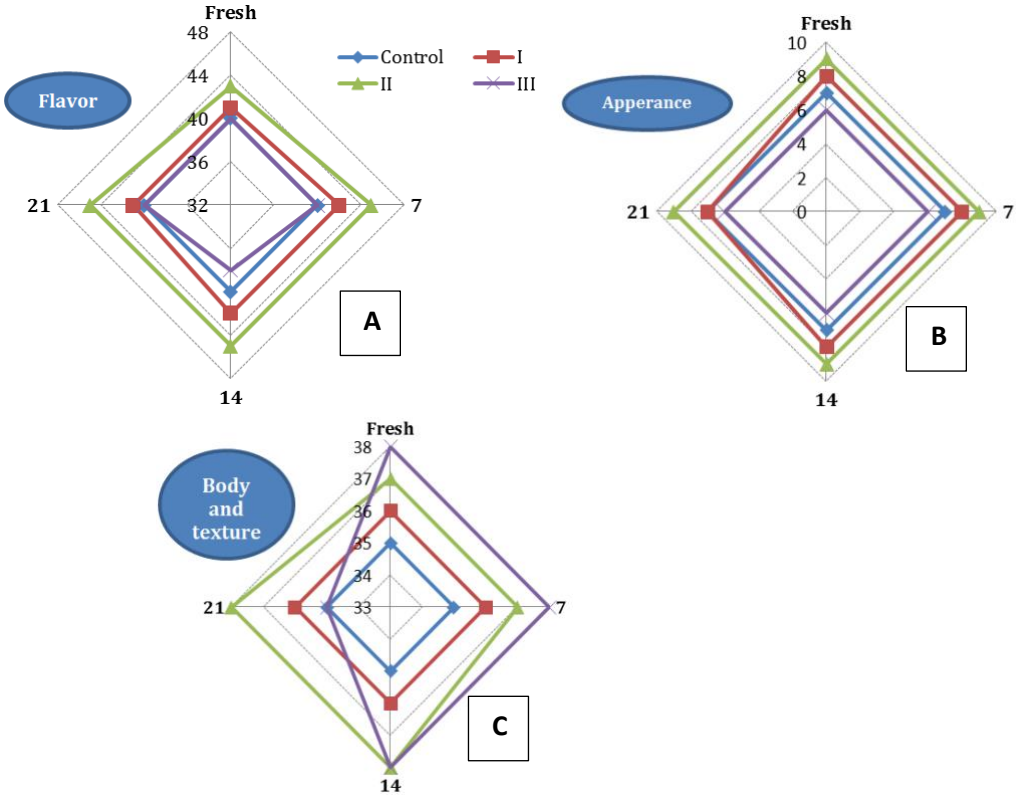


Figure 5. Organoleptic attributes change of algae Ricotta cheese during storage at 5 °C for 21 days. **Control**: Ricotta cheese without algae emulsion, **I**: Ricotta cheese supplemented with 0.25% algae emulsion, **II**: Ricotta cheese supplemented with 0.5% algae emulsion, **III**: Ricotta cheese supplemented with 1% algae emulsion.

3.11. Amino acids content and chemical score of algae Ricotta cheese

Data of Table 5 showed that most of amino acids, including glutamic acid, serine, glycine, histidine, arginine, proline, methionine, cysteine, isoleucine, and phenylalnine, were increased in the Ricotta cheese when *D. salina* algae emulsion formula was added at a level of 0.5% in comparison of untreated cheese. On the other hand, control Ricotta cheese and algal

cheese had comparable TAA, EAA, and EAA/TAA ratio. Fig. 6 demonstrates that the chemical score of various amino acids, including methionine + cysteine, histidine, and isoleucine, increased when algal emulsion formula was added to Ricotta cheese compared to control Ricotta cheese. The fortified Ricotta cheese used in this study with *D. salina* microalgae had high contents of essential amino acids, which are vital to human nutrition and health. Different amino acids have a variety of vital roles in several functional processes, including cell signaling, gene expression, DNA synthesis, immunological response, and food intake and metabolism, in addition to serving as the building blocks for proteins (Manjarín *et al*., 2020; Ryan *et al*., 2021).

Table 5. Amino acids content of algae Ricotta cheese.

Amino acids (mg/g protein)	Ricotta cheese treatments	
	Control	**Best algae cheese (II)**
Aspartic acid	80.02	79.98
Glutamic acid	180.45	184.71
Serine	49.63	57.32
Glycine	19.09	22.83
Histidine	25.79	33.09
Arginine	34.76	41.51
Threonine	119.24	112.75
Alanine	66.18	46.89
Proline	89.61	115.23
Tyrosine	55.28	49.03
Valine	55.61	46.04
Methionine	30.64	31.5
Cysteine	2.86	3.8
Isoleucine	24.95	32.62
Leucine	38.39	36.2
Phenylalnine	12.95	17.78
Lysine	65.00	37.75
Total amino acids (TAA)	950.45	949.03
Total essential amino acids (EAA)	288.09	276.49
EAA/TAA ratio	0.303	0.291

II: Ricotta cheese supplemented with 0.5% algae emulsion formula.

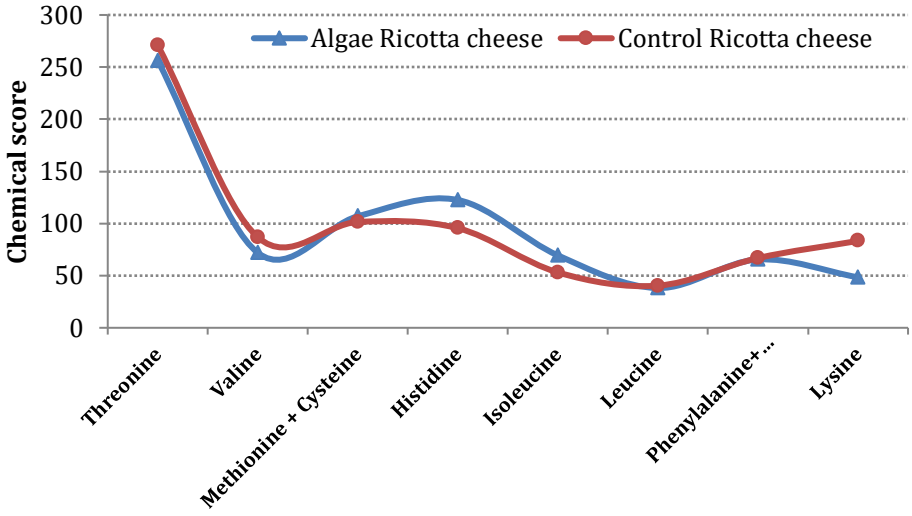
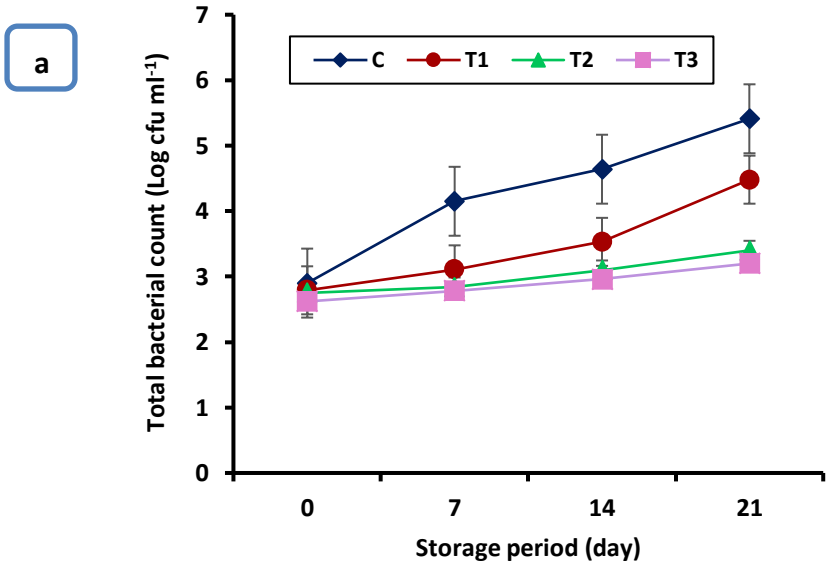


Figure 6. Chemical score of algae Ricotta cheese.
Algae Ricotta cheese: Ricotta cheese supplemented with 0.5% algae emulsion formula.

3.12. Microbiological analysis of algae Ricotta cheese

A slight increase in total bacterial count (TBC) during storage period in algae Ricotta samples was recorded. The TBC was significantly lower ($p \leq 0.05$) in algae Ricotta cheese samples than in control sample (Fig. 7a). The yeast and mold counts showed a different trend in control sample than II and III algae Ricotta samples cheese (Fig. 7b). In the control sample, it appeared after 14 days of storage, whereas in the algae Ricotta cheese samples II and III it appeared only on the 21st day of storage period. There were significant differences between all treatments at the end of the storage period ($p \leq 0.05$).

Coliform bacteria were not detected in all samples either fresh or during the refrigerated storage period. This may be due to the high sanitation conditions during manufacturing and cold storage.



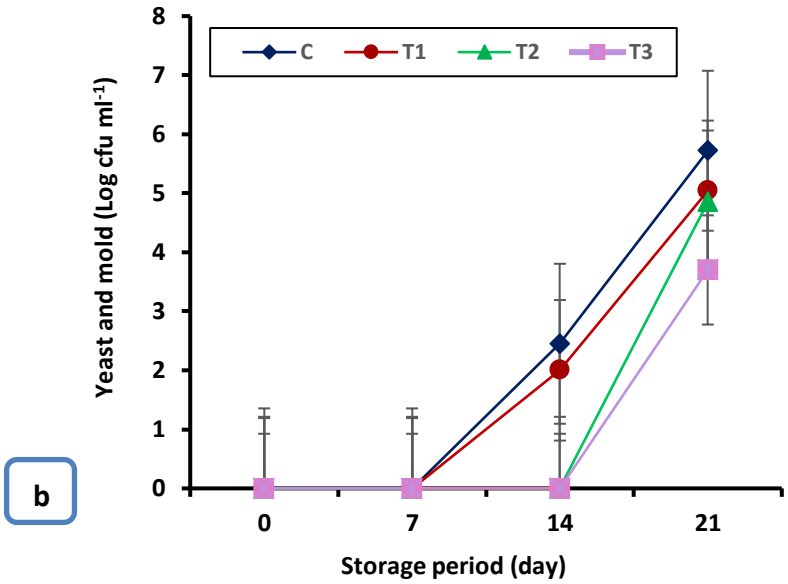


Figure 7. Microbiological changes of algae Ricotta cheese during storage at 5 °C for 21 days. **(a)** Total bacterial count (TBC), **(b)** yeast and mold count.
C: Ricotta cheese without algae emulsion, **T1**: Ricotta cheese supplemented with 0.25% algae emulsion, **T2**: Ricotta cheese supplemented with 0.5% algae emulsion, **T3**: Ricotta cheese supplemented with 1% algae emulsion.

4.Conclusion

Adequate total phenolics, total flavonoids, and phenolics components, particularly gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, catechin, naringenin, quercetin, rutin, and hesperetin, are present in *D. salina* microalgae, which have the potent ability to scavenge free radicals. *D. salina* microalgae contain significant amounts of several amino acids, including threonine, lysine, histidine, valine, and phenylalanine, which are necessary for human growth and health. Therefore, the incorporation of *D. salina* algae/mint essential oil emulsion into Ricotta cheese processing led to improve the final characteristics of such cheese including more antioxidant activity, protein content, mineral profile, and good sensorial acceptability. Hence, *D. salina* microalga could be utilized as a promising functional ingredient of plant origin for the improvement of conventional dairy items, resulting in inventive and more sustainable products.

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EVALUATION OF MICROBIAL QUALITY OF UNFERMENTED COCONUT SAP WITH DIFFERENT COLLECTION METHODS

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https://doi.org/10.34302/crpjfst/2023.15.4.7

Article history:
Received: 31 December 2022
Accepted: 10 October 2023

Keywords:
Microbial quality;
Unfermented coconut sap;
Universal primer.

ABSTRACT

Coconut (*Cocos nucifera*) sap is one of the natural drinks, being traditionally tapped from unopened inflorescences of the coconut palms. In the present study, microbial quality of coconut sap that was collected using three methods; Treatment 1: application of hal bark (*Vateria copallifera)* to the 4L clay pots (TM), Treatment 2: Novel sap collection method (NSM), Treatment 3: application of 5g of hal bark in to 30 cm X 50 cm polythene bag in 4L clay pots (MTM) were evaluated. Sap was collected for 12 h duration and Total Plate Count (TPC) and Yeast and Mold Count (YMC) were determined. Colonies were isolated and preserved. DNA extractions of microbes were done by CTAB (Cetyl trimethylammonium bromide) method with modification. ITS1 forward and ITS4 reverse primers were used to identify yeast species and 27 forward and 1492 reverse primers were used for the bacterial species in Polymerase Chain Reaction **(**PCR). Amplified products were separated using 1.5 % agarose gel and purified DNA was sent into Macrogene Korea for sequencing. Four types (A, B, C and D) of distinct microbial colonies were isolated from the differently collected coconut sap samples. DNA homology data revealed that, A is *Naumannella halotolerans* only presented in NSM. B and C *Serratia marcescens, are Achromobacter xylosoxidans* contained in TM and MTM. *Saccharomyces cerevisiae* (D) was found in all the collection system. The collection method affects for the microbial quality and quantity of unfermented coconut sap.

1.Introduction

Coconut (*Cocos nucifera*) sap is the nutritive phloem fluid that oozes out from the coconut inflorescence after a special extraction process called tapping. The sap is collected at 12 h or 6 h time intervals. Freshly collected coconut sap (*meera*) is a rich source of sugars, minerals and vitamins (Barh and Mazumdar, 2008). It undergoes natural fermentation by wild microorganisms after contacting with atmosphere and is used for product development. The fermented coconut sap ("*Raa"*) is a popular alcoholic beverage among rural people. Natural fermentation of coconut sap starts with lactic acid fermentation and followed by alcoholic fermentation and finally

acetic acid fermentation. Seventeen (17) species of yeasts and seven (7) genera of bacteria have been identified in the naturally fermented coconut sap (Atputharajah *et al.*, 1986).

In order to reduce the rate of fermentation, lime ($Ca(OH)_2$) and bark of Hal tree (*Vateria copallifera*) are put into clay pots from ancient time (Ratnasooriya *et al.*, 2006). However, these substances has negative impact on odour, taste and color of coconut sap. In addition, the traditionally collected sap is generally contaminated by insects, ants, dust particles and pollen due to the gap between clay pot and coconut inflorescence (Navaratne, 2015). Therefore, novel sap collection method was introduced by Coconut Research Institute (CRI) of Sri Lanka to reduce the fermentation creating sealed equipment with an ice box. This new collection device comprises of a fixing unit, a pipe connector, a male adaptor, a flexible hose for translocation of sap and a cooling compartment. The fixing unit has a soft skin to protect the inflorescence without damaging while providing a sealed condition to avoid contaminations. The cooling compartment with ice box prevents the growth of microorganisms due to its low temperature (<15^{o}C). Therefore, the collection method can affect to the microbial quality and quantity of the coconut sap due to having different mode of contact with the coconut inflorescence and different environment.

Molecular approach and biochemical (traditional) approaches can be utilized to microbial identification. The traditional approaches are time-consuming, labour intensive and often subjective. The applications of molecular methods avoid the weaknesses in the culture-dependent methodologies. DNA extraction is crucial step in molecular DNA analysis. Within the different protocols for DNA extraction, CTAB (cetyl trimethyl ammonium bromide) method is commonly used for plant DNA extraction (Doyle and Doyle 1987). Amplification of universal region of microbial genome is used for identification of bacteria. ITS1 forward (5' TCCG TAG GTGAACCTGCGG 3') and ITS4 reverse primers (5' TCCTC GCTTA TTGATATGC 3') (White *et al.*, 1990) were used for yeast species and 27 forward (5'-AGAGT TTGA TCCTGGCTCAG-3') and 1492 reverse (5'-CGGTTACCTTGTTACGACTT-3') primers were used for bacteria species (Miller *et al.*, 2013). This research was conducted to evaluate the microbial quality and quantity of unfermented coconut sap collected from three types of collection methods by using modified plant DNA extraction protocol for bacterial DNA extraction.

2. Materials and methods

2.1. Materials

The experiment was conducted during the period from February to August 2017. Coconut sap was collected from twenty four coconut palms (Tall ×Tall variety, 45 years old) in Bandirippuwa Estate of Coconut Research Institute, Lunuwila. The novel sap collection devices were fabricated at Coconut Processing Research Division, Coconut Research Institute, Lunuwila.

2.2. Methods

2.2.1. Collection of coconut sap sample

One type of sap collection method was applied in to one block of coconut palms which is consisted with eight number of trapping trees. Three rotations were done among blocks to collect samples. Three types of treatments were Treatment 1: Traditional method- application of Hal bark to the clay pots of 4L (TM), Treatment 2: Novel sap collection method (NSM), Treatment 3: Modified traditional method - application of 5g of Hal bark in to 30 cm X 50 cm polythene bag in clay pots of 4L (MTM). The environment temperature was fluctuated from 27^{o}C to 31.4 ^{o}C during sap collection from 6.00 pm to 6.00 am.

Coconut sap from each treatment was used for microbial studies. Aliquot of coconut sap sample was diluted and inoculated to the sterilized plate with Nutrient Agar (NA) media for Total Plate Count (TPC) and Potato Dextrose Agar (PDA) for Yeast and Mold Count (YMC). TPC and YMC were enumerated using standard

protocols and isolated pure cultures were preserved at -20ºC in glycerol.

2.2.2. Microbial DNA extraction

The pure colonies were grown in nutrient broth, in 24 hrs in a shaking incubator (Grant Instruments™ ES-80) at 28ºC. The broth was filled into sterilized 1.5ml micro centrifuge tube in Sigma 1-15K and centrifuged at 5000 rpm for 5 min to separate bacteria and yeast. The supernatant was removed and dry pellet was taken to extract DNA using modified CTAB method. The composition of modified CTAB buffer was 10 %of 1M Tris HCl, 4 % of 0.5 M EDTA, 8.19 % of NaCl, 2 % of CTAB at pH 8. Then, 750 µl of buffer solution was added into the tube with pellet and incubated at 65ºC for 30 min (Memmert 0810).

Equal volume (750µl) of phenol: chloroform (1:24) was added to the tubes and mixed and centrifuged at 13,000 rpm for 15 min. Supernatant was collected and Phenol: chloroform extraction step was repeated. Two third (2/3) volume of ice cold iso-propenol was added to the supernatant and mixed well. Samples were kept in refrigerator (4±2 ºC) for 2hrs and centrifuged at 13,000 rpm for 10 min. Supernatant was removed and the pellet was washed with 20 µl of 70 % alcohol. The mixture was centrifuged at 10,000 rpm for 10 min and pellet was collected removing supernatant. The dried pellet was dissolved in 20µl of TE buffer and stored at -20 ºC. The concentration and quality of extracted DNA were measured by Nano drop 2000C.

2.2.3.PCR amplification with universal primers

For bacteria isolates 27 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 reverse (5'-CGGTTACC TTGTTA CGACTT-3') primers was used for amplification using thermo cycler (Takara Bio Inc TP 600). The temperature profile of PCR machine was 95ºC in 15 min initial denaturation), followed by 31 cycles of 95ºC 1min denaturation, 55.5ºC 2min annealing and 72ºC 2 min (extension) Samples were kept at 72ºC for 10 min for final extension.

For the Yeast amplification universal primers of ITS1 forward (5' TCCG TAG GTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') reverse primers were used with temperature profile of 9 ºC in 15 min (initial denaturation), followed by 94ºC 1 min denaturation process, 55.5ºC 45 s annealing, 72ºC 1.5 min extension, 72ºC 10 min final extension, 4ºC cooling with 35 cycles for yeast ITS1/ITS4 primer amplification.

2.2.4. Identification of PCR products

Amplified products were separated using 1.5 % (w/v) agarose gel with 100 bp ladder and amplified bands were observed under a transilluminator (Cell Bioscience). The bands were excised and purified with wizard PCR clean-up system. The purified products were sent to Macrogen Inc. Korea for sequencing.

2.2.5. Data analysis

Parametric data was analyzed using Mini Tab 16 using ANOVA. Sequences were blasted and compared with data in NCBI data bank.

3. Results and discussions

3.1. Quantity of Microbes in different collection systems

3.1.1. Total plate count and Yeast count

Microbial counts obtained by colony forming units (CFU) were different in three methods. TPC of sap collected from traditional method (TM) ($1.19*10^7$) and poly bag collection method ($5.2*10^6$) did not show a significant difference ($p<0.05$). The seal environment of NSM with its cooler compartment has retarded the microbial contamination. Therefore, the bacteria and yeast count of NSM showed a significantly ($p<0.05$) lower number of TPC (3.64×10^6) and YMC (7.67×10^6).

3.2. Colony characteristics of isolates

Four types of distinct microbial colonies were isolated from the sap of three different methods (Figure 1; A, B, C and D). A, B and C colonies were bacteria while D colony was a yeast strain. Different types of color patterns and culture characteristics were observed in these

four different isolates (Figure 1). Type "A" isolate was observed in samples collected from NSM while B and C isolates were observed in sap samples of TM and MTM. D colony was observed in samples of all collection methods. The A isolate was light yellow in colour while B isolate had an attractive bright purple colour. the C isolate produced gummy or jelly like colonies. White colour D isolate showed a budding growth and it could be identified as S*accharomyces* strain.

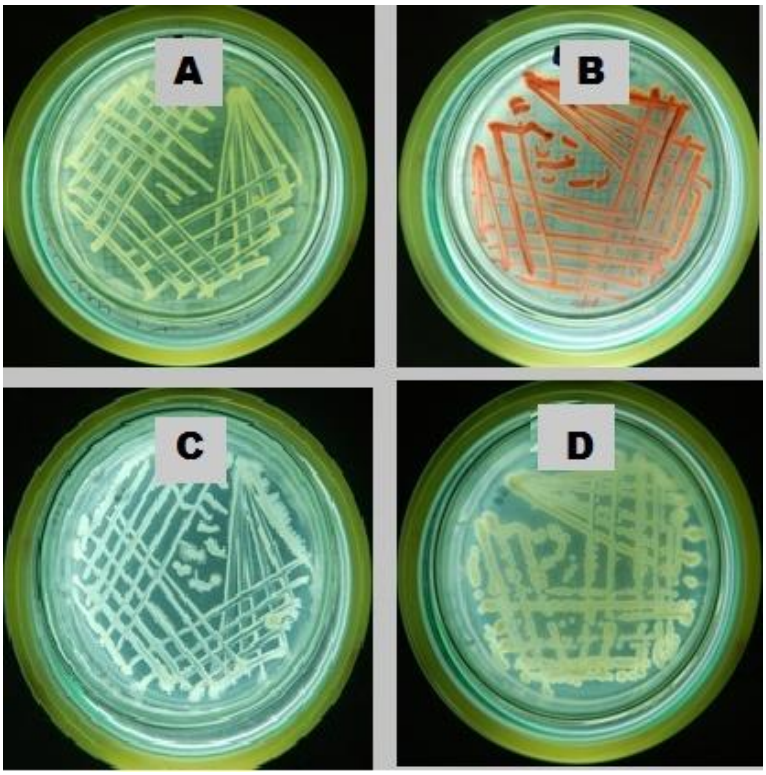

Figure 1. Microbial cultures Isolated from unfermented coconut sap grown in NA (A, B and C) and PDA(D) media

3.3. Quality of extracted DNA by modified CTAB method

The concentrations of DNA of three different isolates revealed that the modified CTAB method for microbial DNA extraction is a feasible method to extract bacterial and yeast DNA with only a few steps while using the same chemicals used for plant DNA extraction. DNA concentrations of three isolates were 3155.1ng/µl, 1567.5ng/µl, 1013.0ng/µl and 3800.3ng/µl for A, B, C and D respectively.

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA and a ratio of ~2.0 is generally accepted as "pure" for RNA (Glasel, J.A, 1995). DNA concentration of 260/280 showed that, all values were over the limit of 1.8. The values of 260/280 were 1.84, 1.96, 2.14 and 2.13 for A, B, C and D respectively showing a high quality without contaminants**.** Expected 260/230 values are commonly in the range of 2.0 - 2.2. If the ratio is appreciably lower than expected, it indicates the presence of contaminants which absorb at 230 nm. The ratio of 260/230 of A, B, C and D were 2.52, 1.58, 2.11 and 2.03 respectively. Gel electrophoresis of PCR products clearly showed that, A B and C isolates extracted from the coconut sap amplified with the 27 forward and 1492 reversed primers and observed as thick bands. The yeast species were amplified with ITS1 forward and ITS4 reversed primers. Homology search results of the sequenced data are represented with accession

number, identity, query cover and isolated collection system (Table: 1).

Table 1. Homology results of the sequenced data

Isolate	Accession Number	Homologous genome / Organism	Identity %	Query Cover %	Isolated collection system
(A)	KC429590.1	*Naumannella halotolerans*	95	94	NSM
(B)	FJ360759.1	*Serratia marcescens*	100	98	TM and MTM
(C)	KP236255.1	*Achromobacter xylosoxidans*	99	98	TM and MTM
(D)	Z75578	*Saccharomyces cerevisiae*	96	69	NSM, TM and MTM

TM: Traditional method- application of Hal bark to the clay pots of 4L, NSM: Novel sap collection method (NSM), MTM: Modified traditional method - application of 5g of Hal bark in to 30 cm X 50 cm polythene bag in clay pots of 4L

3.4. Identification of microorganisms in unfermented coconut sap

Four different isolates A,B, C and D were *Naumannella halotolerans, Serratia marcescens, Achromobacter xylosoxidans and Saccharomyces cerevisiae* respectively. Seventeen strains of yeast of *Saccharomyces* (10 strains), *Pchia* (2 strains), *Torulopsis* (2 strains), *Candida* (2 strains), *Wingae* (1 strains) have been isolated from the coconut toddy (fermented sap) in Sri Lanka. Perera *et al*, (1978) found four strains of *Saccharomyces cerevisiae* and four strains of *Saccharomyces exguus* as the most predominant species. It has been revealed that the fresh sap of *Cocos nucifera* in India contains microorganisms such as *Kloeckera apiculata*, *Schizosaccharomyces pombe*, *Candida glabrata* and in fermented *toddy Kloeckeraa piculata*, *Schizosacc haromyces pombe*, *Pichiaangophorae* and *Bacillus firmus* (Kalaiyarasi *et al.,* 2013). The current study reveals that, the changes in collection methods and climatic condition affect microbial community in coconut sap.

Naumannella halotolerans, is a Gram-positive, non-motile, non-spore forming aerobic coccus of the family Propioni bacteriaceae. The organism was previously isolated from a pharmaceutical room and food (Gernot *et al*, 2012). It is one of the endophytic bacteria (plant beneficial) that colonizing on the shoot-tips of banana and it has shown antagonistic activity against the Panama wilt pathogen (*Fusarium oxysporum)* (Sivamani and Gnanamanickam, 1998).

Serratia marcescens is a gram-negative, rod shaped, bright purple color bacterium in the family Enterobacteriaceae. This bacterium is found abundantly in the environment and is a phloem-colonizing, Squash bug -transmitted bacterium and is the causal agent of cucurbit yellow vine disease. *Serratia marcescens* has become an important nosocomial pathogen and it has an antibacterial characteristic (Lapenda, 2015) due to its Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1Hpyrrole) (Moraes, 2009). When, the isolated culture of *S. marcescens* was tested for diffusion method with 2% phenolic extracts of Hal bark, it (B) didn't show antimicrobial property for phenolic compounds of hal bark compared with isolation A (Figure: 2).

Results emphasized that, antimicrobial property of hal bark does not affect the growth of *S. marcescens* due to its prodigiosin.

Unhygienic practices of clay pot and hal bark collection can be the main routes of contamination of *S. marcescens* in to coconut sap.

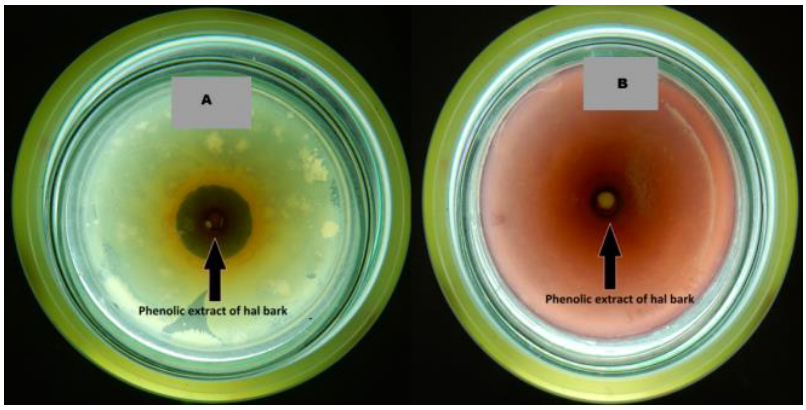

Figure 2. Growth of sap containing microbes A (*Naumannella halotolerans*) and B (*Serratia marcescens*) with phenolic extracts of hal bark

The organism C was identified as *Achromobacter xylosoxidans*. It is a Gram-negative, aerobic, rod shaped motile bacterium. It is generally found in wet environments. *A. xylosoxidans* is considered as an opportunistic pathogen and cause various diseases like bacteremia, meningitis, urinary tract infections, abscesses, osteomyelitis, corneal ulcers, prosthetic valve endocarditis, peritonitis, and pneumonia in both immunocompetent and immunocompromised hosts (Holmes *et al*, 1977). *Saccharomyces cerevisiae* is a unicellular eukaryote that can reproduce both asexually and sexually (Kumar and Srivastava, 2016) and it also known as 'budding yeast' or 'baker's yeast', belongs to kingdom Fungi. The species is widespread and commonly found in fermented fruits, delicious trees and high sugar environment such as nectar and sap. *Saccharomyces cerevisiae* is the main microorganism that has been identified in alcoholic fermentation and odorants production in coconut toddy. It has higher potential to production of alcohol at fairly high temperature (Chandrani, 1998) by converting the sugary sap to alcohol (Amoa-Awua *et al*, 2007).

Three types of sap collection methods did not produce a fully sterile condition. Thus, it facilitates contamination with natural microorganisms. But NSM restricted the colonization of nosocomial pathogen or opportunistic pathogen in unfermented coconut sap compared to other two collection methods. Further, hygienic practices are needed to be applied to increase the quality of coconut sap. The pathogenic nature of isolates restricts the direct consumption of unfermented coconut sap and it needs to go through thermal treatment during the production process to eliminate opportunistic pathogens. However, microbial analysis should be an essential step in processing of sap based products.

4. Conclusions

Microbial DNA can be extracted through modified CTAB method. Three types of unfermented sap samples were contaminated with *Saccharomyces cerevisiae. Naumannella halotolerans* is present in novel sap collection system. *Serratia marcescens* and *Achromobacter xylosoxidans* are identified in sap samples collected from clay pot and poly-bags including *hal* bark. Unfermented sap can't be directly consumed as in the case of fermented sap and it needs to be undergo production processes such as thermal treatment.

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ASSESSMENT OF PHYTOCHEMICAL, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *EVERNIA PRUNASTRI* SPECIES COLLECTED FROM ALGERIA

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https://doi.org/10.34302/crpjfst/2023.15.4.8

Article history:
Received :15 May 2023
Accepted :30 November 2023

Keywords:
Lichens;
Evernia prunastri;
Antioxidant activity;
Antibacterial activity;
Staphylococcus aureus.

ABSTRACT

In this study, the phytochemical screening of lichen samples of *Evernia prunastri* collected from three different regions of Algeria (Jijel sample (JS), Setif sample (SS) and M'sila sample (MS)) was investigated. The phenolic and flavonoid contents of lichen extracts obtained by maceration in methanol were determined. The antioxidant activities of these extracts were measured by determining the total antioxidant capacity (TAC), the ferric reducing antioxidant power (FRAP) and the free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Similarly, antibacterial activity was determined by solid medium diffusion and liquid medium microdilution methods against Gram-positive and Gram-negative bacteria. The qualitative phytochemical analysis of lichen samples revealed the presence of some compounds such as saponins, flavonoids, alkaloids. For quantitative analysis, the MS methanolic extract from M'Sila region showed the highest values for phenolic and flavonoid compounds which are equal to 929.3 mg/100g and 56.34 mg/100g, respectively. This extract also shows the best antioxidant activities with the three tested methods. For antibacterial activity, the best effect was obtained for the methanolic extract from Jijel region (JS) against the methicillin resistant *Staphylococcus aureus* strain with an inhibition zone of 35.33 mm and an MIC of 0.058 mg/mL. However, the three *Evernia prunastri* methanolic extracts (JS, SS and MS) were found to be inactive against Gram-negative bacteria. The obtained results indicate that the studied extracts have interesting antioxidant and antibacterial activities, which is probably due to the presence of phenolic and flavonoid compounds.

1. Introduction

Lichens are cryptogams that are present in all terrestrial ecosystems, including extreme ecosystems. They represent unique life forms- a symbiosis between a fungus (mycobiont) and an alga and/or a cyanobacterium (photobiont). They typically grow on rocks and non-fertile soils and as epiphytes on trees and leaves (Stamenković *et al.,* 2011; Chahra *et al.,* 2016).

For centuries, lichens have been used in the nutrition of many animals and humans during famines. Many edible lichens species such as *Dermatocarpon miniatum*, *Lobaria pulmonaria*, *Umbilicaria esculenta*, *Alectoria asiatica* are used in China as dishes after a simple transformation. They are also used as

spices, dyes in textiles, odorant in the perfume industry and for medicinal purposes to treat kidney, respiratory and liver infections and also as antiseptic (Mitrović *et al.,* 2011; Aoussar *et al.,* 2021; Muthu *et al.,* 2021; Zhao *et al.,* 2021).

Lichens are considered as a potential source of new biologically active compounds. They produce specific chemicals, phenols, that are very different from those synthesized by the rest of the plant species: about 1050 compounds in all and more than 550 compounds that are specific to lichens. Lichenic substances include aliphatic, cycloaliphatic, aromatic and terpene components (Herrero-Yudego *et al.,* 1989; Buçukoglu *et al.,* 2013; Kalidoss *et al.,* 2020). These metabolites are key components in the bioactivity of lichen extracts that are of great importance to modern pharmacy and medicine. Various biological activities of some lichens are known, such as antimicrobial, antiviral, antitumor, anti-inflammatory, analgesic, antipyretic, antiproliferative and antiprotozoal activities (Kosanic *et al.,* 2011; Mitrović *et al.,* 2011). Lichens as valuable sources of natural antioxidants and antimicrobial agents have been extensively studied. A strong antioxidant power of some lichen species was demonstrated in several studies (Kosanic *et al.,* 2011; Mitrović *et al.,* 2014).

The increasing development of microorganism resistance to conventional antibiotics and the problem of treatment of induced infections have prompted researchers to find other antimicrobial alternatives in lichens. Antibacterial activity against Gram-positive and Gram-negative bacteria, as well as antifungal activity are demonstrated for many lichen species (Mitrović *et al.,* 2011), such as *Evernia prunastri* (L.) Ach., *Ramalina fastigiata* (Pers.) Ach. and *Cladonia rangiformis* Hoffm. collected in northeastern Algeria which showed a very interesting antibacterial activity against *Staphylococcus aureus* strain. This activity is certainly due to predominant compounds such as lichenic acids which have already proved their antibacterial efficacy (Brakni and Ali Ahmed, 2018). Usnic acid as a pure substance has been formulated in creams, toothpastes, mouthwashes, deodorants and cosmetics, sunscreen products (Behera *et al.,* 2005).

According to the study of Miara *et al.* (2013), some species including *Evernia prunastri* (L.) Ach., were proposed as new species for the Algerian medicinal flora. Indeed, these species do not appear in the main works on therapeutic plants in Algeria, including the works of Baba-Aissa (1999) and those of Beloued (2009). According to these authors, other studies should be undertaken in the future, especially the phytochemical and pharmacological aspects of these new species.

Thus, in this context we propose through this work for the investigation of new natural sources with potential therapeutic effects from a species belonging to the group of lichens which is *Evernia prunastri* (L.) Ach. To the best of our knowledge, no study has already considered the study of this Algerian species, especially the one collected from the three cited regions namely Jijel (North-East Algeria), Setif and M'Sila (Steppe of Algeria). The aim of this study is to investigate the qualitative and quantitative phytochemistry and to determine the antibacterial and antioxidant activities of these three samples of *Evernia prunastri*.

2. Materials and methods

2.1. Lichen collection and preparation

The thallus of three samples of the lichen JS, SS and MS were collected respectively in the regions of Texenna (Jijel, North-East Algeria), Aïn Lahdjar (Setif, Algerian Steppe) and BouSaada (M'Sila, Algerian Steppe).

The lichen material was identified by Dr. Samira Salem (Laboratory of biotechnology, environment and health, Jijel university, Algeria). The intact thalli of *Evernia prunastri* (Figure 1) were dried at 40 °C and ground in an electric grinder into a fine powder and passed through very fine porosity sieves (250 µm). It was then put into boxes and stored for later use.

Figure 1. *Evernia prunastri* (L.) Ach. sample

2.2. Phytochemical analysis

The lichen powder was tested for the presence of saponins, alkaloids, tannins, flavonoids and terpenoids. Qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

2.2.1. Phytochemical screening

Saponins test (Frothing test)

Saponins were identified according to the method described by Banso and Adeyemo (2006). For this purpose, an aliquot of 0.5 g of different lichen powders was mixed in a test tube containing 3 mL of hot distilled water (100°C), then the whole was shaken vigorously and continuously (1 min) to observe the persistence of a foam.

Flavonoids test (Cyanidine test)

This test was performed according to the method of Tiencheu *et al.* (2021). An aliquot of 0.5 g of each lichen powder was mixed with 2 mL of methanol, and then 1mL of concentrated sulfuric acid was added. Finally, magnesium chloride powder (MgCl2) was added to this mixture. The observation is done after 1 minute and a positive result is shown by an effervescence and a brick-red coloration.

Tannins test (Ferric chloride test)

This test was performed according to the method of Banso and Adeyemo (2006). An aliquot of 0.5 g of each lichen powder was mixed with 10 mL of distilled water and filtered. To the filtrate, two drops of 5% iron (III) chloride reagent (FeCl3) were added. The blue-black or blue-green coloration or the formation of a precipitate indicates the presence of tannins.

Alkaloids test (Wagner's test)

This test was performed according to the method of Tiencheu *et al.* (2021). An aliquot of 0.5 g of lichen powders was mixed with 5mL of 1% HCl. After incubation in a water bath for 5 minutes, the mixture was filtered. Two grams (2 g) of potassium iodide and 1.27 g of iodine were dissolved in 5 mL of distilled water and the solution was diluted to 100 mL with distilled water. Two drops of this iodine solution were added to the filtrate; a brown colored precipitate indicates the presence of alkaloids.

Terpenoids test (Salkowski test)

The method described by Ayoola *et al.* (2008) was used to search for terpenoids. To 0.5 g of each lichen grind, 2 mL of chloroform was added. Three milliliter of concentrated sulfuric acid was carefully added to form a layer. A reddish-brown coloration of the interface indicates the presence of terpenoids.

2.3. Lichen extraction

Crude extracts of lichens were prepared as described by Stojanović *et al.* (2010). Approximately 60 g of each lichen powder was subjected to maceration extraction in 600 mL of methanol (80%) for 48 h at room temperature and then the extracts were filtered and concentrated through a rotary evaporator at 40 °C. The obtained dry extracts were stored at 4 °C until use.

2.3.1. Phenolic compounds content

Total phenolic compounds were determined by the Folin-Ciocalteu method (Turkmen *et al.*, 2006). The obtained results were determined from the regression equation of the gallic acid calibration curve (ranging from 0.005 to 0.05 mg/mL) prepared previously and expressed as mg gallic acid equivalents (GAE) per hundred grams of the lichen powder. In this method, 1 mL of methanolic extract of lichens diluted 10-75 times with methanol was mixed with 1 mL of 3-fold diluted Folin-Ciocalteu reagent. Two milliliters of a 35% sodium carbonate solution

were added to the mixture, shaken thoroughly and diluted to 6 mL by adding 2 mL of distilled water. The mixture was allowed to stand for 30 minutes and the formed blue color was measured at 700 nm using a spectrophotometer (Analytik Jena, Specord 50 plus).

2.3.2. Flavonoid content

The flavonoid content of the extracts was determined by spectrophotometry (Djeridane *et al.*, 2006), using the method based on the formation of a flavonoid-aluminum complex, having the maximum absorbance at 430 nm. An aliquot of 1.5 mL of methanolic extract of lichens was mixed with 1.5 mL of a 2% methanolic solution of aluminum chloride. After incubation at room temperature for 15 minutes, the absorbance of the reaction mixture was measured and the flavonoid content is expressed as mg quercetin equivalent (QE) per 100 grams of the lichen powder.

2.3.3. Antioxidant activity

Evaluation of total antioxidant capacity (TAC)

The total antioxidant capacity of the methanolic extracts was assessed by the phospho–molybdate method (Prieto *et al.*, 1999). A 0.5 mL aliquot of diluted lichen methanolic extract was placed in a tube with 5 mL of molybdate reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 minutes. After cooling to room temperature, absorbance was measured at 695 nm against a blank. Antioxidant capacities were expressed as mg ascorbic acid equivalent (AAE) per 100 grams of the lichen powder.

Ferric reducing antioxidant power (FRAP) assay

The reducing power of the methanolic extract was determined according to the method of Gülçin *et al.* (2002). One milliter of diluted lichen methanolic extract was mixed with phosphate buffer (2.5 mL; 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL a 1%). The mixture was incubated at 50°C for 20 min. A 2.5 mL aliquot of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The top layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. The reducing power is expressed as mg ascorbic acid equivalent (AAE) per 100 grams of the lichen powder.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Antioxidant activity was measured as free radical scavenging potential in a methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Tepe *et al.* (2005). Fifty microliters of lichen methanolic extract was added to 5 mL of 0.004% DPPH methanolic solution. After an incubation period of 30 minutes at room temperature, the absorbance was read against a blank at 517 nm. The free radical inhibition by DPPH in percent (I %) was calculated as follows:

$$\mathbf{I\ \% = [(A\ blank - A\ sample) / A\ blank] \times 100}$$

A blank: is the absorbance of the control reaction (containing all reagents except the test compound),

A sample: is the absorbance of the test compound.

The results were determined from the regression equation of the calibration curve and are expressed as mg ascorbic acid equivalent (AAE) per 100 grams of the lichen powder.

2.3.4. Antibacterial Activity of methanolic extracts in solid medium

The antibacterial activities of the methanolic extracts were tested against three bacterial strains (methicillin resistant *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) by the solid media diffusion method. After swabbing the bacterial strain (load of 10^8 colony forming units (CFU)/mL) onto Mueller-Hinton agar, Whatman No. 1 paper discs (6 mm diameter) were placed on the surface. Then, 10 µL of each methanolic extract and penicillin as a control (30 mg/mL) were placed on each disk. After incubation at 37 °C for 24 h, the diameters of the inhibition zones around the

discs were measured. The tests were performed in duplicate and the result is expressed as the mean of the standard deviation of the results of both tests (Bekka-Hadji *et al.,* 2022).

2.3.5. Antibacterial Activity of methanolic extracts in a liquid medium

The minimum inhibitory concentrations (MIC) of the methanolic extracts were determined by the microdilution method (Bekka-Hadji *et al.,* 2022). The different concentrations of methanolic extracts and penicillin (0.015 - 15 mg/mL) were carried out in a 96-well round-bottomed microplate. All the tests were carried out with Mueller-Hinton broth at the rate of 100 µL per well. Finally, each well was inoculated with 100 µL of a bacterial suspension (the final load in each well is 10^7 CFU/mL). After 24 h of incubation at 37°C, the MIC corresponded to the minimum concentration showing no bacterial growth.

2.4. Statistical analysis

All data were performed using ORIGIN PRO version 93 E software for Windows. All experiments were performed in duplicate and data obtained from the analysis are expressed as mean ± standard (SD). Statistical differences were analyzed by one-way analysis of variance (ANOVA) at $p < 0.05$ and Fisher LSD test.

3. Results and discussions

3.1. Phytochemical screening

The phytochemical screening, allows to highlight the presence of secondary metabolites at the level of the studied lichen species. The detection of these chemicalcompounds is mainly based on precipitation reactions and specific color change.

The phytochemical evaluation of the different grinds of *Evernia prunastri* species revealed the presence of some chemical constituents such as: alkaloids, flavonoids, saponins, terpenoids, but we note also the absence of tannins (Table 1).

Results reported by Rashmi and Rajkumar (2014), on several lichen species, confirm the presence of at least one known constituent (saponins, alkaloids, terpenoids, flavonoids, etc.) in methanolic extracts. According to these authors, phytochemical analysis performed on lichen extracts revealed the presence of constituents known for their medicinal and physiological activities. The usefulness of lichens is due to the range of secondary compounds they produce.

Table 1. Phytochemical screening of the lichen grind of *Evernia prunastri* samples

	Interferences		
	JS	**SS**	**MS**
Test for saponins (Frothing test)	+	+	+
Test for flavonoids (Cyanidine test)	+	+	+
Test for tannins (Ferric chloride test)	-	-	-
Test for alkaloids (Wagner's test)	+	+	+
Test for terpenoids (Salkowski test)	+	+	+

+ : presence, - : absence, JS: Jijel sample, SS:Setif sample, MS: M'Sila sample.

3.2. Phenolic and flavonoid contents

The contents of phenolic compounds and flavonoids in the methanolic extracts of lichens were determined. The obtained results are presented in Figure 2 (a, b).

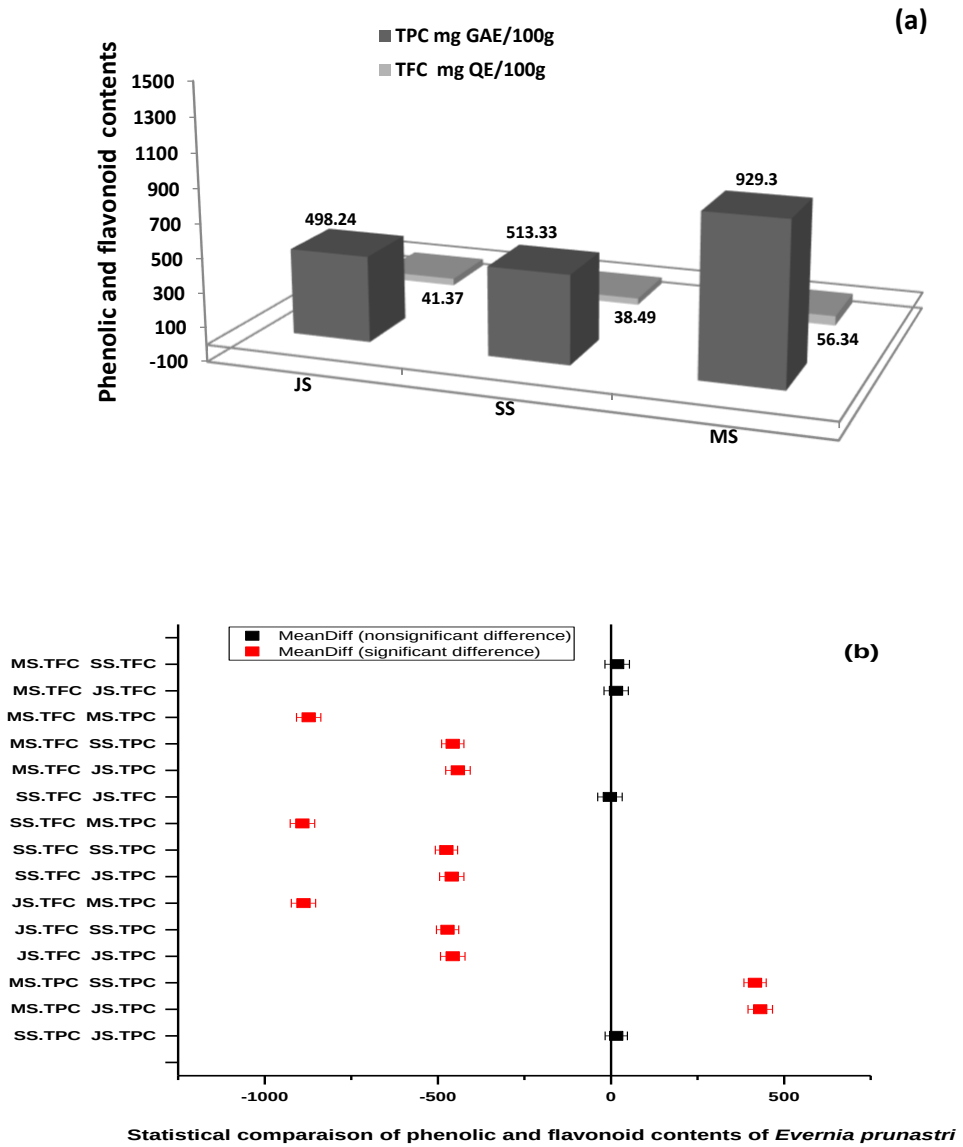

Figure 2. Phenolic and flavonoid contents of methanolic extracts of *Evernia prunastri*s amples **(a)**. Statistical comparison of phenolic and flavonoid contents of *Evernia prunastri* **(b)**. JS: Jijel sample, SS: Setif sample; MS: M'Sila sample; TPC: Total phenolic content; TFC: Total flavonoid content; GAE: Gallic acid equivalents; QE: Quercetin acid equivalents. Results are expressed as mean ± SD of two experiments for TPC and TFC tests.

The content of phenolic compounds is significantly higher for the lichen from M'Sila (929.3 mg GAE/100g) in comparison with those from Jijel and Setif (498.24 and 513.33 mg GAE/100g, respectively). Mitrović *et al.* (2011), found a phenolic compound content of 80.73 mg/g of the extract of *Evernia prunastri* species from Serbia. This is higher than our phenolic compound result. Similarly, Shcherbakova *et al.* (2021) obtained the highest content (73 mg gallic acid/g extract) for hexane extract of *Evernia prunastri* species collected in Mari El Republic of the Russian Federation. However, Stojanović *et al.* (2010) found a

content of 18.24 µg/g extract for *Evernia prunastri* species from Bojaninevode (Serbia), which is less significant than our result. In comparison with another species of lichen *Xanthoria parietina* collected in Algeria (Boumerdes region), the content of phenolic compounds was found to be 13.9 mg/100g (Bouchenak *et al.*, 2020), which is also lower than our obtained phenolic compound result.

The results of flavonoid contents are presented in Figure 2 (a), the lichen extract from M'Sila region exhibited also the highest content (56.34 mg QE/100g) compared to the other two studied lichen extracts. The flavonoid contents of our extracts are lower than that of lichen *Evernia prunastri* from Serbia (27.46 mg/g) (Mitrović *et al.*, 2011).

3.3. Antioxidant activities of *Evernia prunastri* methanol extracts

The antioxidant capacity (TAC), reducing power (FRAP) and free radical scavenging activity (DPPH) of the lichen methanolic extracts from Jijel, Setif and M'Sila samples were determined. The results obtained are presented in Figures 3, 4 and 5.

3.3.1. Total antioxidant capacity

From the results presented in Figure 3 (a, b), we note that the antioxidant capacity of the methanolic lichen extract of M'Sila (1063.35 mg AAE/100g) is significantly higher compared to those of Jijel and Setif.

The total antioxidant capacities of our extracts are higher than that found by Stojanović *et al.* (2010) for *Evernia prunastri* species (Bojanine vode, Serbia) and which is in the order of 0.60 µg/g. This antioxidant power observed in the extracts may be mainly due to the richness of the extracts in phenolic compounds and it also depends on the chemical nature of the bioactive molecules.

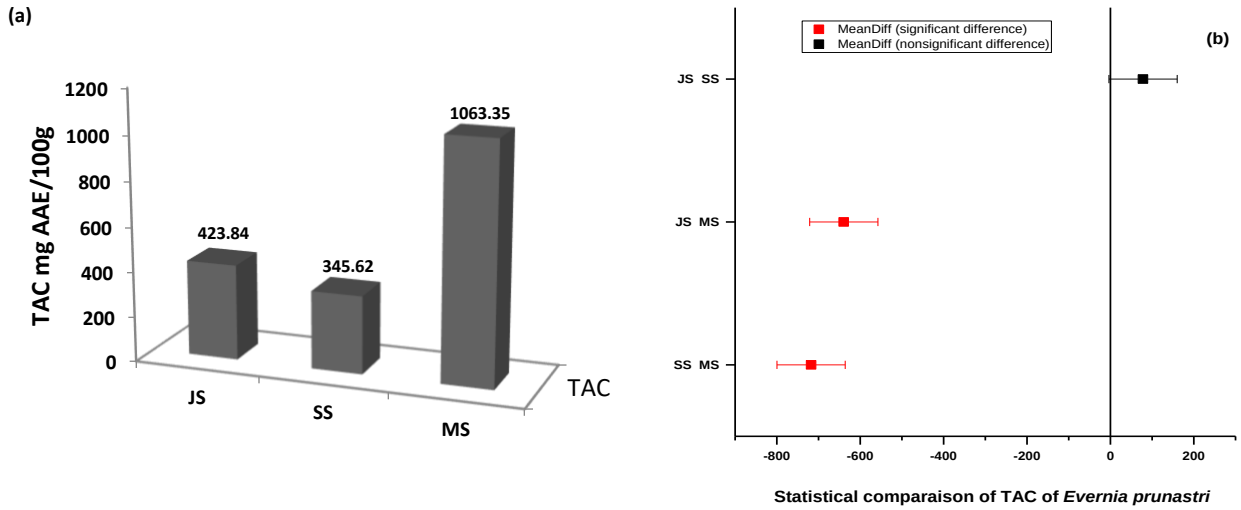

Figure 3. Total Antioxidant Capacity of methanolic extracts of *Evernia prunastri* samples **(a)**. Statistical comparison of TAC contents of *Evernia prunastri* **(b)**. JS: Jijel sample; SS: Setif sample; MS: M'Sila sample; TAC: Total antioxidant capacity; AAE: Ascorbicacid equivalents. Results are expressed as mean ± SD of two experiments for TAC and tests.

3.3.2. Reducing power

The results illustrated in Figure 4 (a, b), revealed that the reducing power of M'Sila lichen sample (277.66 mg AAE/100g powder) is the most important one.

The reducing power of Iron from our extracts is higher than that found by Stojanović *et al.* (2010) for *Evernia prunastri* species from Serbia (35.5 µg/g).

The reducing power of the methanolic

extract of M'Sila lichen sample is slightly higher than that found by Bouchenak *et al.* (2020) who worked on the methanolic extract of *Xanthoria parietina* species (206 mg AAE/100g) from Boumerdes region (Algeria).

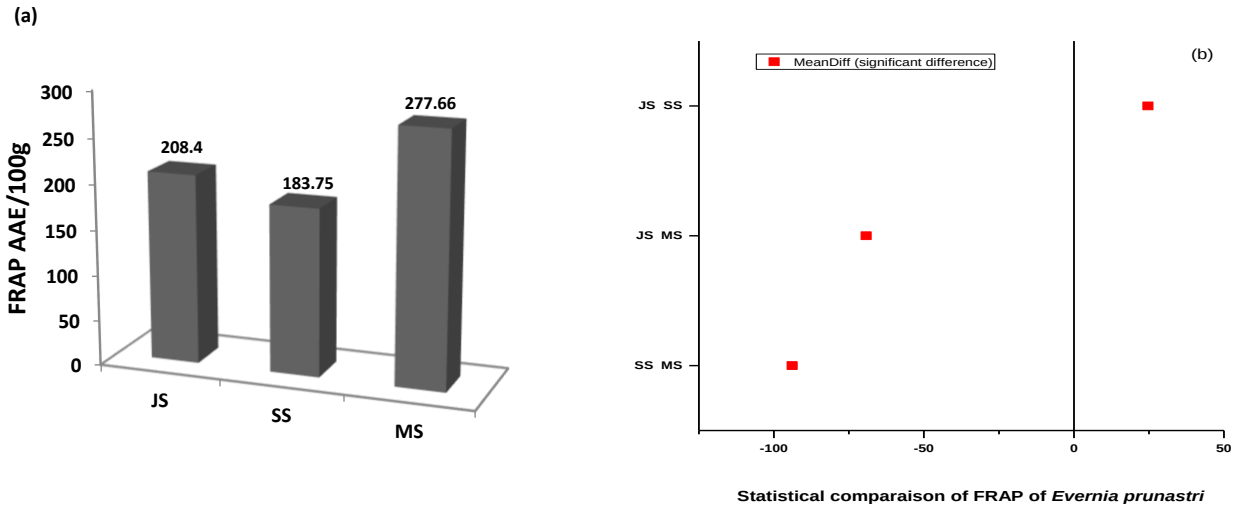

Figure 4. Reducing power of the methanolic extracts of *Evernia prunastri* samples **(a)**. Statistical comparison of FRAP of *Evernia prunastri* **(b)**. JS: Jijel sample; SS: Setif sample; MS: M'Sila sample; FRAP: Ferric reducing antioxidant power; AAE: Ascorbic acid equivalents.Results are expressed as mean ± SD of two experiments for FRAP test.

3.3.3. DPPH scavenging activity

According to our results (Figure 5), the DPPH inhibition rates recorded in the presence of the various methanolic lichen extracts are very weak compared to the antioxidant capacity and the reducing power.

The methanolic extract of the *Evernia prunastri* species growing in the M'Sila region yielded the highest level (7.67 mg AAE/100 g powder). This extract has a high concentration of total phenolic and flavonoids compounds, which correlates with antioxidant activity.

According to Kosanic *et al.* (2013), the high antioxidant activity of the tested lichen extracts correlated with a high content of total phenols, suggesting that phenols are the main agents of their antioxidant activity.

Phenols are very important constituents because of their scavenging capacity due to their hydroxyl groups. These compounds may contribute directly to the antioxidant action. It is suggested that phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Gülçin *et al.,* 2002). Similarly, flavonoids are a class of secondary metabolites possessing important antioxidant and chelating properties. The antioxidant activity of flavonoids depends on the structure and substitution of hydroxyl groups (Stanković, 2011).

The region of M'Sila constitutes the capital of the Hodna region. The territory of Hodna covers an area of 6951 Km^2, in the heart of one of the largest sets of semi-arid and steppe areas that exist in North Africa. This region is set back from the southern shores of the Mediterranean between the Tellian Atlas in the north and the Saharan Atlas in the south (Mili *et al.,* 2019). This leads us to explain that the production of bioactive substances endowed with antioxidant activity have tendency to be synthesized in conditions of drought and intense light so that the organism can protect itself against adverse conditions.

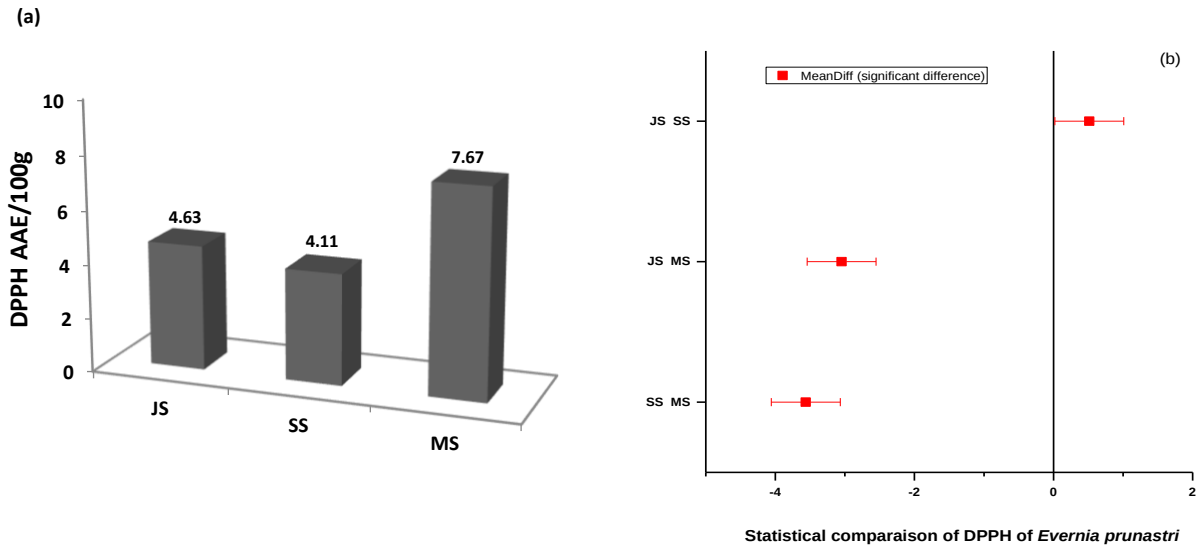

Figure 5. DPPH scavenging activity of the methanol extracts of *Evernia prunastri* samples **(a)**. Statistical comparison of DPPH of *Evernia prunastri* **(b)**. JS: Jijel sample; SS: Setif sample; MS: M'Sila sample; DPPH: 2,2-diphenyl-1-picrylhydrazyl; AAE: Ascorbic acid equivalents. Results are expressed as mean ± SD of two experiments for DPPH test.

3.4. Antibacterial Activities

The antibacterial activities of methanolic extracts of *Evernia prunastri* against three bacteria (methicillin resistant *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) were determined. The obtained results were expressed by the diameters of the inhibition zones (IZDs) and the minimum inhibitory concentrations (MICs). The results are presented in Table 2.

The results show that the methanolic extracts of lichen have a very high antibacterial activity against one of the tested bacterial strain (methicillin resistant *Staphylococcus aureus).* The diameters of the obtained zones of inhibition and the minimum inhibitory concentrations values range from 6 to 35.33 mm and from 0.058 to 15 mg/mL, respectively.

3.4.1. Antibacterial activity of methanolic extracts in solid medium

According to the obtained results, the best activity was obtained for the methicillin-resistant *S. aureus* (MRSA) strain with a diameter of 35.33 mm for the methanolic extract of Jijel lichen. Penicillin used as a control gave a diameter of 35 mm (Table 2, Figure 6).

These results are similar to those of Kiran *et al.* (2013) who found that acetone and chloroform extracts obtained from different samples of the lichen *Evernia prunastri* species from Turkey show antibacterial activity against several strains (*Staphylococcus*, *Enterococcus* and *Bacillus*) with zones of inhibition ranging from 5 to 21 mm for chloroform extracts and from 6 to 20 mm for acetone extracts. For *Staphylococcus* strains, the reported inhibition zones varied from 9 to 20 mm. Likewise, Brakni and Ali Ahmed (2018) investigated the sensitivity of bacteria to different extracts of lichen (*Evernia prunastri* (L.) Ach., *Ramalina fastigiata* (Pers.) Ach. and *Cladonia rangiformis* Hoffm.) collected in the region of Seraïdi (North-East of Algeria). The obtained results showed that the *Staphylococcus aureus* strain was very sensitive to all extracts and more precisely to the methanolic extract of *Evernia prunastri* (an inhibition zone of 43 mm). *Escherichia coli* was sensitive to the chloroformic and acetonic extracts of *E. prunastri* and *R. fastigiata* and to the methanolic and aqueous extracts of *C.*

rangiformis. *Klebsiella pneumonia* was insensitive to all the extracts of lichen, *Proteus mirabilis* and *Pseudomonas aeruginosa* are slightly sensitive to some extracts of *R. fastigiata.*

Table 2. IZDs (mm) and MICs (mg/mL) of the methanolic extracts of *Evernia prunastri* samples and Penicillin control against bacterial strains.

IZDs and MICs of samples lichens and control		**Methicillin resistant *S. aureus*ATCC43300**	***E. coli* ATCC25922**	***P. aeruginosa* ATCC27853**
JS	I Z D MIC	35.33 ± 1.15 0.058	7 ± 0 7.5	8.5 ± 0.7 7.5
SS	I Z D MIC	27.33 ± 1.15 0.12	NZ 7.5	7 ± 0 7.5
MS	I Z D MIC	23.67 ± 1.53 0.12	8 ± 0 7.5	7 ± 0 7.5
P (control)	I Z D MIC	35 ± 1 1.88	20.50 ± 0.35 0.47	NZ >15

JS: Jijel sample; SS: Setif sample; MS: M'Sila sample; P: penicillin; IZD: Inhibition zone diameter (mm) including disk diameter of 6 mm and values are given as mean ± standard deviation; NZ: No inhibition zone; MIC: minimal inhibitory concentration in mg/mL.

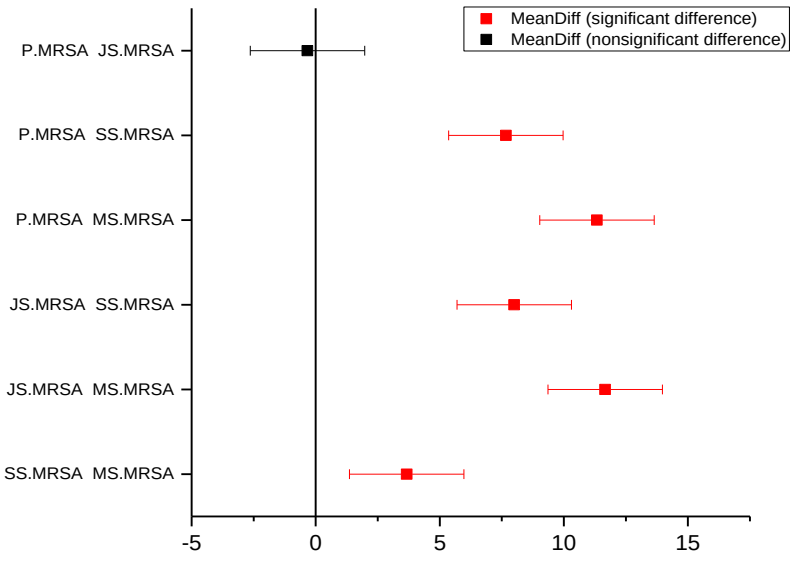

Figure 6. Statistical comparaison of IZDs of the methanolic extracts of *Evernia prunastri* samples and penicillin control against MRSA strain. MRSA: Methicillin resistant *S. aureus* ATCC 43300

3.4.2. Antibacterial activity of methanolic extracts in liquid medium

The MICs of methanolic extracts and penicillin are those obtained in microplate wells before the first growth, i.e. when no growth is visible to the naked eye.

The lowest minimum inhibitory concentrations are those obtained with the methanolic extract of Jijel against *S. aureus* which is of 0.058 mg/mL. However, the highest

MIC was obtained against *E. coli* and *P. aeruginosa* species which is of 7.5 mg/mL and this was observed for all the lichen extracts. In comparison with penicillin, the lowest MIC is retained for *E. coli* with a value of 0.47 mg/mL (Table 2).

Kosanic *et al.* (2013) found that acetone-based extracts of *E. prunastri* from Serbia inhibited all tested microorganisms, but at somewhat higher concentrations. The obtained MICs for the extract of this lichen vary from 6.25 to 25 mg/mL, while for the isolated compound (usnic acid) from this lichen extract, the MICs vary from 0.25 to 1 mg/mL.

The obtained MIC for the *S. aureus* strain is 12.5 mg/mL, while usnic acid gives an MIC of 0.5 mg/mL. This shows that our results are more interesting because the MIC of our extracts is reduced by almost 2 times compared to that of usnic acid. In another study done by Mitrović *et al.* (2011) on methanolic extracts of *E. prunastri* from Serbia, the authors obtained MICs ranging from 0.0391 to 10 mg/mL against all tested strains. The highest MICs (2.5 to 10 mg/mL) were observed for Gram-negative bacteria and the lowest (0.0391 to 0.156 mg/mL) for Gram-positive bacteria. For *S. aureus* strains, the MIC obtained was 0.156 mg/mL. which is close to our MIC result value.

The interesting effect of the tested extracts is probably due to their richness in usnic acid and other active compounds.

The secondary metabolites of lichens are of biological interest for humans as pharmaceuticals. The biosynthesis and the pathways involved in their regulation have been reviewed by a number of authors and many studies have focused on their biological role as protectors of thalli against various stresses and enabling them to withstand unfavorable environmental conditions for their growth (Aoussar *et al.,* 2020).

Shcherbakova *et al.* (2021) showed that the dichloromethane and hexane extracts were both active against *S. aureus* (MICs of 4 and 21 μg/mL, respectively) but they were less active against Gram-negative bacteria and yeasts. The acetone extract showed activity against both *S. aureus* (MIC of 14 μg/mL) and *C. albicans* (MIC of 38 μg/mL).

Several studies have reported the antimicrobial activity of usnic acid, a constituent isolated from lichens, against Gram-positive bacteria including vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA). In addition, usnic acid is reported to be more effective than penicillin ointments in the treatment of external wounds and burns. Similarly, synergistic action was observed in combination with gentamicin, while antagonism was observed with levofloxacin. The combination with erythromycin showed an indifferent effect (Behera *et al.,* 2005; Araújo *et al.,* 2015).

4. Conclusion

In conclusion, we can say that the tested lichen extracts presented potent antioxidant activities and antibacterial activities especially against *S. aureus*. The substances contained in the extracts included alkaloids, saponins, flavonoids, etc.

This is the first study reporting the *in vitro* biological activities of species of *E. prunastri* collected in three different regions of Algeria. The obtained results of the present study indicate that methanolic extracts obtained from these species are potential sources of natural antibacterial and antioxidant biomolecules. This could be important for their use as antibacterial agents against *Staphylcoccus* infections or as food preservatives against oxidation reactions.

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Acknowledgements : We would like to express our thanks to the Department of Applied Microbiology and Food Science, Faculty of Natural and Life Sciences, University of Jijel (Algeria) and to the Algerian Ministry of Higher Education and Scientific Research.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

QUANTITATIVE DETERMINATION OF PORK MEAT RESIDUE IN CATTLE MEAT MIXTURES USING DROPLET DIGITAL PCR

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https://doi.org/10.34302/crpjfst/2023.15.4.9

Article history:
Received: 6 April 2023
Accepted: 6 December 2023

Keywords:
Species identification;
Droplet digital PCR;
Authentication;
Quantification;
Pork meat.

ABSTRACT

Meat adulteration is an important economic and social problem worldwide. Therefore, the accurate identification and quantification methods for species substitutions in meat products are needed. In this study, a precise quantitative method was performed to identify the minimum content of pork (*Sus scrofa*) meat in the mixture of pork: cattle (*Sus scrofa :Bos taurus*) using the droplet digital Polymerase Chain Reaction (ddPCR) method. Experiments were conducted by using a series of dilutions for heat-treated and raw meat mixtures. The detection limits in DNA fragments of this study were 0.65 copies/µL for heat-treated and 0.1 copies/µL for raw meat samples. Based on these results, the sensitivity explaining minimum pork meat in meat mixtures was 0.1% for heat-treated and 0.001% for raw samples. The results showed that the ddPCR method is effective for identifying and quantifying pork meat in meat products and has potential to be applied for other meat species also.

1. Introduction

Determining the origin and contents of meat and meat products is an important issue worldwide because of authenticity problems. The most frequent meat adulteration is the use of different animal origins as a meat source. The use of different meat origins without declaring them on the label causes some religious and health problems and creates unfair competition. Consequently, species identification is gradually gaining importance (Hossain et al. 2019).

The need for safe and rapid methods for determining animal species in foods is becoming crucial. The methods used for meat species' identification are generally protein and DNA-based analyses. The most frequent protein-based methods used for species identification in meats products are electrophoretic, immunochemical and chromatographic (Alikord et al. 2018). However, these methods have some disadvantages because of the denaturation of proteins during the heating process (Fajardo et al. 2010). The DNA-based methods are more advantageous from this point because the DNA present in animal tissues could resist to some physical factors such as heating process. DNA molecules are more resistant to the heat process in comparison to proteins and large numbers of DNA cells led to increase the efficiency of DNA based methods (Alikord et al. 2018). Moreover, the amount of mitochondrial DNA (mtDNA) changes variably according to the tissue type used for a species' identification application. Therefore, the use of nuclear DNA is reported as more effective than mtDNA for reliable

quantification results (Floren et al. 2015). Spectroscopic techniques are used also for the determination of authentication in meat products being rapid and non-destructive methods (Sajali et al. 2021). However, they have some disadvantages of showing lack of specificity for identification of resembling meat species and heat-treated materials due to the denaturation of proteins (Fajardo et al. 2010).

DNA-based polymerase chain reaction (PCR) technique is a rapid, sensitive, highly specific, and cheaper alternative method used to identify animal origins even in processed foods (Amaral et al. 2015; Soares et al. 2013). However, quantitative data about the target DNA is another important point for evaluating the adulteration. Improvements in PCR techniques have created the real-time PCR (qPCR) which can detect quantitatively the presence of DNA (Sajali et al. 2021). The real-time PCR technique allows monitoring and measuring the amplified products along each amplification cycle. The presence of PCR products is identified with fluorescent molecules during the real-time process (López-Andreo et al. 2005). For this purpose, two different fluorescent chemistries have been used, namely universal fluorescent dyes, such as SYBR green, and sequence-specific DNA probes like the hydrolysis TaqMan dual labelled probes. Recently, the real-time PCR method was used successfully for the species identification of some different meat species (Kang et al. 2021; Köppel et al. 2020; Li et al. 2021; Liu et al. 2021; Ren et al. 2017).

Droplet digital PCR (ddPCR) has emerged in recent years as an advanced technique that enables detecting and quantifying DNA at trace levels. The principle of this method is to count the number of identifications in a sample by using excessive dilutions of nucleic acids and Poisson statistics into multiple individual PCR amplification cells. In every cell, individual PCR assays are performed, and positive PCR amplifications are monitored with fluorescent target-specific probes. It is reported that the ddPCR method is more sensitive and precise than other quantitative real-time PCR methods (Basanisi et al. 2020; Cao et al. 2020; Dimond et al. 2022). ddPCR permits quantifying the absolute number of nucleic acids in several thousand of individual compartments by portioning and without the use of standard curves. In addition, ddPCR permits the possibility of determining and quantifying very few nucleic acid contents in a sample (Hudecova 2015). The ddPCR has been used in some studies for the development of methods for determining meat authenticity. Ren et al. (2017) compared the two methods (real-time PCR and ddPCR) for determining meat adulteration and they reported that the ddPCR method is more accurate and easier compared to real-time PCR. In their study, they detected 1% (w/w) sheep and 0.1% chicken meat in meat mixtures. Wang et al. (2018) developed a ddPCR method for determining goat meat and sheep meat derivatives in commercial meat products. Floren et al. (2015) reported a limit of quantification (LOQ) of 0.01% and a limit of detection (LOD) of 0.001% for beef, horse meat and pork in processed meat products. Cai et al. (2017) used a duplex ddPCR method to detect and quantify beef and pork materials in meat products. They reported LOD/LOQ of 0.1 ng/μL for beef and 0.5 ng/μL for pork. Noh et al. (2019) applied ddPCR for determining Alaska pollock (*Gadus chalcogrammus*) content in seafood products.

The real-time PCR technique has some limitations related to the accurate measurements. The impurities and contaminant levels in DNA solution can affect the amplification efficiency and the copy numbers based on Ct values from real-time PCR (Hudecova 2015). Digital droplet PCR (ddPCR) exhibits an effective single molecule counting principle to detect very small amounts of genetic material, thus providing precise and sensitive determinations that can eliminate the effect of contaminants without the use of standard curves. Besides, the reports on the application of ddPCR to the quantification of meat species in food is still limited. Hence, the objective of this study was to determine the minimum quantity of pork meat in heat-treated and raw pork:cattle meat mixtures of different concentrations using

ddPCR as an alternative new method for quantitative identification of meat species.

2. Materials and methods

2.1. Materials

Fresh lean cattle (*Bos taurus*) meat was provided by a local butcher (Yamaner, Bolu, Turkey) and pork (*Sus scrofa*) meat was provided by a local market (İstanbul, Turkey). They were immediately stored at -18°C in 1 kg plastic containers until use. All chemicals and reagents used were analytical grades.

2.2. Sample preparation

Frozen meats were thawed at 4°C and the fat and connective tissues removed. They were ground to 1-2 mm with a chopper. To avoid contaminations, the knives and the chopper were cleaned carefully and treated with DNA decontamination solution (20% bleach) after each grounding process between the meat species. Next, a series of pork:cattle binary meat mixtures were prepared at different ratios such as 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2.5%, 5% and 10% (Table 1). To provide the homogeneity, meat mixtures were homogenized separately in a laboratory blender (Waring Commercial 8010ES, USA) for 2-3 minutes. The knives and the container of blender were cleaned and treated with DNA decontamination solution (20% bleach) to remove residual DNA between samples. A total of 400 g of meat mixture was prepared for each group and divided into two equal parts. One part of the meat mixture was shaped into standard patties 4 cm in diameter and 0.5 cm in thickness for the cooking process. They were baked in an oven (Memmert UN-800) at 120°C (75°C internal temperature). The second part was used for the direct DNA isolation.

Table 1. Meat mixtures

No	% Cattle Meat	Cattle Meat (g)	Pork Meat (%)	Pork Meat (g)
1	0	0	100	200
2	90	180	10	20
3	95	190	5	10
4	97.5	195	2.5	5
5	99	198	1	2
6	99.5	199	0.5	1
7	99.9	199.8	0.1	0.2
8	99.99	199.98	0.01	0.02
9	99.999	199.998	0.001	0.002
10	99.9999	199.9998	0.0001	0.0002
11	99.99999	199.99998	0.00001	0.00002
12	100	200	0	0

2.3. DNA extraction

Before the DNA isolation, a 25 mg of meat sample from each meat mixture was homogenized with 75 µL PBS (Phosphate Buffered Saline) (pH: 7.3) in a 1.5 mL microcentrifuge tube. After the homogenization, the DNA isolation was performed according to the manufacturer's instructions (Qiagen DNeasy Blood & Tissue Kit, USA). The amount and quality of DNA were determined by measurement of absorbance at 260 nm and 280 nm using a nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000). The ratio of absorbance of the A260/280 values which are between 1.7 and 2.0 was selected. The DNA samples of each group were diluted to 25 ng/µL in the elution Buffer AE (Qiagen DNeasy Blood & Tissue Kit, USA) and then were tested directly.

2.4. Primers and probes

Primers and probes were retrieved from Floren et al. (2015) (Table 2) to target the

chromosomal coagulation factor II (*F2)* gene to amplify fragments of 96 bp for and 97 bp for cattle and pork, respectively. All primers and probes were synthesized by Metabion GmbH (Germany). The hydrolysis probes were labeled with Carboxyfluorescein (FAM) as the indicator for pork and labeled with Hexachlore-6-carboxyfluorescein (HEX) for cattle. For verifying the specificity of the primers, BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) was used.

Table 2. Primers and probes sequencing used for ddPCR

Primer name[a,b]	Sequence (5´-3´)	Chromosome	Position Genomic[c]
Sus_F2_For	5'-CTGCCAGCGGGCTGGGAATA -3'	2	SSC2:17167390-17167410
Sus_F2_Rev	5'-GGAGTTGACTCTGGAATAAGAAAT TG -3'	2	SSC2:17167460-17167486
Sus_F2_FAM	5'-FAM-CGCCCCCGCCCCCAGGGTCT - BHQ1-3'	2	SSC2:17167438-17167457
Bov_F2_For	5'-CCTGTCTGCTGAGACGCCG-3'	15	BTA15:76998246-76998265
Bov_F2_Rev	5'- GTGGTAGAGTTGATTCTG GAATAGAAAGCAT -3'	15	BTA15:76998310-76998341
Bov_F2_HEX	5'-HEX -CCCCGCCACCCGCAGTGTCT-BHQ1-3'	15	BTA15:76998274-76998293

[a]F2: coagulation factor II (F2) gene
[b] Sus: pork; Bov: cattle
[c] Bovine genome: Btau_4.6.1; Porcine genome: Sscrofa_10.2

2.5. Droplet digital PCR and analysis

Following the DNA extraction and concentration adjustment for the PCR assay, the digest step, droplet generation, PCR step, and droplet reading were performed, respectively. Samples of meat mixtures were prepared in a manner so that the DNA concentrations of each sample were 25 ng/µL. Firstly, 0.5 µL *Bam*HI (New England Biolabs GmbH, Germany) containing 0.055 µL BSA (Bovine Serum Albumin) (Thermo Scientific, 23209, USA) in 11 µL 1x ddPCR supermix (#1863024, BioRad Laboratories, USA) was added to the 1 µL template DNA and incubated at 37°C for 1 hour. After the digestion, 0.9µM of each primer and 0.25µM of each hydrolysis probes were added. A total of 10 µL of the dUTPs enzyme solution was added and the final volume was completed with 22µL of nuclease and protease-free water (AppliChem GmbH, Germany).

A total of 20 µL from each mixture of 12 different groups and non-template control (NTC) samples were loaded using a multichannel pipette into an eight-channel single-use droplet generating generator cartridge (#1864007, BioRad Laboratories, USA). Droplets were generated from a droplet generator (QX200; BioRad Laboratories, USA). The droplets are aqueous substances surrounded by oil and contain a surfactant that prevents oil droplets from clustering together. After droplet generation, the droplets were transferred gently onto a 96-well plate (40 µL for each well) with a multichannel pipette and the PCR amplifications were performed using a thermal cycling protocol in a thermal cycler (C1000 Touch, BioRad, USA) according to the amplification protocol of Floren et al. (2015) stated in Table 3. After the PCR amplification, the plate containing the droplets was placed in the droplet reader (QX200, BioRad Laboratories, USA), which examined each droplet by counting positive reactions one-by-one using the two-color determination method (FAM and HEX) for determining the number of species at the lowest concentration in the

mixture. Positive droplets which comprised at least one replica of the target DNA molecule, represented an increased fluorescence compared to negative droplets. The measurement of the positive and negative droplets for each sample was performed using the Absolute Quantification (ABS) method Quanta Soft™ 1.7.4. software which adapts the results to the Poisson algorithm for defining the preliminary concentration of the target DNA molecules by copies/µL (Biorad 2018).

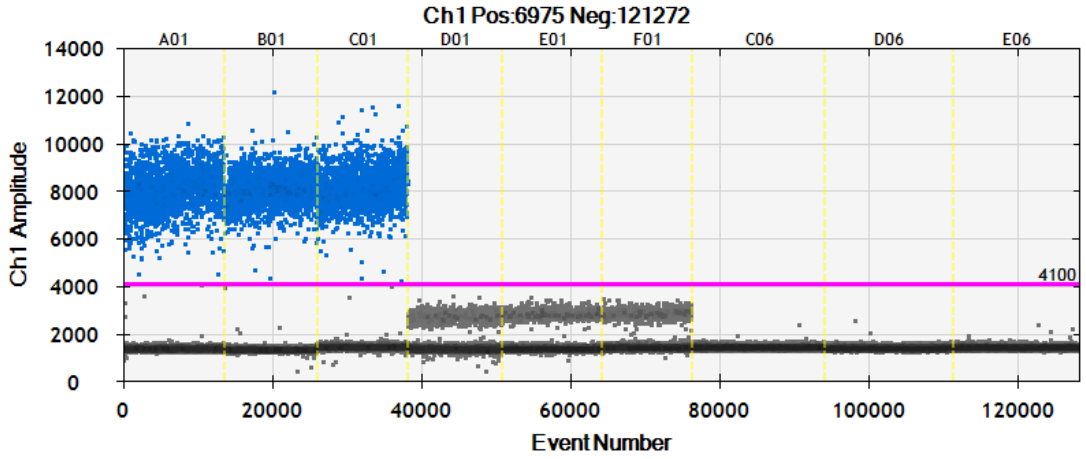
Table 3. Reaction conditions of ddPCR

Step	***F2***
Initial denaturation (°C/min)	95/10
Cycles	50
Denaturation (°C/s)	95/30
Primer annealing (°C/s)	55/10
Primer extension (°C/s)	68/20
Inactivation (°C/min)	98/10

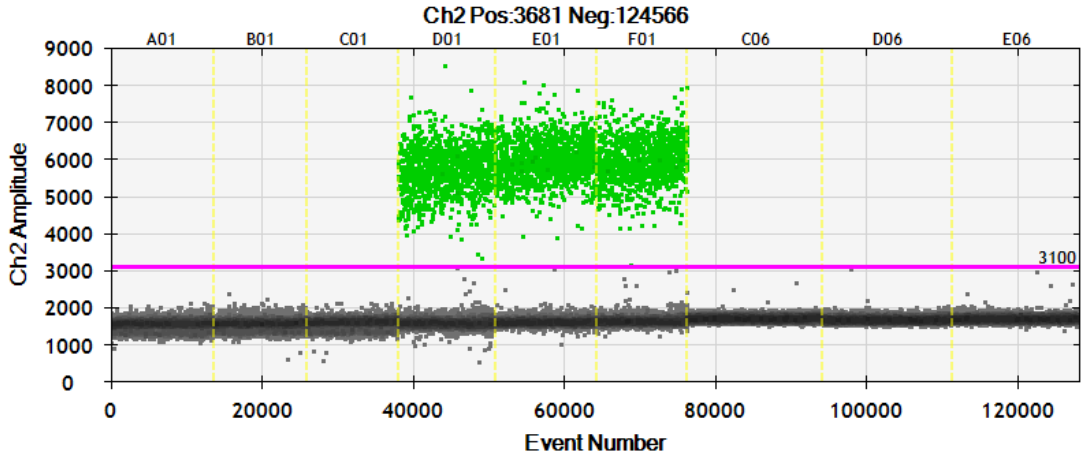
3. Results and discussions

3.1. The tests of specificity of primers and probes

The specificity assays for primers and probes have been performed by Floren et al. (2015) by using DNA from different horse, cattle, and pig breeds. In addition to this, we made some ddPCR analysis for investigating the probable cross-contaminations. As shown in Fig. 1, there is no cross-contamination between the amplitude of droplets (Lanes A01, B01, C01) showing the reaction signals of 100% pork DNA (FAM, blue-stained). In Fig. 1a, there is no cross-contamination also between the amplitude of droplets (Lanes D01, E01, F01) showing the reaction signals of 100% cattle DNA (HEX, green-stained). On the other hand, there were not any FAM or HEX fluorescence signals in (Lanes C06, D06, and E06) showing the reaction of signals in the NTC samples (Fig. 1b). The results showed that this analysis can be performed for reliable quantification of cattle and pork species in meat mixtures for sensitivity of primers and probes.



(a)



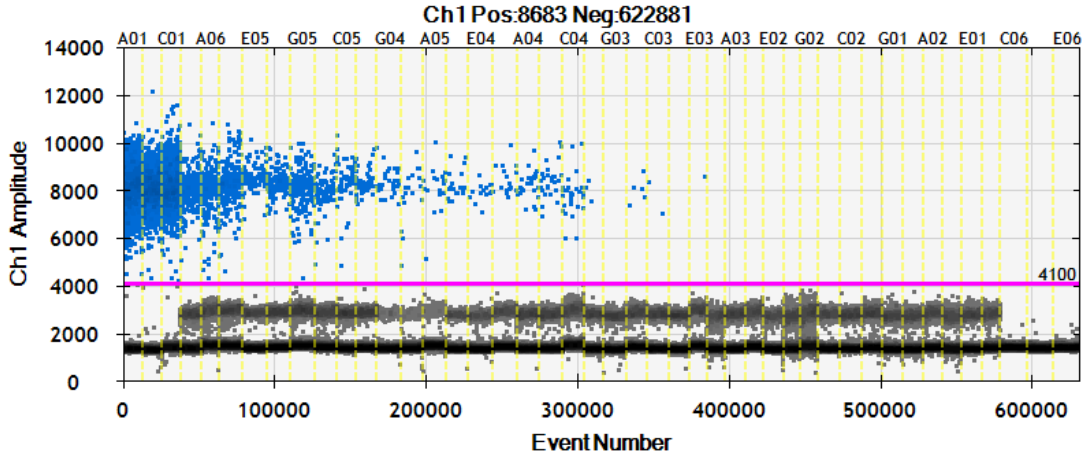
(b)

Figure 1. The specificity results of ddPCR assays. (a) The ddPCR assays for pork; and (b) The ddPCR assays for cattle. The horizontal axis represents the event number of the meat mixture. The vertical axis represents the amplitude of samples. Lanes: A01, B01, C01: 100% pork; D01, E01, F01: 100% cattle; C06, D06, E06: NTC.

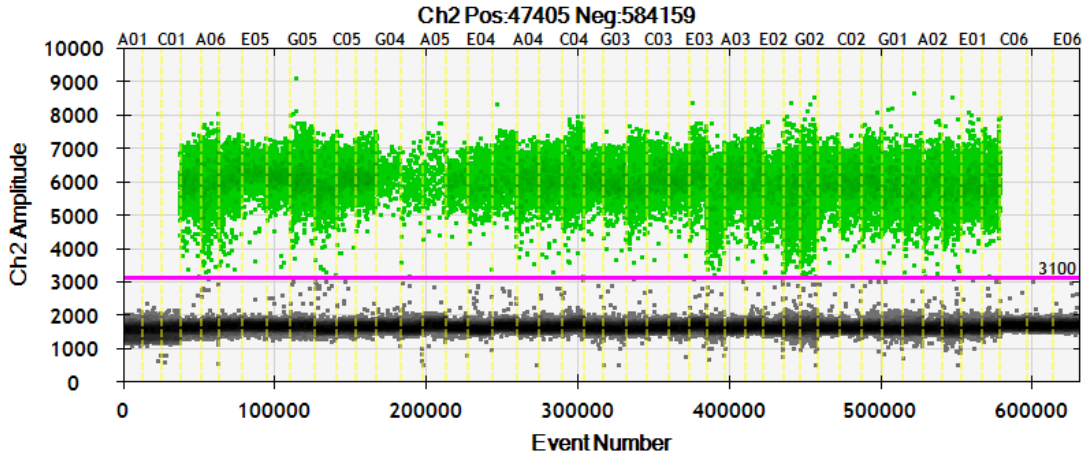
3.2. ddPCR results for raw meat mixtures

At this study, the copy numbers were obtained by ddPCR assays based on the weight proportions of meat mixtures. The analysis was performed in triplicate for each sample. At the end of the ddPCR process for the raw meat mixtures, the numbers for the mean of the droplets were 14,075 and standard deviations were 1785 droplets, respectively (Fig. 2). As a result of the ddPCR process, 0.1 copies/µL of the sample 0.001% pork meat was observed as a minimum copy number corresponding to 2 positive droplets (Fig. 2a).

According to the ddPCR analysis results of droplets for the raw meat mixture sample of 0.001% pork, 2 positive droplets were detected in 14,213 droplets as shown at the lane C03 (Fig. 2a). Also, in the parallel analysis 1 positive droplets was detected in 11,579 droplets as shown at the lane E03 (Fig. 2a).



(a)



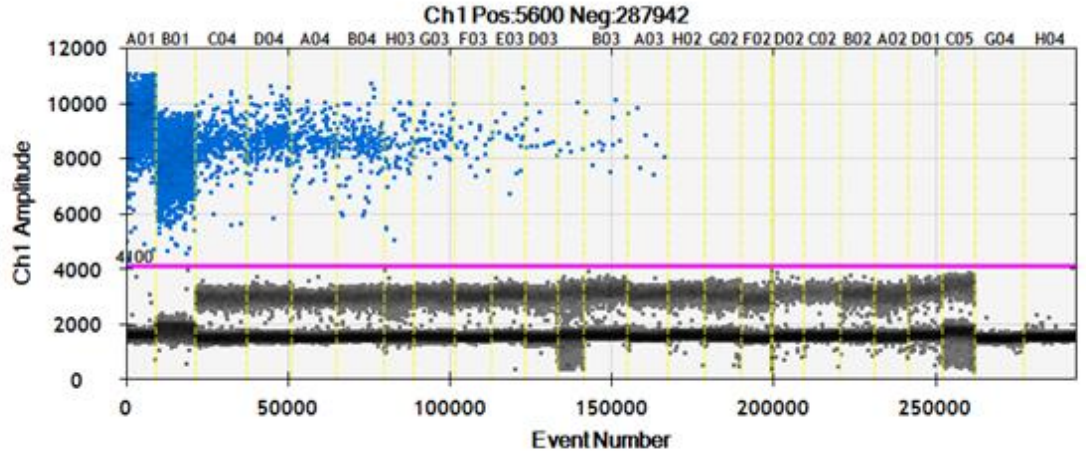
(b)

Figure 2. The results of ddPCR assays in raw meat samples. (a) The ddPCR assays for pork; and (b) The ddPCR assays for cattle. The horizontal axis represents the event number of the meat mixture. The vertical axis represents the amplitude of samples. Lanes: C03: 2 positive droplets detected; E03: 1 positive droplet detected; C06, E06: NTC.

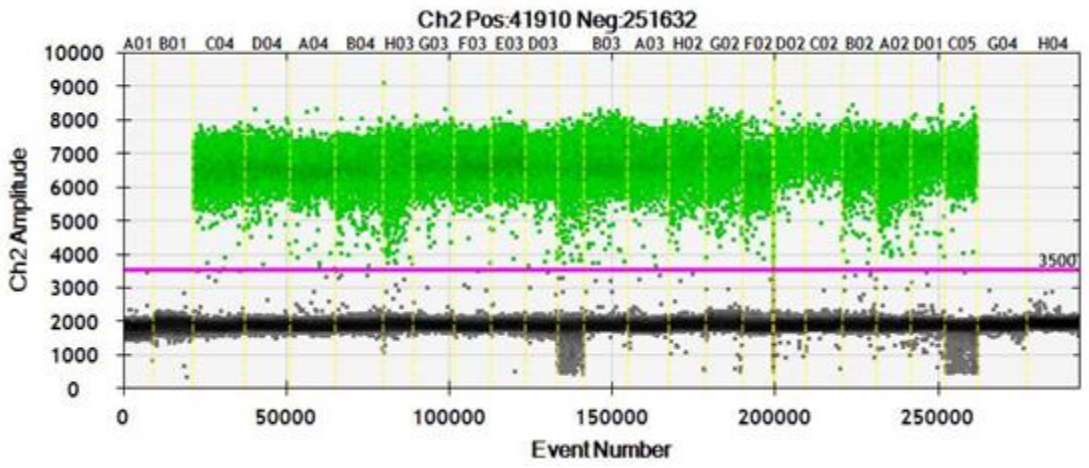
The means and standard deviations of ddPCR results for raw meat mixtures were given in Table 4. The experiments conducted for NTC samples were resulted in no positive droplet (no signal) that showing no contamination in the experiment (Fig. 2a and Fig. 2b, Lanes C06 and E06; Table 4).

3.3. ddPCR results for heat-treated meat mixtures

The copy numbers were obtained by ddPCR assays based on the weight proportions of heat-treated meat mixtures. The analysis was performed with a duplicate for each sample. At the end of the ddPCR process for heat-treated meat mixtures, the numbers of the mean of the droplets were 13,852 and standard deviations were 1375 droplets (Fig. 3). As a result of the ddPCR process, 0.65 copies/µL of the sample 0.1% pork meat was observed as a minimum copy number corresponding to 7 positive droplets (Fig. 3b).



(a)



(b)

Figure 3. The results of ddPCR assays in heat-treated samples. (a) The ddPCR assays for pork; and (b) The ddPCR assays for cattle. The horizontal axis represents the event number of the meat mixture. The vertical axis represents the amplitude of samples. Lanes: A03: 7 positive droplets detected; B03: 12 positive droplets detected; G04, H04: NTC.

According to the ddPCR analysis results of droplets for the heat-treated meat mixture sample of 0.1% pork, 7 positive droplets were detected in 12,666 droplets as shown in the lane A03 (Fig. 3a). Also, in the parallel analysis 12 positive droplets were detected in 13,632 droplets as shown in the lane B03 (Fig. 3a). This result could be explained by the denaturation effect of heat treatment by the cooking process on DNA fragments. The means and standard deviations of ddPCR results for heat-treated meat mixtures were given in Table 5. The experiments conducted for NTC samples were resulted in no positive droplet (no signal) that showing no contamination in the experiment (Fig. 3a and Fig. 3b, Lanes G04 and H04; Table 5).

Many research studies based on the qPCR or real-time PCR have been reported for species identification and quantification in meat products as follows. Ren et al. (2017) reported the detection of 1% (w/w) sheep meat in sheep: chicken meat mixture and 0.1% chicken in the meat mixture. They compared real-time PCR and ddPCR methods and they reported that the ddPCR method is more accurate and easier compared to the real-time PCR method. Ulca et al. (2013) reported a 0.1% detection limit of pork meat in raw and cooked beef, chicken and turkey meat products using commercial PCR kits. Ali et al. (2015) practiced a multiplex PCR assay to identify five meat species (cat, dog, pig, monkey, and rat) in meatballs (cooked at 121°C) and they reported a detection limit of 1%. Kesmen et al. (2009) performed a species-specific PCR analysis for the identification of pork, horse meat, and donkey meat in cooked sausages. The lower detection level that reported was 0.1% for each other. Köppel, Zimmerli, and Breitenmoser (2009) developed a quantitative multiplex PCR method for detection of pork, beef, chicken, turkey, horse meat, sheep and goat meat and the minimum detection level reported by this study was 2%. Mousavi et al. (2015) reported a detection limit of 0.1% for identifying of chicken and donkey in raw meat samples due to a species-specific PCR study.

In our study, minimum detection of pork in pork:cattle meat mixtures were stated as 0.1% for heat-treated and 0.001% for raw samples. Compared to the results obtained by previous reported works based on the qPCR or real-time PCR, the results of our ddPCR study were more sensitive, especially for heat-treated samples.

Table 4. ddPCR analysis results of raw binary meat mixtures

Sample No	Pork meat (%)	Copies / µL		Copies / Well (20 µL)		Positives Droplets		Accepted droplets	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0*	-	-	-	-	-	-	17273.00	489.79
2	0.0000001	-	-	-	-	-	-	13307.00	678.05
3	0.000001	-	-	-	-	-	-	14245.33	889.02
4	0.00001	-	-	-	-	-	-	12033.00	1394.88
5	0.0001	-	-	-	-	-	-	12281.00	1415.62
6	0.001	0.09	0.09	1.80	1.71	1.00	1.00	13139.00	1382.62
7	0.01	0.23	0.21	4.60	4.26	2.67	2.52	13823.67	938.62
8	0.1	2.10	0.00	42.00	0.00	26.33	1.53	14823.67	1025.50
9	0.5	1.20	0.26	24.00	5.29	15.67	2.08	15565.67	1347.99
10	1.0	1.70	0.52	34.00	10.39	22.00	7.81	15273.67	743.98
11	2.5	5.36	0.67	107.33	13.32	62.67	13.43	13751.67	1528.57
12	5.0	11.70	4.07	234.00	81.46	156.33	52.60	15863.00	317.51
13	10	24.36	2.60	487.33	51.94	282.67	64.61	13665.67	1834.16
14	100	237.33	11.02	4746.67	220.30	2325.00	140.62	12741.00	848.46
	NTC	-	-	-	-	-	-	12735.00	670.35
	Total	18.94	1.30	378.78	25.91	192.96	19.08	14034.76	192.96

* : Cattle (100%)
SD : Standard deviation.
NTC: None Template Control.
- : not detection

Table 5. ddPCR analysis results of heat-treated binary meat mixtures

Sample No	Pork meat (%)	Copies / µL		Copies / Well (20 µL)		Positives Droplets		Accepted droplets	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0*	-	-	-	-	-	-	10917.50	277.89
2	0.0000001	-	-	-	-	-	-	11231.00	3003.79
3	0.000001	-	-	-	-	-	-	11836.00	927.72
4	0.00001	-	-	-	-	-	-	10484.50	235.47
5	0.0001	-	-	-	-	-	-	10408.50	958.13
6	0.001	-	-	-	-	-	-	11447.50	2704.68
7	0.01	-	-	-	-	-	-	11166.50	185.97
8	0.1	0.83	0.25	16.50	4.95	9.50	3.54	13149.00	683.07
9	0.5	0.90	0.14	18.00	2.83	7.00	2.83	8878.50	1747.26
10	1.0	2.20	0.42	44.00	8.49	20.50	3.54	11074.50	521.14
11	2.5	5.60	0.71	112.00	14.14	50.50	3.54	10808.50	2202.64
12	5.0	12.35	1.06	247.00	21.21	149.00	7.07	14323.50	516.90
13	10	20.30	1.41	406.00	28.28	254.50	6.36	14926.50	1413.51
14	100	284.00	5.66	5680.00	113.14	2309.00	325.27	10782.50	1707.66
	NTC	-	-	-	-	-	-	15577.50	495.68
	Total	21.75	0.64	434.90	12.87	186.67	25.15	11880.80	1172.10

* : Cattle (100%)
SD : Standard deviation.
NTC: None Template Control.
- : not detection

4. Conclusions

Regarding the authenticity problems in the world, the (qPCR) is already a more prevalent method for identification and quantification of meat species compared to ddPCR, because of its lower costs. Additionally, the ddPCR method would require more time which could be attributed to the droplet reader process for analyzing individual droplets. However, ddPCR provides the measurements with higher precision, sensitivity, and repeatability which is related to the number of partitions ensuring concurrent template amplification and of being capable of detecting the smallest traces of nucleic acids. This study was performed in order to identify minimum pork meat quantity in raw and heat-treated meat mixtures (cattle:pork) using an alternative ddPCR method. The main purpose of this study was to prove the usability of ddPCR for the quantification of meat species with higher precision and sensitivity. Consequently, the absolute limits of detection (aLOD) of the ddPCR were determined as 0.65 copies/µL and the absolute limits of quantification (aLOQ) of ddPCR were determined as 13 copies/µL for heat-treated samples. The (aLOD) results were defined as 0.1 copies/µL and the (aLOQ) results were defined as 2 copies/µL for raw meat samples. According to these results, the minimum pork meat concentration in meat mixtures (cattle:pork) samples was identified with a precision of 0.001% (for raw samples) and 0.1% (for heat-treated samples). Our study showed that ddPCR could be an effective alternative tool usable for law enforcement authorities to control and prevent food adulteration. Furthermore, this method has the potential of being adapted for the quantifying of various other meat species.

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Acknowledgments

This work was supported by research grant 2012.09.01.528 from Scientific Research Projects at BAIB University. Ö. E. was the project coordinator. The authors would like to thank Scientific Industrial and Technological Application and Research Center for using some laboratory opportunities.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

DRYING CHARACTERISTICS, CHEMICAL CONSTITUENTS, VOLATILES PROFILES OF DIFFERENT ROOT SYSTEMS FROM HAIRY FIG (*FICUS HIRTA VAHL.*) AND ANTIOXIDANT ACTIVITY

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https://doi.org/10.34302/crpjfst/2023.15.4.10

Article history:
Received: 11 November 2023
Accepted: 12 December 2023

Keywords:
Hairy fig root;
Chemical constituents;
Volatile profiles;
Antioxidant activity.

ABSTRACT

Hairy fig (*Ficus hirta* Vahl.) root is a traditional medicinal and food homologous plant in China. Due to the lignified roots are extremely difficult to cut and package, postharvest treatment is very difficult. Therefore, when the roots of the hairy fig are used as a soup ingredient, the root cortex is simply peeled off and used.In order to promote the development and utilization of hairy fig root, chemical composition, volatile profiles and antioxidant activities of entire root (ER), root cortex (RC) and root internal tissue (RIT) of hairy fig were investigated. Hairy fig root was rich in fat, protein, soluble sugar, polyphenols, flavones and other nutrients, which had significant difference in ER, RC and RIT ($P<0.05$). Chemical components and nutrients were highest in RC, and lowest in RIT, as well as psoralen and bergapten. In ER, RC and RIT, content of psoralen was 0.70, 0.77, 0.32 mg/g, and 0.27, 0.42, 0.14 mg/g for bergapten. Phosphorus, potassium, magnesium, calcium were the main minerals, and arginine was the main free amino acid. There were 47 volatile components detected in ER, RC and RIT, where aldehydes and heterocyclic compounds were the dominant components. The relative level of aldehydes was 16.98~34.09%, and 6.71~45.23% for heterocyclic compounds. In addition, total relative amount of volatile components was 75.68% in RC, but 57.94% in RIT. From chemical components, nutritional components and volatile components content, the quality of RC was better than ER and RIT. The results might be related to chemicals accumulation position and the physiological structure of different parts of plants. According to antioxidant activity, ER, RC and RIT have scavenging activity of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals. The half inhibitory concentration (IC50) of RC on ABTS, DPPH and hydroxyl radical were 3.51, 0.08 and 1360.40 mg/mL, respectively, which were lower than ER and RIT. Based on the drying process of the samples, the RC and RIT exhibited the best drying efficiency. All in all, from the point of view of saving energy and quality, RC not only had better quality but need less energy to dry. These results provided a reference for the development and utilization of hairy fig root cortex as a raw material in medicine, functional food or dietary additives.

1.Introduction

With more than 800 species of trees, shrubs, hemiepiphytes, climbers, and creepers in the tropics and subtropics worldwide, Ficus is one of the largest genera of angiosperms. There are 23 species of lithophytes and hemiepiphytes in the ficus family that form aerial and creeping root systems (Ronsted, et al., 2008). Among these, hairy fig (*Ficus hirta* Vahl.) is one of the important member in the family of Moraceae (Shu, et al., 1975). It widely distributed in southern China and it's known as Wuzhimaotao in Chinese language (Dai, et al., 2018). It is a popular herbal medicine and a food ingredient among Hakka people in China. Hairy fig is a wild edible plant in Assam and Meghalaya,India. It is popular as a folk medicine in Vietnam for the treatment of hepatitis, nephritis, mastitis and rheumatism (Yi, et al., 2013) . Hairy fig root has a pleasant fragrance and medicinal potential for the treatment of hepatitis, anticancer, antioxidation and improving fatigue resistance in China (Au, et al., 2008; Zeng, et al.,2012; Liang, et al., 2021).

In recent years, many studies have been done on the hairy fig root for understanding its chemical compositions. From the stem and root extracts of hairy fig, hundreds of various therapeutically important phyto-compounds such as phenylpropanoids, flavonoids, terpenoids, sterols and volatile oils have been identified (Wang and Chen, 2013; Cheng, et al., 2017; Ye, et al., 2019). Hairy fig root is rich with polyphenols (197.16 mg/g) and flavonoid (9.10 mg/g) (Gui, et al., 2018; Chen, et al., 2021). The content of volatile oils in root cortex and root internal tissue of hairy fig was 0.3 and 0.25 mL/Kg, respectively. The popular linear furanocoumarins such as psoralen and bergapten were considered as the most valuable quality evaluation indexes component (Liu, et al., 2004; Wei, et al., 2005). The content of psoralen and bergapten in hairy fig root was 0.0008-1.187mg/g and 0.0039-0.034mg/g, respectively (Cai, et al., 2019; Chen, et al., 2022).

According to various reports, different plants have a distinct distribution of metabolites in their roots. Paeoniflorin and its derivatives are primarily found in the cortex and phloem of Paeonia suffruticosa root, although they are also present in Paeonia lactiflora's xylem rays (Li, et al., 2021). As well as in Tripterygium root, the quinone methides compound, celastrol is observed only in periderm, but the intense signals of sesquiterpene pyridine alkaloids are detected throughout the root cortex (Lang, et al., 2016). The antibacterial activity of root cortex extract in Berberis heteropoda Schrenk is superior than root internal tissue against Pseudomonas aeruginosa and Enterococcus faecalis, whereas their antibacterial activity against Escherichia coli and Staphylococcus aureus is comparable (Zhu and Li, 2018). Periplocin and 4-methoxysalicylaldehyde are crucial index component in Periploca sepium Bunge, but they are found in greater concentrations in the root cortex than the root internal tissue of the plant (Zhang, et al., 2012). It is indicated that the accumulation of plant metabolites are varying in different parts of herbal root.

Various studies have been done on the hairy fig root and revealed the presence of numerous bioactive phyto molecules. Nevertheless, the studies were a general approach to understand the contents in the entire root. But based on the various studies (Zhang, et al., 2012; Lang, et al., 2016; Zhu and Li, 2018; Li, et al., 2021), it is understood that each root parts possess different level of bioactive molecules. So the current study has been designed to analyse the two common forms (entire root (ER) and root cortex (RC)) and the new root part, root internal tissue (RIT). Chinese Pharmacopoeia (1977 edition) mention that hairy fig root with thick cortex and nice fragrance can be an improved product, but no studies have shown that whether root cortex has higher content of active ingredients or antioxidant activity. Although, Liu et al. (2004) find that the content of volatile oil and psoralen in root cortex is higher than that of root internal tissue. Another study report that the contents of psoralen, polyphenols and flavonoids in the thin root are higher than thick root (Gui, et al., 2018). But there is no systematic study about the

difference of chemical component and antioxidant activity in different part of hairy fig root.

In this paper, the drying curves of ER, RC and RIT of hairy fig root were analyzed under the hot air drying condition at 80℃, and the energy required to dry the different parts was calculated. Then, the differences of chemical components, volatile flavor components and antioxidant activities in different parts of hairy fig root were detected, and the relationship between chemical components and microscopic structure were analyzed. The results from this study will be useful not only at the scientific level to find out the effective parts accurately but also to encourage farmers, large-scale breeders and the pharmaceutical industry to explore the development and utilization of hairy figs.

2.Materials and methods

2.1.Plant Materials

Fresh hairy fig (HF) roots were collected from the experimental farm of the Chinese Academy of Tropical Agricultural Sciences (CATAS; 19°32′55″N, 109°28′30″E) in Danzhou City. The entire root (ER)of HF was separated to root cortex (RC) and root internal tissue (RIT), and the three samples were dried in a hot air drying oven (GZX-9240MBE, China) at 80 ℃ to constant weight. Hairy fig plant, entire root, root cortex and root internal tissue of hairy fig were shown in Figure 1. Then the dried samples were ground into powder and sieved through 100 mesh sieves. The hairy fig root powder was collected and stored at -20℃ prior to further use.

Figure 1. Hairy fig plant (A) and entire root (B) , root cortex (C) and root internal tissue (D) of hairy fig

2.2.Chemicals and reagents

Acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Psoralen and bergapten were purchased from Aladdin (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Chemical Co.LTD,

(St. Louis, MO, USA). Gallic acid, rutin (analytical grade) was purchased from sinopharm chemical reagent Co., LTD. (Shanghai, China). Folin-Ciocalteu was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Methanol, ethanol was purchased from Xilong Scientific Co., Ltd. (Guangdong, China). Ultrapure water (~18.2 MΩ, 25 ℃) obtained from a Master-S plus UVF ultra-pure water system (Shanghai Fushite environmental protection technology Co., Ltd, China). Glucose (Glu) and phenol and other chemicals and reagents used were analytical grade.

2.3.Drying curve

The moisture contents of fresh and dried samples were analyzed by an oven drying method at 105 °C (AOAC, 2005). The samples were weighed every 30 min until the weight of sample became constant. The initial and final moisture contents of the samples were recorded. The drying rate is expressed as the amount of evaporated moisture over time. It is calculated as

$$\textbf{Drying rate} = \frac{MC_1 - MC_2}{t_1 - t_2} \quad (1)$$

where MC_1 and MC_2 are moisture contents of sample (g water/g dry matter) at time t_1 and t_2 (h), respectively (Akpinar and Toraman, 2016).

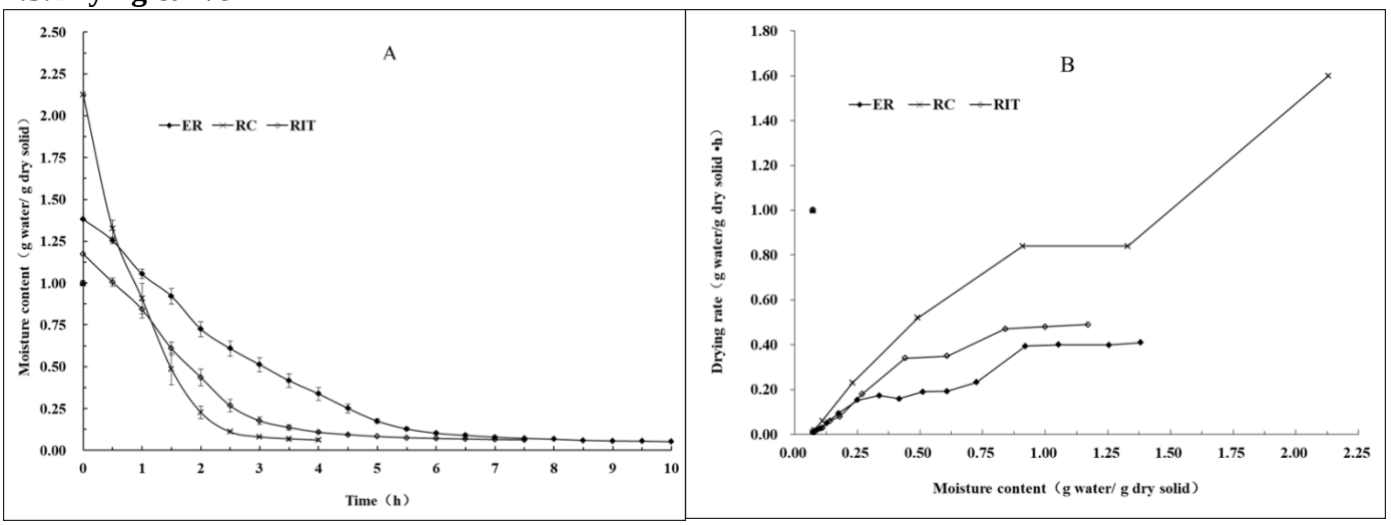


Figure 2. Drying curves (A) and drying rate (B) of ER, RC and RIT of hairy fig root dried by hot air at 80 ℃. ER, entire root; RC, root cortex; RIT, root internal tissue.

2.4.Chemical composition analysis

Chemical compositions including ash (AOAC 938.08), protein (AOAC 2001.11) and fat (AOAC 920.39) were determined according to the methods recommended by AOAC (2005). The soluble sugar was determined by phenol-sulfuric acid method with glucose as a standard (Rover, et al., 2014).

2.5.Extraction for phenolic, flavonoid, psoralen, bergapten analysis

2 g hairy fig powder was extracted three times by 60% ethanol (*V/V*) with ultrasonic at 40 KHz (SB5200DTS, Ningbo Scientz Biotechnology Co., Ltd., Zhejiang, China) for 0.5 h. The extraction solutions were combined to be filtered and concentrated to 100 mL and stored at -80 ℃ for phenolic, flavonoid, psoralen, bergapten analysis.

2.6.Determination of total phenolic and total flavonoid

The total phenolic concentration (TPC) and total flavonoid concentration (TFC) were determined according to the method reported by Chumroenphat et al. (2021). Briefly, the 60% ethanol extract of hairy fig (0.1 mL) was added Folin–Ciocalteu reagent (0.5 mL) and 10%

Na_2CO_3 (1.5 mL). The TPC of the sample was quantified by constructing standard curves with different concentrations of gallic acid at 760 nm (UV-2600, Shimadzu Co., Kyoto, Japan). TFC determination was performed by adding 5% $NaNO_2$ (0.15 mL) and 10% $AlCl_3$ (0.15 mL) solutions to the 60% ethanol extract (1 mL), and then adding 1 M NaOH (1 mL) for color development after avoiding light for 5 min. TFC in the sample was quantified by making standard curves with different concentrations of quercetin at 506 nm (UV-2600, Shimadzu Co., Tokyo, Japan). psoralen, bergapten analysis. TPCs and TFCs were expressed as equivalents of gallic acid and rutin per kilogram of dry matter, expressed as mg GAE/g and mg RE/g, respectively.

2.7. Determination of psoralen and bergapten

Psoralen and bergapten content were determined by high performance liquid chromatography (HPLC), discribed by Yang et al. (2010) with small modification, an Agilent 1200 liquid chromatography system (Agilent, USA) equipped with a quaternary solvent delivery system, an autosampler and a column compartment was used for all analysis. The chromatographic separation was performed on an Agilent Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm), and the column temperature was kept at 35℃. The mobile phase consisted of ultrapure water and acetonitrile (65:35,V/V) with a flow rate of 1.0 mL/min, Psoralen and bergapten were monitored at 245 nm.

The appropriate amounts of psoralen and bergapten were separately weighed and dissolved in methanol to make the stock solution containing 0.80 mg/ml of psoralen and bergapten. A series of working solutions of these analytes were freshly prepared by diluting mixed standard solution with methanol at appropriate ratios to yield concentrations of 3.20, 16.00, 80.00 and 400.00 ug/mL. Hairy fig extract was filltered with 0.45 μm millipore filter. The psoralen and bergapten composition were calculated according to the standard curve.

2.8. Determination of mineral elements

Mineral elements were determined according to the method described by Zhang et al. (2022). Briefly, 0.25 g sample powder was mixed with 6.0 mL nitric acid. Then the mixture was predigested for 1 h. After that, 2 mL hydrogen peroxide were added to digest thoroughly in the Anton Paar multiwave pro microwave digestion instrument (Graz, Austria). Then mineral elements were determined by Perkin Elmer Nexion 300X Inductively coupled plasma mass spectrometry (ICP-MS) (Waltham, MA, USA). The content of each element was calculated with reference of standard curve.

2.9.Determination of free amino acid

The free amino acid were profiled using LC-MS with slight modification of Zhang et al. (2022). Briefly, 0.2 g sample powder was ground with liquid nitrogen and 3 mL 50% acetonitrile contained 0.1% hydrochloric acid (V/V), and stored at 4 ℃ for 30 min after vortexed. Then the extracts were centrifuged at 13800×g for 10 min at 4 ℃. The supernatant was extracted 3 times and filtered through a 0.22 μm filter before analysis. The LC-MS system consisted of an high-performance liquid chromatography (Ultimate3000, Thermo Fisher, USA) system with a high resolution mass spectrometer (Exactive Plus, Thermo Fisher, USA). Chromatographic separation was achieved using an Poroshell SB-Aq column (3×150mm, 2.7 μm; Agilent), kept at 30 ℃ with 0.3 mL/min flow rate. The mobile solutions were water with 0.1% formic acid (A) and acetonitrile (B). The following gradient was employed: 0-2 min, 0-2% B; 2-5 min, 2-8% B; 5-5.5 min, 8-80% B; 5.5-8 min, 80% B, the initial conditions were maintained for 2 min to equilibrate the column. The injection volume was 2 μL. MS detection was performed in the positive mode with Full MS/AIF mode. The ion spray voltage was set to 3,500 V, and the source temperature was set to 320 ℃. The nebulizer and heater gases were maintained at 55 psi. The content of each amino acid per gram of sample in dry weight was calculated by establishing a standard curve of the standard substance.

2.10. Analysis of volatile components by headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS)

1.0 g of hairy fig powder was taken into an extraction bottle and added 10 mL distilled water. The sample vials were preheated using the solid-phase microextraction unit at 100 ℃ for 20 min, and then solid-phase microextraction (SPME) fibers (50/30 μm PDMS/CAR/DVB; Supelco Co., PA, USA) were inserted into the headspace of the sample and extracted for 30 min. Volatile components were qualitatively determined using a 7890B-5977 GC–MS system equipped with a HP-5MS column (0.25μm, 30.0 m × 250 μm; Agilent Co., CA, USA). The initial column temperature was 40 ℃ (maintained for 3 min), followed by an increase up to 120 ℃ at a rate of 5℃/min, and then again hiked up to 280 ℃ at 10 ℃/min, then it maintained for 5 min). The injector temperature was 250 ℃, and the carrier gas was He with a flow rate of 1.0 mL/min. The mass spectrometer was set to electron ionization (EI) mode, with an ion source temperature of 230 ℃, quadrupole temperature of 250 ℃, and scanning quality range of 45-400 m/z. The volatile compounds were identified by mass spectra from NIST 17.0 database (Liu, et al., 2022).

2.11.In vitro antioxidant assays

Radical scavenging activity (ABTS assay)

The radical scavenging activity of ABTS radical of the 60% ethanol extracts of ER, RC and RIT was assessed as described by Euch et al. (2015). The radical solution consisted of a mixture of equal volumes of 2.45 mM of potassium persulphate and 7 mM ABTS, kept in the dark for 12-16 h at room temperature and further diluted with ethanol in a ratio 1:70 to obtain a 0.700 ± 0.003 absorbance at 734 nm.

A mixture of 0.1 mL of diluted sample extracts and 900 mL of ABTS solution was incubated in the dark for 6 min. The absorbance was then measured at 734 nm, and the ascorbic acid (Vc) as a positive control. The ABTS radical scavenging activity of the extract was expressed as the inhibition concentration (IC_{50}) i.e., the concentration of the extract required to decrease the initial ABTS radical concentration by 50% (IC_{50}) under the specified experimental condition. The ability to scavenge the ABTS radical was evaluated according to the following formula:

$$\text{ABTS radical scavenging effect (\%)} = \left[\mathbf{1} - \frac{\boldsymbol{A}}{\boldsymbol{A_0}}\right] \times 100 \quad (2)$$

Where: $\boldsymbol{A_0}$: control absorbance; A: sample absorbance.

2.11.Radical scavenging activity (DPPH assay)

DPPH assay was conducted according to Oke-Altuntas et al. (2017). Serial dilutions of each 60% ethanol extracts of ER, RC and RIT were prepared. A diluted sample (100 mL) and 900 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in ethanol solution (5×10^(-5)M) were mixed. After 30 min of incubation in the dark, the absorbance at 520 nm was measured, Vc as a positive control. The antiradical activity was expressed as (IC_{50}). The ability to scavenge the DPPH radical was calculated as follows:

$$\text{Radical scavenging effect (\%)} = \left[\mathbf{1} - \frac{\boldsymbol{A}}{\boldsymbol{A_0}}\right] \times 100 \quad (3)$$

Where: A_0: control absorbance; A: sample absorbance.

2.12.Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity of 60% ethanol extracts of ER, RC and RIT was assayed according to zhang et al. (2012), and Vc is used as a positive control. The reaction mixture, containing 1 mL of 60% ethanolic extracts, was incubated with 0.3 mL of $FeSO_4$ (8 mM), 1 mL of salicylic acid (3 mM) and 0.25 mL of H_2O_2 (20 mM) at 37 ℃ for 30 min. The reaction was cooled to room temperature by keeping in the water. Then 0.45 mL distilled water was added into the mixture to make the end volume 3.0 mL, then centrifuged at 3000×g

for 10 min. The absorbance of the supernatant was measured at 510 nm, and 1 mL of solvent solution was used instead of 60% ethanol extracts solution as a control. The extract activity against hydroxyl radicals was expressed as (IC_{50}), the concentration of the compound required to decrease the initial hydroxyl radical concentration by 50%. Hydroxyl radical scavenging activity was calculated as follows：

Hydroxyl radical scavenging effect (%) = $\left[\mathbf{1}-\frac{\boldsymbol{A}}{\boldsymbol{A_0}}\right]$*100. (4)

Where: A_0: Control absorbance; A: sample absorbance

2.13.Fourier-transform infrared (FT-IR) spectroscopy

Dried samples powder was mixed with KBr powder evenly and pressed into a pellet and measured on FT-IR spectroscopy (IS50, Thermo Fisher Scientific, MA, USA) in the wave number range from 400 cm-1 to 4000 cm^{-1}.

2.14.Statistical analysis

All obtained data were expressed as mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA). Microsoft Office 2007 and SPSS 17.0 software were used for drawing and data calculation. Principal component analysis and partial least squares-discriminate analysis (PLS-DA) using Omics share 6.4.5 online analysis (Genedenovo Co.ltd, Guangzhou, China). Differences at $P<0.05$ were taken as statistically significant.

3.Results and Disscusion

3.1.Drying characteristics

The entire root were separated into root cortex and root internal tissue (Figure 1). The initial moisture content of ER, RC and RIT was 56.22%, 66.02% and 51.10% w.b., respectively, while the final moisture content of the dried products was in the range of 6.21-7.64 % w.b are presented in Table 1. Drying curves and drying rate of ER, RC and RIT are presented in Figure 2. ER, RC and RIT were needed 10, 4, and 7.5 hours, respectively, to reach a certain moisture content (Table 1). From Figure 1B, RC had higher drying rate, followed by RIT and ER. The falling drying rate period occurred through the whole drying process except for at the beginning 0.5 h of ER and RIT and 0.5-1.0 h of RC, in which the constant rate period was also observed when moisture content between 1.00-1.50 g water/g dry solid. The results indi cated that in hairy fig root drying process, the surface water was evaporated faster than the diffusion of water inside the sample to the surface and there was no saturated water surrounding the sample surface. Therefore, diffusion is the mechanism describing the removal of water in this process (Akpinar and Toraman, 2016).

Table 1. Drying time, moisture content of the fresh and dried Hairy fig root

Samples	Drying time (h)	Moisture content (% w.b.)	
		Fresh	Dried
ER	10	56.22 ± 0.69^{b}	7.64 ± 0.07^{c}
RC	4	66.02 ± 0.25^{c}	6.21 ± 0.06^{a}
RIT	7.5	51.10 ± 0.14^{a}	7.27 ± 0.01^{b}

* ER, entire root; RC, root cortex; RIT, root internal tissue; w.b., wet base.

3.2.Chemical composition

Chemical contents such as fat, protein, ash, total polyphenols (TPC) and total flavonoids (TFC) in RC was higher than in RIT and ER, yet, the total soluble sugar was higher in RIT (4.48%). As results shown in Table 2, ER, RC and RIT of hairy fig root have significant differences ($p<0.05$) in fat, protein, ash, total polyphenols and total flavonoids content. The content of fat, protein, ash, TPC and TFC in RC

was 8.48%, 9.27%, 8.39%, 3.42 mg/g and 3.16 mg/g, respectively, and it was 8.43%, 4.70%, 1.63%, 1.56 mg/g and 0.78 mg/g in RIT. The lowest content of fat and total soluble sugar in ER might be due to the physical or chemical reactions which occurred as a result of the long drying time.

Table 2. Chemical composition in hairy fig root

Parameter	**ER**	**RC**	**RIT**
Fat（%）	5.73±0.21^{a}	8.48±0.39^{c}	8.43±0.11^{b}
Protein (%)	5.88±0.13^{b}	9.27±0.22^{c}	4.70±0.08^{a}
total soluble sugar(mg/g)	3.98±0.13^{a}	4.12±0.12^{b}	4.48±0.15^{c}
Ash (%)	3.55±0.05^{b}	8.39±0.11^{c}	1.63±0.04^{a}
TPC (mg GAE/g)	2.61±0.20^{b}	3.42±0.10^{c}	1.56±0.03^{a}
TFC（mg RE/g）	1.89±0.01^{b}	3.16±0.02^{c}	0.78±0.01^{a}
Psoralen	0.70±0.00^{b}	0.77±0.00^{c}	0.32±0.00^{a}
Bergapten	0.27±0.00^{b}	0.42±0.00^{c}	0.14±0.00^{a}

*ER, entire root; RC, root cortex; RIT, root internal tissue. Different letters in the right superscript of the same line showed significant differences (P< 0.05).

Table 3. Mineral elements in hairy fig root

Mineral Elements	**Content (mg/kg)**		
	ER	**RC**	**RIT**
Macro-minerals			
Phosphorus	1325.82±82.22^{b}	2490.23±164.99^{c}	1050.91±71.31^{a}
Sodium	24.18±1.51^{a}	46.88±3.73^{b}	23.16±2.27^{a}
Potassium	4303.08±120.97^{b}	8179.86±157.58^{c}	3127.37±192.88^{a}
Magnesium	1139.88±69.39^{b}	2403.03±211.14^{c}	714.46±8.71^{a}
Calcium	5099.40±83.60^{b}	11456.80±346.95^{c}	2910.42±117.61^{a}
Micro-minerals			
Copper	2.63±0.04^{b}	4.74±0.14^{c}	1.89±0.06^{a}
Zinc	81.46±1.44^{c}	76.57±1.62^{b}	64.63±3.02^{a}
Manganese	478.67±4.69^{b}	1063.67±69.50^{c}	255.66±30.49^{a}
Iron	206.98±8.09^{b}	655.60±18.51^{c}	50.07±2.02^{a}
Other-minerals			
Nickel	2.26±0.03^{c}	1.94±0.06^{a}	2.16±0.02^{b}
Aluminum	1570.60±60.92^{c}	563.68±10.41^{b}	701.86±31.04^{a}
Rubidium	26.02±1.08^{b}	42.61±0.89^{c}	17.42±1.17^{a}
Tin	0.02±0.00^{b}	0.04±0.00^{c}	0.02±0.00^{a}
Boron	6.05±0.21^{b}	12.47±0.85^{c}	3.43±0.22^{a}

* Different letters in right superscript of the same line showed significant differences (P<0.05). ER, entire root; RC, root cortex; RIT, root internal tissue.

The content of other chemical composition were protein, ash, TPC and TFC between RC and RIT. The results showed that primary and secondary metabolites were accumulated more in RC, which was similar to the results reported by Aielloa et al. (2015).

Psoralen and bergapten were common plant secondary metabolites in Lemon juice (Jungen,

et al., 2023), celery (Beier, et al., 1983) and *Psoralea corylifolia* L (Guo, et al., 2005). They were regarded as marker compounds in hairy fig (Liu, et al., 2004; Wei,et al., 2005). Content of psoralen in ER, RC and RIT was 0.70, 0.77 and 0.32 mg/g, respectively, and content of bergapten was 0.27, 0.42, 0.14 mg/g. This result was consistent with Aielloa et al. (2015) whose conclusion that the root cortex as the main accumulation site for marker compounds. As we known, psoralen and bergapten were belong to furanocoumarins. It was reported that coumarins in primary root of A. *dahurica* were concentrated in the periderm, cortex, and phloem, whereas they were concentrated in the phloem in lateral roots (Gao and Li, 2023), which provided guidance for further analysis of the accumulation of psoralen, bergapten and other active ingredients in hairy fig root.

3.3.Analysis of Mineral Elements

Fourteen mineral elements were detected in ER, RC and RIT (Table 3). All minerals had significant difference between ER, RC and RIT ($P<0.05$), except for sodium in ER and RIT ($p>0.05$). Calcium and potassium were two main macro-minerals in hairy fig root. Calcium was the highest mineral in ER and RC, whose content was 5099.40 and 11456.80 mg/kg. In RIT potassium content was the highest for 3127.37 mg/kg. Manganese was the highest micro-minerals which was 478.67, 1063.67 and 255.66 mg/kg in ER, RC and RIT, followed by iron, zinc and copper. As other-minerals, Aluminum was the highest mineral, the next was rubidium, boron, nickel and tin. Compared to the amount of elements in the roots of sweet potato, the content of calcium, magnesium, phosphorus were higher in hairy fig root (Md Mokter, et al., 2022). Content of potassium in RIT was similar to sweet potato root reported by Senthilkumar et al. (2020) and Sanoussi et al. (2016). From Table 3, the results showed that hairy fig root had abundant mineral elements, especially in RC. Moreover, this could explain why ash content in RC was the highest.

3.4.Analysis of amino acid composition

Different levels of ten free amino acids were detected in the test-parts of hairy fig root (Table 4). Total free amino acids in ER, RC and RIT were 2219.48, 2656.81 and 2052.72 mg/Kg, respectively, with significant differences ($P<0.05$). Only five essential amino acids (histidine, threonine, valine, methionine and phenylalanine) and five nonessential amino acids (aspartic acid, glutamic acid, arginine, glycine and serine) were detected. The content of methionine was 0.27 and 5.05 mg/Kg in ER and RC, respectively, but it was not detected in RIT. The content of arginine was 2013.51(ER), 2405.76 (RC) and 1853.13 mg/Kg (RIT), which was the highest among all detected free amino acids. Besides arginine, the main free amino acids in ER were aspartic acid (110.59 mg/Kg) and phenylalanine (24.27 mg/Kg). Aspartic acid (81.60 mg/Kg), valine (50.44 mg/Kg), and phenylalanine (44.55 mg/Kg) were three main amino acids in RC. The content of aspartic acid (122.17 mg/Kg) and valine (19.35 mg/Kg) in RIT was also noticeable. So, from Table 4, we could see that amino acid content was lower in hairy fig root, and fewer amino acid varieties were detected. Yang et al. (2019) reported that sixteen amino acids were detected in Codonopsis pilosula root, the lowest content was 0.01% for methionine and the highest content was 1.19 % for arginine, which were lower in hairy fig root. But it had similar results that arginine was highest and methionine was lowest in root.

3.5.Analysis of volatile profiles

Table 5 showed the presence of a total of 47 volatile compounds in hairy fig root, including twelve aldehydes, seven alcohols, four ketones, one acids, two esters, thirteen hydrocarbons, and three other compounds. Total relative amount of volatile compounds in entire root, root cortex and root internal tissue were 65.08%, 75.68% and 58.16%. Hydrocarbons were the most abundant volatile compounds, accounting for 32.5% of all volatile compounds. However, the highest relative content of volatile compounds in ER and RIT were aldehydes, which was 34.09%

and 27.69%, respectively, while heterocyclic compounds were the highest relative content of 45.23% in RC. The second largest group of volatiles were heterocyclic compounds for ER (16.20%), aldehydes for RC (16.98%), and alcohols for RIT (13.56%). Moreover, the relative content of six compounds in three samples were consisted of hexanal, nonanal, methyl salicylate, decanal, (E)-2-decenal and ficus in with a significant difference.

Table 4. Amino acid compositions in hairy fig root

Amino Acid	Content (mg/Kg)		
	ER	**RC**	**RIT**
Histidine	16.99±0.01^{b}	13.91±0.01^{a}	18.52±0.00^{c}
Threonine	13.14±0.01^{b}	19.88±0.01^{c}	9.67±0.00^{a}
Valine	13.05±0.05^{a}	50.44±0.03^{c}	19.35±0.06^{b}
Methionine	0.27±0.02^{a}	5.05±0.05^{b}	-
Phenylalanine	24.27±0.28^{b}	44.55±0.02^{c}	12.43±0.00^{a}
Aspartic acid	110.59±0.06^{b}	81.60±0.03^{a}	122.17±0.08^{c}
Glutamic acid	4.42±0.00^{b}	7.45±0.01^{c}	0.45±0.01^{a}
Arginine	2013.51±0.24^{b}	2405.76±6.31^{c}	1853.13±27.60^{a}
Glycine	4.27±0.02^{b}	5.78±0.01^{c}	3.13±0.01^{a}
Serine	19.04±0.01^{b}	22.39±0.01^{c}	13.87±0.01^{a}
Total	2219.48±0.07	2656.81±0.65	2052.72±3.09

* "-" represented not detected, different letters in right superscript of the same line showed significant differences (P< 0.05).ER, entire root; RC, root cortex; RIT, root internal tissue.

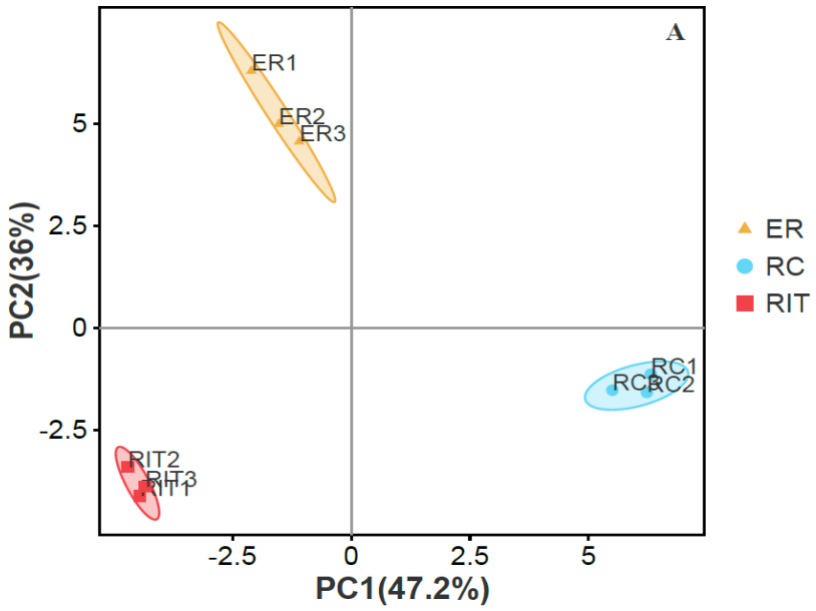
Table 5. Volatile compounds identified from the hairy fig root in GC-MS

NO.	Retention Time(min)	Compound Name	CAS#	Match	Relative Amount (%)		
					ER	**RC**	**RIT**
1	5.88	Hexanal	66-25-1	91	12.27±1.75^{c}	2.81±1.30^{a}	6.46±1.08^{b}
2	8.33	1-Hexanol	111-27-3	83	-	-	10.81±1.52
3	9.26	2-Heptanone	110-43-0	80	-	0.55±0.11	-
4	9.63	Heptanal	111-71-7	86	0.57±0.13	-	-
5	9.91	Oxime-, methoxy-phenyl-	222-86-6	86	2.43±0.56^{b}	2.02±0.33^{b}	1.41±0.21^{a}
6	10.36	Phenol, 2-ethyl-	90-00-6	80	-	-	0.30±0.03
7	12.06	Benzaldehyde	100-52-7	95	5.20±0.67^{a}	5.74±0.67^{a}	2.93±0.05^{a}
8	12.72	1-Heptanol	111-70-6	83	-	-	1.03±0.23
9	13.67	Furan, 2-pentyl-	3777-69-3	91	1.84±0.48^{a}	2.15±0.45^{a}	1.92±0.32^{a}
10	14.56	2,4-Heptadienal, (E,E)-	4313-03-5	87	1.85±0.15^{a}	2.34±0.08^{b}	1.61±0.26^{a}
11	15.47	1,3-Hexadiene, 3-ethyl-2-methyl-	61142-36-7	91	1.04±0.19^{a}	0.89±0.25^{a}	-
12	15.64	Benzyl alcohol	100-51-6	97	-	0.34±0.03	-

13	15.79	Phenylethyl Alcohol	100-51-6	94	-	1.08±0.09^{b}	0.29±0.07^{a}
14	15.97	3-Octen-2-one	1669-44-9	93	1.36±0.54^{a}	-	1.08±0.06^{a}
15	16.82	2-Octenal, (E)-	2548-87-0	91	7.30±1.60^{b}	2.62±0.36^{a}	4.17±0.09^{a}
16	17.51	3,5-Octadien-2-one	38284-27-4	93	-	0.78±0.04^{a}	2.29±0.80^{b}
17	17.67	(5-Ethylcyclopent-1-enyl)methanol	36431-59-1	94	-	-	0.35±0.07
18	19.10	Nonanal	124-19-6	93	2.29±0.13^{c}	1.50±0.24^{a}	1.89±0.36^{b}
19	20.82	trans-3-Nonen-2-one	18402-83-0	81	-	-	0.18±0.03
20	21.16	Benzene, 1,2-dimethoxy-	91-16-7	93	0.43±0.06^{a}	-	0.54±0.05^{b}
21	21.72	2-Nonenal, (E)-	18829-56-6	86	-	-	1.07±0.30
22	22.32	1-Nonanol	143-08-8	91	0.52±0.06^{a}	-	1.08±0.29^{b}
23	22.56	Naphthalene	91-20-3	95	0.76±0.24^{a}	1.60±0.15^{a}	-
24	23.56	Methyl salicylate	119-36-8	97	1.77±0.21^{b}	4.04±0.20^{c}	1.02±0.09^{a}
25	23.91	Decanal	112-31-2	91	0.65±0.04^{c}	0.43±0.02^{a}	0.54±0.05^{b}
26	24.22	2,4-Nonadienal, (E,E)-	5910-87-2	95	1.68±0.16^{b}	1.06±0.08^{a}	1.70±0.17^{b}
27	26.45	2-Decenal, (E)-	3913-81-3	80	1.13±0.18^{b}	0.48±0.07^{a}	1.42±0.07^{c}
28	27.63	Naphthalene, 1-methyl-	90-12-0	91	0.34±0.04^{a}	0.43±0.00^{b}	0.38±0.05^{ab}
29	28.87	2,4-Decadienal, (E,E)-	25152-84-5	81	-	-	5.90±1.07
30	30.82	2(3H)-Furanone, dihydro-5-pentyl-	104-61-0	90	0.69±0.01^{a}	3.46±0.31^{b}	-
31	30.9	2-Dodecenal	4826-62-4	83	1.15±0.45	-	-
32	32.29	Tetradecane	629-59-4	97	0.70±0.08^{a}	0.69±0.04^{a}	0.54±0.12^{a}
33	33.97	Seychellene	20085-93-2	97	0.36±0.05^{a}	0.45±0.08^{b}	-
34	34.36	Pentadecane, 2,6,10-trimethyl-	3892-00-0	81	0.28±0.10	-	-
35	35.08	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]-	17066-67-0	99	0.37±0.10	-	-
36	35.4	Heneicosane	629-94-7	86	0.71±0.10^{b}	0.46±0.11^{a}	0.35±0.02^{a}
37	35.52	Pentadecane	629-62-9	95	0.34±0.09^{a}	0.35±0.00^{a}	0.25±0.02^{a}
38	35.89	2,4-Di-tert-butylphenol	96-76-4	97	1.42±0.57^{a}	0.65±0.19^{a}	0.92±0.06^{a}
39	36.69	Hentriacontane	630-04-6	86	0.34±0.16^{a}	-	0.38±0.07^{a}
40	38.26	Hexadecane	544-76-3	97	0.83±0.30^{a}	0.81±0.15^{a}	0.71±0.11^{a}
41	39.68	Aciphyllene	87745-31-1	97	0.41±0.20	-	-

42	40.7	Heptadecane	629-78-7	96	0.27±0.07[a]	-	0.20±0.01[ab]
43	42.19	1-Naphthalenemethanol, 1,4,4a,5,6,7,8,8a-octahydro-2,5,5,8a-tetramethyl-	19078-37-6	83	0.28±0.02	-	-
44	43.48	Ficusin	66-97-7	98	10.55±2.14[b]	35.02±2.54[c]	2.99±0.77[a]
45	44.43	Phthalic acid, isobutyl nonyl ester	1000309-04-4	90	1.63±0.70[a]	-	0.84±0.20[a]
46	46.18	n-Hexadecanoic acid	57-10-3	98	0.15±0.02[a]	0.35±0.08[b]	-
47	47.83	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy-	484-20-8	98	0.69±0.19[a]	2.58±0.20[b]	0.39±0.18[a]
Aldehydes (12)					34.09±1.46	16.98±0.83	27.69±2.61
Alcohols (7)					0.80±0.02	1.42±0.0.08	13.56±1.00
Ketones (4)					1.36±0.08	1.33±0.07	3.55±0.13
Acids (1)					0.15±0.02	0.35±0.08	-
Esters (2)					3.40±0.54	4.04±0.46	1.86±0.22
Hydrocarbons (13)					6.75±0.09	5.68±0.06	2.81±0.08
Heterocyclic compounds (5)					16.20±3.46	45.23±5.23	6.71±1.05
Others (3)					1.85±0.01	0.65±0.19	1.76±0.06
Total					64.60±5.85	75.68±8.12	57.94±3.73

* "-" is not detected, CAS# refer to the number assigned to each chemical substance by the Chemical Abstracts Service (CAS), an organization under the American Chemical Society. The numbers in brackets after aldehydes, esters, acids, ketones, esters and pyrazines indicated the quantity of substances. Different letters in the right superscript of the same line showed significant differences (P< 0.05).



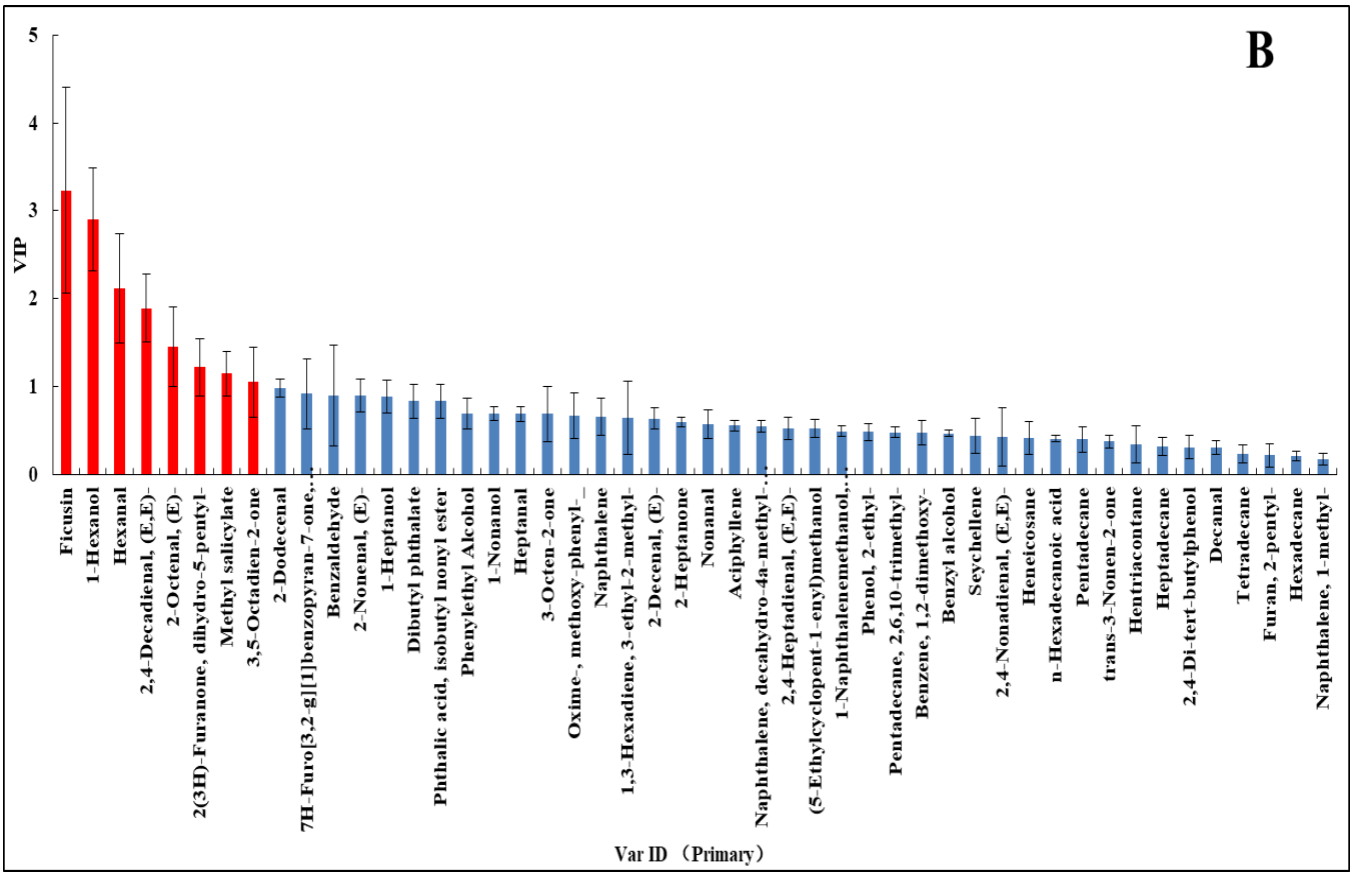


Figure 3. PCA of volatile flavour compounds in different part of hairy fig root(A) and VIP based on PLS-DA (B). The yellow,blue and red ellipse represented the 95% confidence interval in Figure 3A.

The principal component analysis (PCA) of all identified substances showed that the cumulative variance contribution rates of the first two principal components were 47.20% and 36.00%, respectively (Figure 3A). The first and second principal components expressed almost all information of the volatile components in the three samples, they could be used for the comparative analysis of volatile components between different samples. Moreover, the close relative distance between the same group of samples, indicated that the detection repetition of samples was good. The obvious spacing between different groups showed obvious difference in volatile substances among them (Figure 3A). These results pointed that GC-MS could distinguish entire root, root cortex and root internal tissue well by the difference in volatile substances. Figure 3B showed that 8 potential differential metabolites that were screened (VIP≥1, $p< 0.05$) by PLS-DA (Figure 3B), including ficusin, 1-hexanol, hexanal, (E,E)-2,4-Decadienal, (E)-2-Octenal, dihydro-5-pentyl-2(3H)-Furanone, methyl salicylate and 3,5-Octadien-2-one. Hairy fig root has special flavor, it may be related to ficusin, dihydro-5-pentyl-2(3H)-furanone and methyl salicylate, which had high relative amount. The result was similar to Liu et al. (2004) .

3.6.Infrared spectrum analysis

The FT-IR spectra of ER, RC and RIT were shown in Figure 4. The three samples showed 8 characteristic absorption peaks from 4000-400 cm-1. The strong absorption peak at 3372.43 cm-1 was assigned to the O-H vibration of cellulose and hemicellulose (Zhang, et al., 2020). The absorption peak at 2924.77 cm-1 was related to the vibration of C-H from some methylene groups of polysaccharides (Sanoussi,

et al., 2016). The absorption peak around 1736.41 cm-1 and 1630.84 cm^{-1} were indicated to C=O stretching vibration of protein and uronic acid, and the absorption peak at 1383.84 and 1244.28 represented the variable angle vibration of C-H and stretching vibration of C-O, respectively (Cao, et al., 2018). The absorption peak observed at 1026.96 cm^{-1} and 535.69 cm-1 of fingerprint area were associated with the β-glycosidic linkage of polysaccharides (Chen, et al., 2019). On the whole, the FT-IR spectra of hairy fig root were basically the same, which indicated that the organic functional groups of ER, RC and RIT were similar.

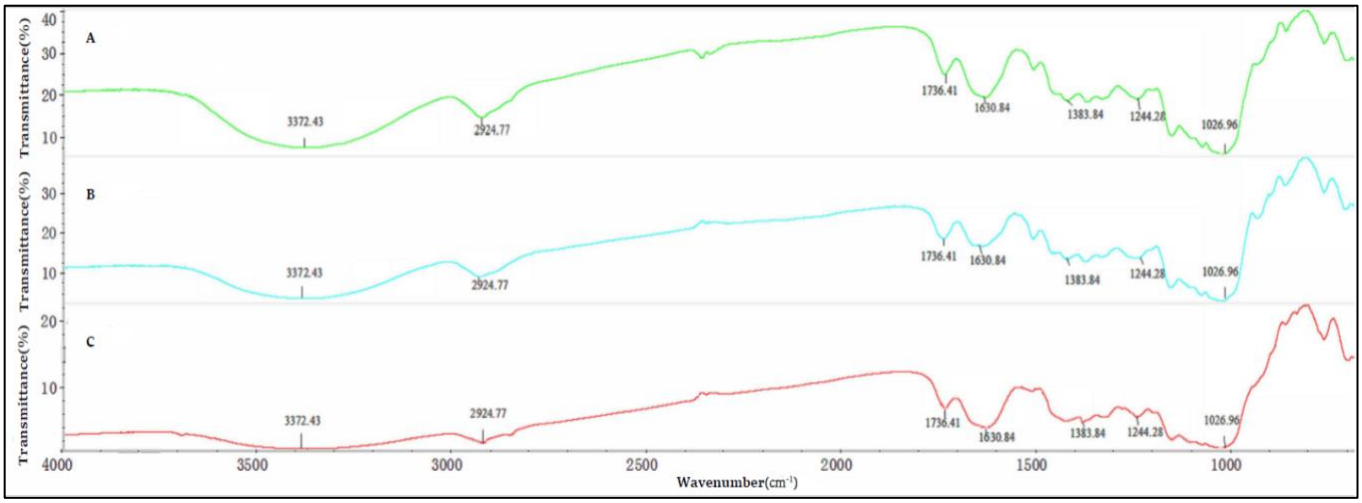


Figure 4. FT-IR spectroscopy of hairy fig root. Entire root (ER), root internal tissue (RIT) and root cortex (RC) were represented byA, B and C.

3.7.In vitro antioxidant analysis

ABTS radical scavenging activity

The radical scavenging ability measured by ABTS assay is given in Table 6 and expressed as IC_{50}. In this experiment, a lower IC_{50} value denotes a higher level of free radical scavenging action. The ABTS radical scavenging rate of ER, RC and RIT were 98.03%, 97.31% and 75.69%, respectively, at 20 mg/mL (Figure 5A). And the corresponding IC_{50} values were 4.25 mg/mL, 3.51 mg/mL and 8.77 mg/mL (Table 6). RC exhibited significantly higher free radical activities than ER and RIT.

Table 6. IC_{50} value of ethanol extract of hairy fig root on ABTS, DPPH and Hydroxyl radical

Parameters		**ER**	**RC**	**RIT**	**Vc***
IC_{50} value	ABTS	4.25 ± 0.07^{b}	3.51 ± 0.06^{a}	8.77 ± 0.35^{c}	12.03±0.01
	DPPH	0.58 ± 0.04^{b}	0.08 ± 0.02^{a}	2.29 ± 0.06^{c}	3.23±0.03
	Hydroxyl	238.47 ± 38.20^{b}	40.80 ± 2.09^{a}	1360.40 ± 380.04^{c}	141.82±1.32

The units of all results was mg/mL except for the data marked with * indicated the unit was μg/mL. Different letters in right superscript of the same row showed significant differences (P<0.05).

3.8.DPPH radical scavenging activity

In DPPH radical scavenging ability, the scavenging rate of ER, RC and RIT at the concentration of 5 mg/mL could reach to 89.57%, 91.82% and 72.60%, respectively (Figure 5B). IC_{50} values of scavenging DPPH

radicals for the ER, RC and RIT were 0.58 mg/mL, 0.08 mg/mL and 2.29 mg/mL, respectively (Table 6) and the free radical scavenging ability were in the following: RC > ER > RIT.

3.9.Hydroxyl radical scavenging activity

In hydroxyl radical scavenging test, the scavenging rate of ER, RC and RIT were the lowest among the three assays. At 50 mg/mL of ER, RC and RIT, the scavenging rate was 28.82%, 64.49 %and 20.47 %, respectively (Figure 5C). And the IC_{50} values of ER, RC and RIT were 238.47 mg/mL, 40.80 mg/mL and 1360.40 mg/mL (Table 6). The radical scavenging activities of different part of hairy fig root followed the order: RC>ER>RIT. This order was in accordance with those of TPC, TFC and bergapten, and the results was in in accordance with previous reports, which was reported that the scavenging capacity was increased by the TPC content (Zhao, et al., 2014; Dong, et al., 2015). Li et al. (2022) found that the DPPH and ABTS radical scavenging capacities were positively correlated with TPC and TFC.

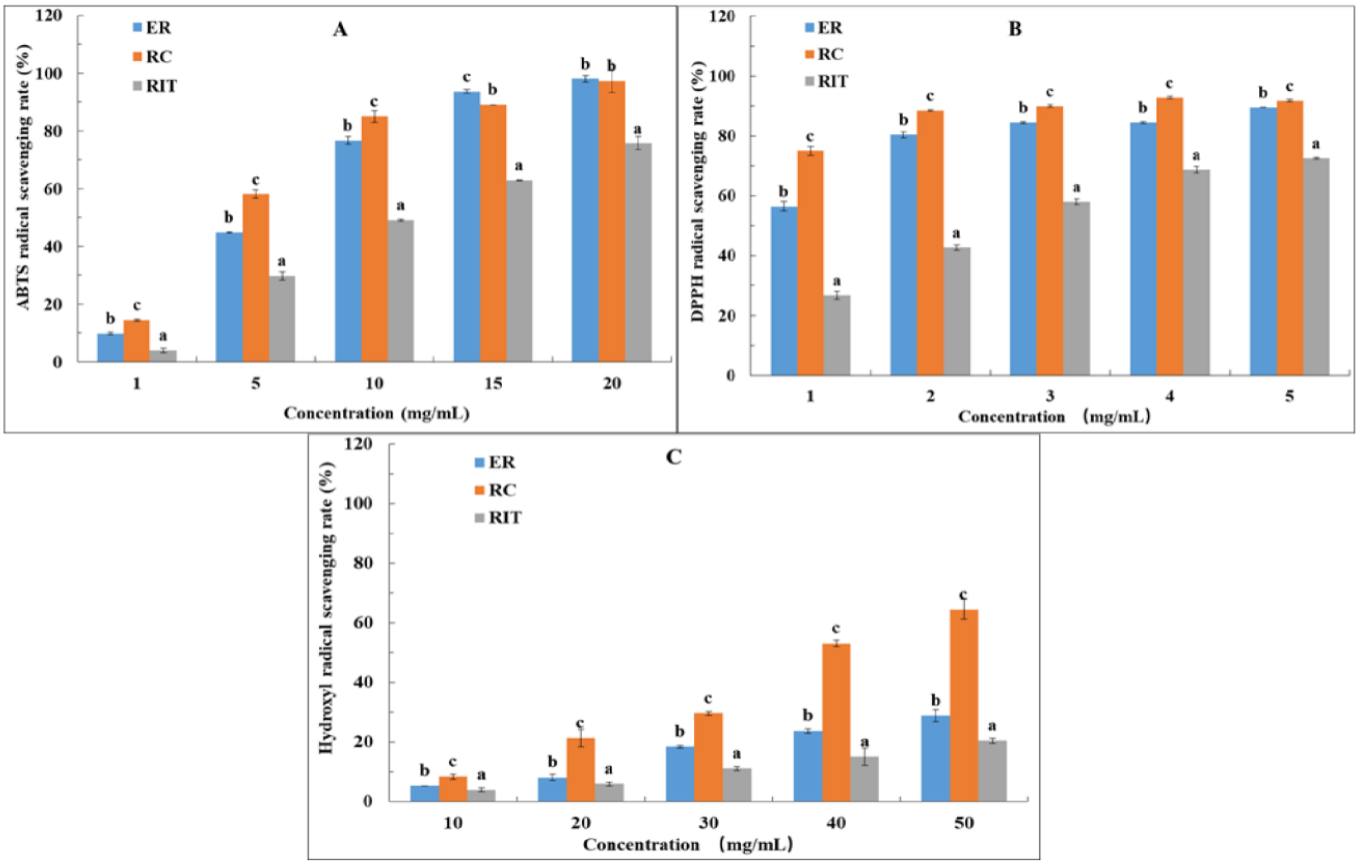


Figure 5. ABTS and DPPH, hydroxyl radical scavenging activities of ER, RC and RIT (A, B, C). The error bar are standard deviations. Different letters on the same concentration of ER, RC and RIT showed significant difference ($P<0.05$), and the significant difference was not analysis among different concentrations of each samples.

Another study emphasize that IC_{50} values of bergapten (23.98 ug/mL) was examined in DPPH assay (S ü zgeç-Selçuk and Dikpınar, 2021), which was indicated that bergapten was contributed to antioxidant activity of hairy fig root. Although, RC had high content of psoralen, but Guo et al. (2005) reported that psoralen had no antioxidant activities at 0.02% and 0.04%

levels. It was indicated that psoralen didn't make contributions to antioxidant activity of hairy fig root. As we known, hydroxyl radical model was suitable to evaluate hydrophilic antioxidants (Süzgeç-Selçuk and Dikpınar, 2021), the results might be explained that there were more hydrophilic substance in RC. So, the results might indicated that TPC, TFC and bergapten were the main components contributed to antioxidant activity.

4.Conclusions

RC and RIT are two important constituent part of hairy fig root. In this article, dry characteristics, chemical components, nutritional compositions, antioxidant activity and structure of ER, RC and RIT were analyzed. The results showed that the time of ER, RC and RIT dried to constant weight was 10, 4, 7.5 h at 80 ℃, respectively. In the whole drying process, decreasing rate drying was the main drying process with a short time constant rate drying. It was indicated that diffusion is the mechanism describing the removal of water in this process, and when hairy fig root was separate into RC and RIT, it was conducive to water diffusion, improve drying efficiency, and save energy and resources. In this study, the results demonstrated that there were more chemical composition, nutritional composition and flavor composition accumulated in RC, also had better antioxidant activity. There were significant differences among ER, RC and RIT of all components and antioxidant activity ($P<0.05$). However, the result of FT-IR was shown that there were no differences among these three samples, which was indicated no new functional group substances appeared in ER, RC and RIT, namely, the types of substances were similar, but the content was different. Results of this study were similar to that the root cortex was the main accumulation site for secondary metabolites which was reported by Aielloa et al. (2015). So, it can be concluded that RC had better quality and biological activity than RIT.

The results from this study proved that RC was the effective part of hairy fig root. It provided a basis for the study of the metabolism and accumulation mechanism of active ingredients in hairy fig root and also providing a reference for the development and utilization of hairy fig root cortex as a raw material in medicine, functional food or dietary additives. Moreover, it could address to scientific researcher, farmers, whole-salers and phyto-pharmaceutical industries that hairy fig root with a thicker cortex was needed to be plant or for sale.

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Acknowledgements

This research was supported by the Hainan Natural Science Foundation of China (321RC657 and 320MS110) and China Agriculture Research System (CARS-33-ZP3).

Conflicts of Interest:

The authors declare that they have no known competing financial interest orpersonal relationship that could have appeared to influence the work reported in this paper.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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STUDY OF ACRYLAMIDE PRECURSOR'S LEVEL IN POTATO TUBERS CORRELATED WITH ACRYLAMIDE LEVEL IN CRISPS

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https://doi.org/10.34302/crpjfst/2023.15.4.11

Article history,
Received: 15 October 2023
Accepted: 17 December 2023

Keywords,
Acrylamide,
Potato tubers,
Reducing sugars,
Asparagine,
Crisps.

ABSTRACT
Potato crisps are a popular snack due to their unique sensory properties and their nutritional value. Wide variations of acrylamide concentration in finished products are caused by different levels of precursors (asparagine and sugars) of acrylamide in the potato's tubers and the conditions of thermal process. The aim of this study was to assess the content of precursors of acrylamide from potatoes tubers, prediction of the amount of acrylamide based on precursors quantity and correlation of predicted values with the acrylamide level from crisps. The principle of the method used to measure reducing sugars and asparagine from potato tubers were based on an enzymatic reaction which is detectable by photometric measurement while the acrylamide level in potato crisps was determined using HPLC system. The relationship between concentration of precursors and acrylamide formation is very important and the prediction of acrylamide level can provide to crisps manufacturer warnings on potatoes batches in which is expected to arise high level of acrylamide concentration, thus allowing to make adjustments in process to mitigate acrylamide formation in potato crisps.

1.Introduction

Many of the food products require thermal processing. Deep-fat frying is one of the oldest and most common cooking method used in the preparation of potato crisps that creates unique texture and flavor of final product (Moreira *et al.*, 1999).

An important quality parameter for potato crisps is the acrylamide content. Acrylamide levels in foods have been subject to a benchmarking system. Based on the monitored results, the European Commission has implemented the Regulation 2017/2158 and establish benchmark levels for the reduction of the presence of acrylamide in food. For potato crisps, recommendation to mitigate the formation of acrylamide as contaminant, include selecting suitable potato varieties, controlling potato storage and transport, monitoring recipe and process conditions, and the indicative value for the presence of acrylamide was set at 750 µg kg^{-1} (parts per billion) (EU 2017/2158).

Acrylamide is formed as a result of reaction between amino acids, namely asparagine and reducing sugars, particularly glucose and fructose as part of the Maillard reaction (Mottram *et al.*,2002, Stadler *et al.*, 2002). During the heating process of potato products at high temperature (>120 °C) and low moisture conditions acrylamide is developed. The amount which is developed is depending on the applied energy (heat) and the quantity of precursors in potato (Moreira *et al.*, 1999, Tareke *et al.*, 2002).

Lipid oxidation, the Maillard reaction and the absorption of oil during deep-fat frying process play an important role in the acrylamide formation. The Maillard reaction is also known as non-enzymatic browning, which is responsible for flavor and aroma in potato crisps. As L-asparagine is the dominant amino acid in potato tissue, the acrylamide formation

depends to the levels of fructose and glucose (Biedermann *et al.,* 2002).

Sugar reduction is an important processing step, as reducing sugars as glucose and fructose together with asparagine are acrylamide precursors and can impact on its production during the deep-frying process (Knol *et al.,* 2005). Reducing sugar content is used to predict potato tuber suitability for potato crisps processing, since it is often an indicator of color development (Marquez *et al.,* 1986).

Since 2002 many researches have been done with focus on acrylamide formation and detection. Studies showed that higher levels of acrylamide in deep-fat fried products may be due to the different composition of potatoes in acrylamide precursors and also this level may be influenced by the storage condition and the conditions of thermal process (Abt *et al.,* 2019, Andačić *et al.,* 2020, Yang *et al.,* 2016, EFSA, 2015, Keramat *et al*., 2011, Becalski *et al*., 2003, Zyzak *et al*., 2003, Haase *et al.,* 2004, Palazoglu *et al.*, 2010, Williams, 2005).

Potatoes may accumulate high levels of reducing sugars during the storage conditions even though the sugar levels were accepted at harvest, therefore the contents of these precursors in potato tubers before processing are important and have to be controlled (Williams, 2005, Martinez et al., 2019).

In literature already there are an important number of strategies to reduce acrylamide (AA) level, and limiting the content of reducing sugars and asparagine is a common goal of all crisps manufacturer.

2. Materials and methods

2.1. Materials

2.2.1. Samples

36 samples from three potatoes varieties were analyzed, Hermes, Opal and Pirol. Potatoes varieties were selected for crisps production due to their low levels of reducing sugars. All the potato varieties were harvested in 2022 and were analyzed from December 2022 till February 2023. The samples were stored in a carefully controlled environment, protected for light and stored at temperature between 8 – 10 °C. For deep-fat frying the vegetable sunflower oil was used.

2.2.2. Precursor analysis

Reducing sugars (glucose and fructose) and asparagine concentrations in potatoes tubers were quantified using Konelab Arena 20 biochemical analyzer. The method is based on an enzymatic reaction which is detectable by photometric measurement. In order to determine the initial reducing sugars and asparagine a 5 kg samples were washed and blended. After blending, 50 g blended potato pulp was mixed with 50 mL of water and approximate 0.3 ml Octanol was added. The solution was homogenized after adding 4 mL of potassium hexacyanoferrate and 4 mL zinc sulfate heptahydrate. The pulp was filled into the 250 mL volumetric flask and flask stand for at least 10 minutes, then filtered. The filtrate was analyzed with Konelab Arena 20 analyzer. 5 analyses were made on each potato sample, and for results interpretation was used the average of the values. As reference material standard solution of D-fructose, D-glucose and L-asparagine from Thermo Fisher were used.

2.2.3. AA quantification in crisps

Before frying, potatoes were peeled, sliced at 1.6 mm average thickness. After peeling they were rinsed in water and the excess water from the potato sliced was removed using absorbent paper. Samples of potatoes were fried using a temperature-controlled fryer AEG FR 5548 fryer with two unites with capacity of 15 L. Potatoes were fried at temperature between 164 – 178 °C. During frying process fryer temperature (inlet and outlet) were recorded and moisture and fat content were analyzed. Sample cooled were grounded in order to analyze the AA level from potato crisps.

The AA level in crisps was determined using an Agilent 1200 model HPLC system.

Thus, 2 grams of potato chips were weighed and grounded into a fine powder, then putted into a 50 mL centrifuge tube. Over grounded sample was added 30 mL ultrapure water, samples were vortex oscillated and ultrasonic vibrated for at least 10 minutes, then centrifuged at 6000 rpm. The resultant 15 mL supernatant

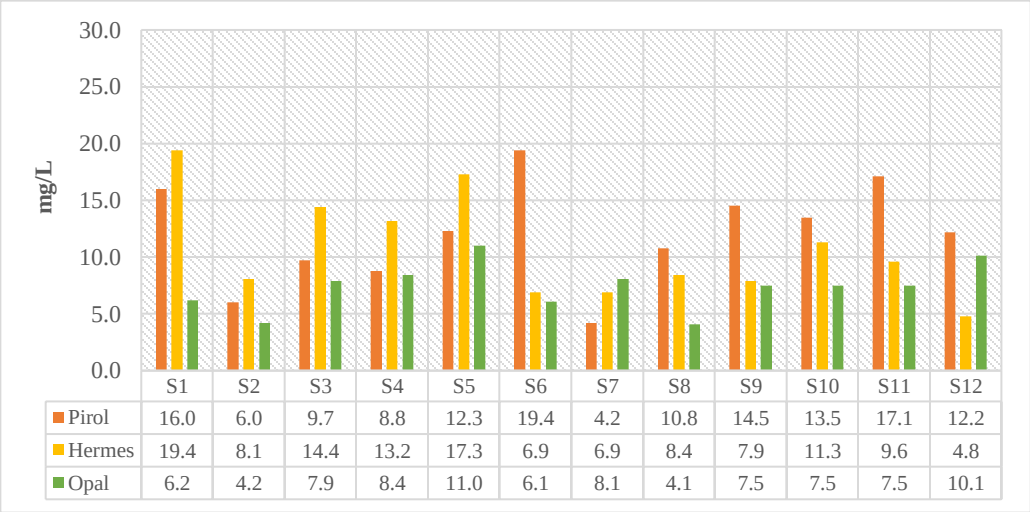
solution was collected and putted into a new 50 mL centrifuge tube mixed with 5 mL n-hexane, followed by vortex oscillated for 1 minute. Then the mixture was centrifuged at 6000 rpm for 10 minutes followed by pipette out of the upper layer of n-hexane. The centrifuge and n-hexane extraction steps were repeated one more time. The resultant 15 mL sample solution was used for determination of AA content using HPLC method. Reagents, solvents and reference materials used were analytical grade.

Controlling of reducing sugar it is a measure to reduce AA level in potato crisps. The results are given in Figure 1, Figure 2 and Figure 3.

Analyzed samples give us information on the state of potatoes samples. Reducing sugars (glucose and fructose) levels were low for all samples from the three varieties analyzed. Fructose levels ranged from 4.1 mg/L for Opal potato variety to 19.4 mg/L for Hermes variety while in case of glucose content ranged from 9.6 mg/L in case of Opal variety to 50.6 mg/L in case of Pirol variety.

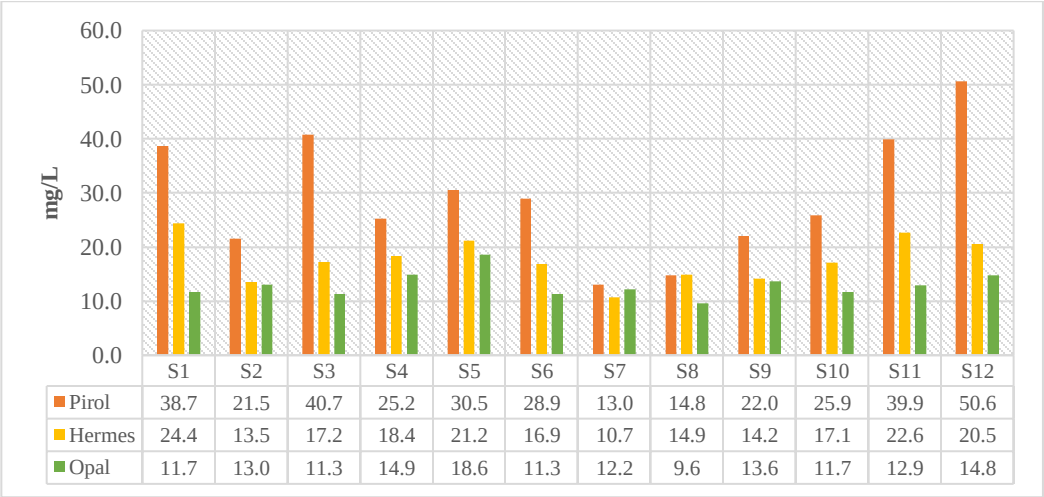
3.Results and discussions

3.1. Precursor analysis



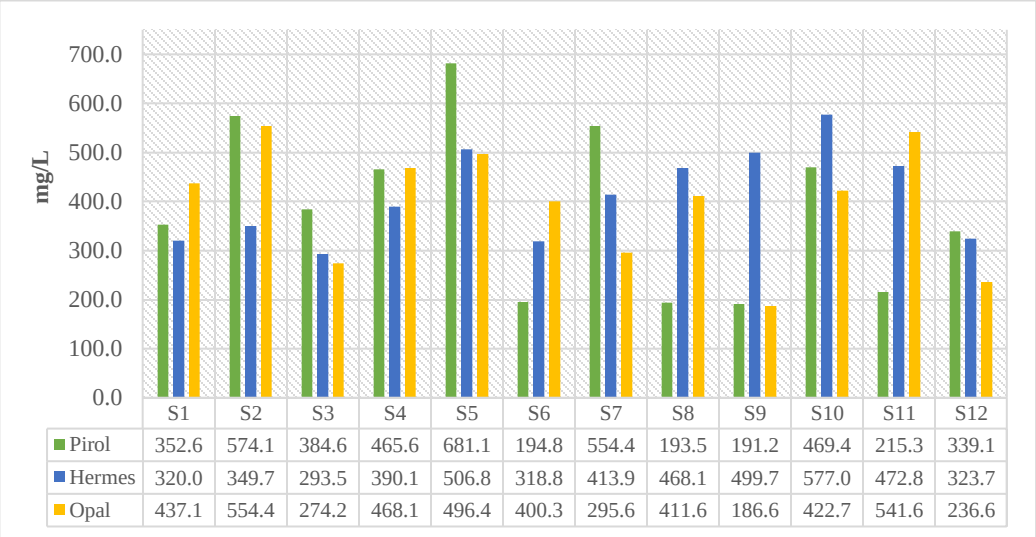
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Pirol	16.0	6.0	9.7	8.8	12.3	19.4	4.2	10.8	14.5	13.5	17.1	12.2
Hermes	19.4	8.1	14.4	13.2	17.3	6.9	6.9	8.4	7.9	11.3	9.6	4.8
Opal	6.2	4.2	7.9	8.4	11.0	6.1	8.1	4.1	7.5	7.5	7.5	10.1

Figure 1. Fructose concentration in potato tubers



	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Pirol	38.7	21.5	40.7	25.2	30.5	28.9	13.0	14.8	22.0	25.9	39.9	50.6
Hermes	24.4	13.5	17.2	18.4	21.2	16.9	10.7	14.9	14.2	17.1	22.6	20.5
Opal	11.7	13.0	11.3	14.9	18.6	11.3	12.2	9.6	13.6	11.7	12.9	14.8

Figure 2. Glucose concentration in potato tubers



	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Pirol	352.6	574.1	384.6	465.6	681.1	194.8	554.4	193.5	191.2	469.4	215.3	339.1
Hermes	320.0	349.7	293.5	390.1	506.8	318.8	413.9	468.1	499.7	577.0	472.8	323.7
Opal	437.1	554.4	274.2	468.1	496.4	400.3	295.6	411.6	186.6	422.7	541.6	236.6

Figure 3. Asparagine concentration in potato tubers

3.2. Acrylamide (AA) level in potato crisps

For AA determination the content obtained was expressed in µg kg^{-1}. The lowest concentration of AA that can be determined with an acceptable level of repeatability precision and trueness was 20 µg kg^{-1}.

The amount of acrylamide is strongly related to the moisture content of the product. Moisture content has a strong influence on crisps browning and acrylamide formation. The inside of crisp product is not as strongly exposed to high temperature and the outer layer is dried first. During frying process fryer temperature (inlet and outlet) were recorded and moisture and fat content were analyzed. Potatoes were fried at temperature between 164 – 178°C. For the analyzed samples the moisture content ranged from 1.3% to 1.8% and the fat content ranged from 30% to 39%.

The AA concentration in samples of potato crisps for each variety are shown in Figure 4, Figure 5 and Figure 6. All the crisps' samples analyzed contained AA levels higher than the limit of quantification (20 µg kg^{-1}).

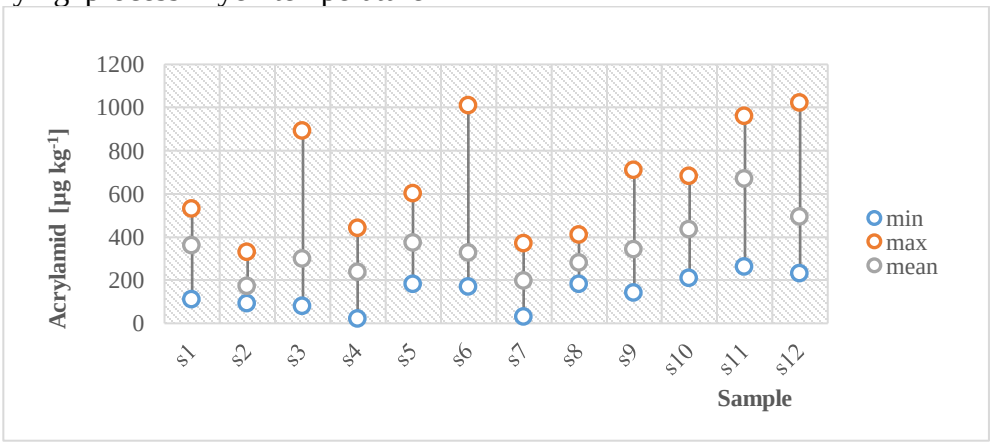


Figure 4. Concentration of AA in crisps samples from Pirol variety

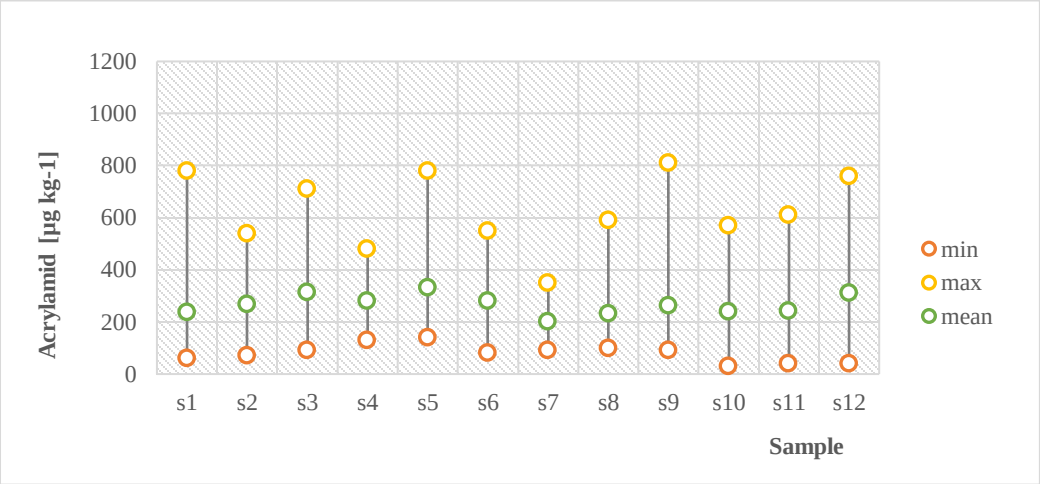


Figure 5. Concentration of AA in crisps samples from Hermes variety

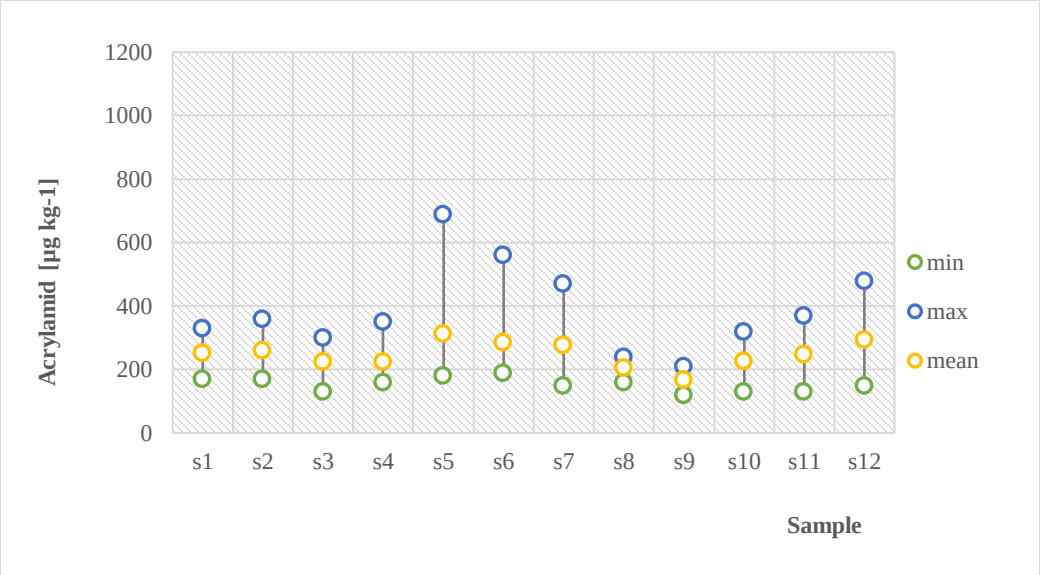


Figure 6. Concentration of AA in crisps samples from Opal variety

All the mean values obtained not exceed the acrylamide indicative value of 750 µg kg^{-1} recommended. Data distribution showed a variability in the AA content through samples and were registered some maximum values of AA (case of Pirol potato variety, samples s3, s6, s11, s12, and Hermes potato variety, samples s9 and s12) exceed the indicative level, values which may be due to the processing conditions applied.

Acrylamide level ranged from the minimum of 20 µg kg^{-1} to maximum 1020 µg kg^{-1} in case of Pirol potato crisps.

In case of Hermes potato crisps minimum value ranged from 30 µg kg^{-1} to maximum 810 µg kg^{-1}.

3.3. AA prediction and AA concentration

The reduction of AA concentration in case of potato crisps is based on reducing the precursors (reducing sugars and asparagine) present in the potato tubers. The reducing sugars level and the asparagine level may be reduced in manufacturing process by blanching the potatoes before deep frying process. The amount of acrylamide depends also by frying temperature which should be low and not higher than 175°C (EU 2017/2158) and the frying time.

The AA concentration correlated with predicted AA level in samples of potato crisps for each variety are shown in Figure 7, Figure 8 and Figure 9, where each value represents the mean.

The predicted AA level was calculated using the data presented in 3.1 for reducing sugars and asparagine from potato samples. Additional information about samples (moisture content, time and temperature of frying) was taken into account to establish a relationship with the acrylamide predicted level.

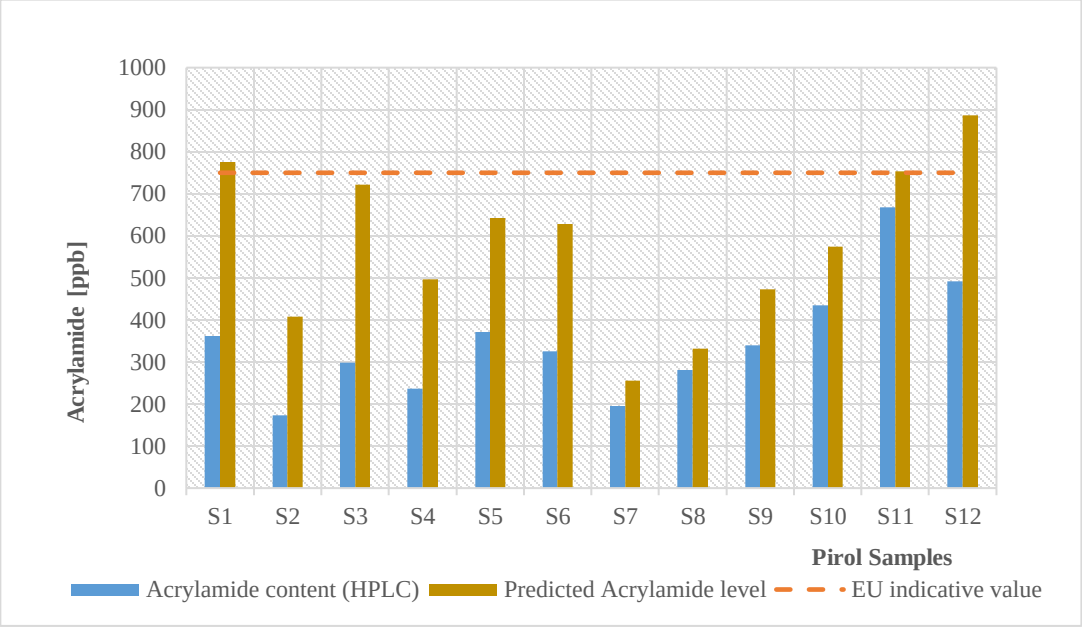


Figure 7. AA concentration and predicted AA level - Pirol variety

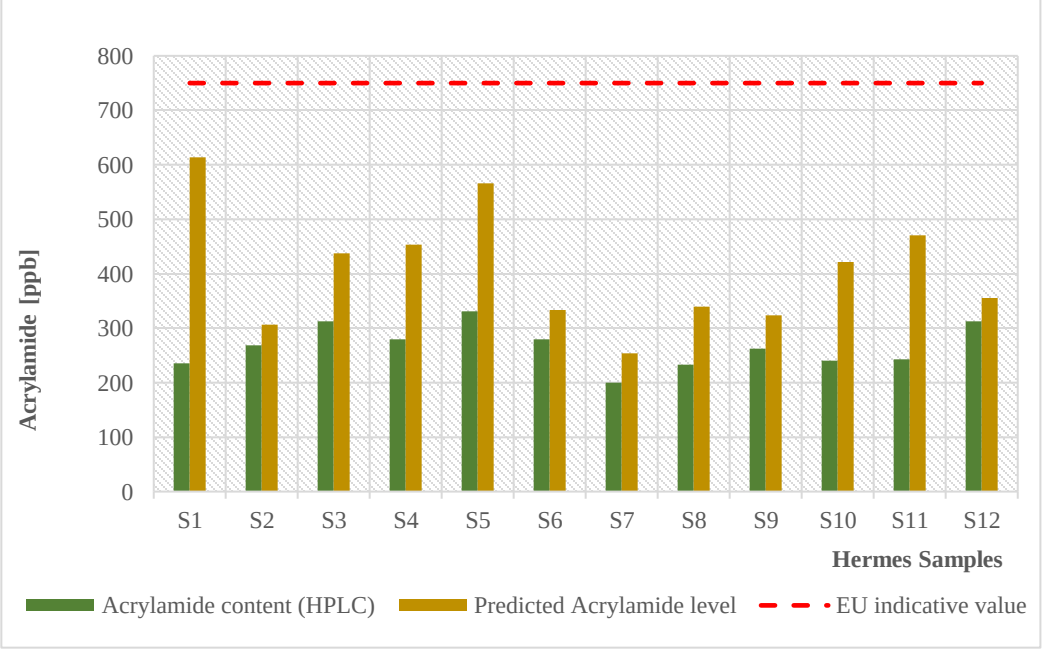


Figure 8. AA concentration and predicted AA level - Hermes variety

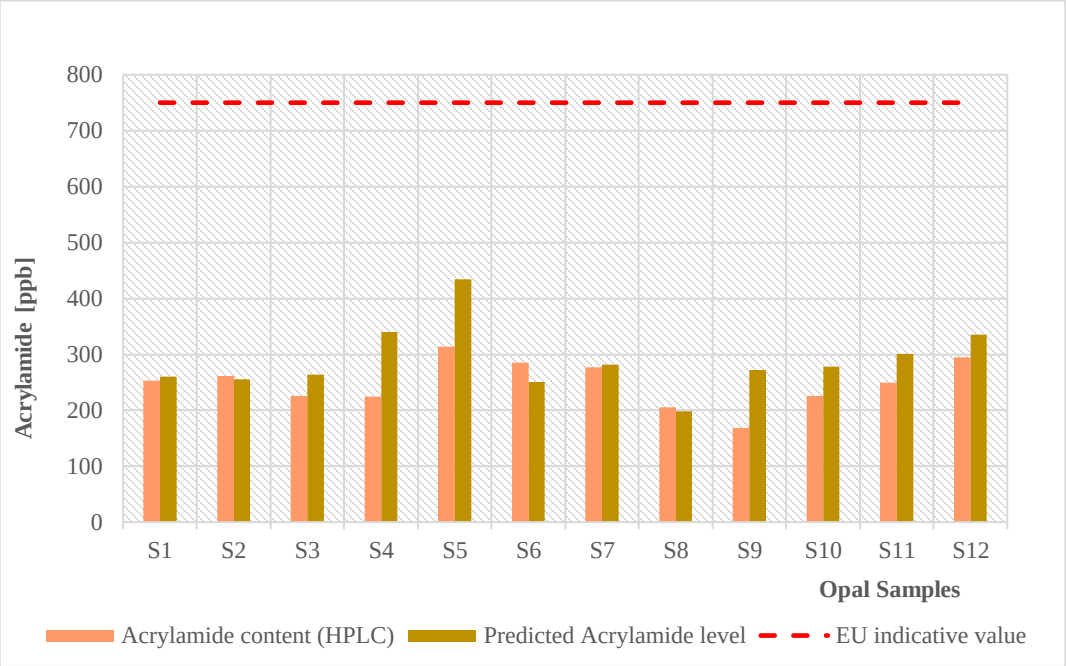


Figure 9. AA concentration and predicted AA level - Opal variety

The obtained values for the predicted AA level were higher with 5% to 20% than acrylamide concentration determinate with HPLC and this can provide warnings that characteristics of the potatoes tubers which will be used in process have an increased probability of high acrylamide in crisps, and thus this information allowing process adjustment (e.g., blanching before frying, adjustment of frying temperature, time of frying) to mitigate acrylamide formation in finished product.

4. Conclusions

Concerns related to concentration of AA in potato crisps are well known. Levels of reducing sugars and asparagine in potato tubers are the main factors contributing to the formation of AA in crisps.

For determination and detection of AA in potato crisps are available methods based on the principles of chromatography and mass spectrometry including high performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography tandem mass spectrometry (LC-MS/MS), and gas chromatography-mass spectrometry (GC-MS) (Andrzejewski *et al*., 2004, Chen et al., 2012). All these methods are used to provide information about acrylamide concentration in finished product, so the purpose of this study was to analyze the content of acrylamide precursors in potato tubers and in the case of a batch of potatoes likely to develop acrylamide to be able to adjust the process.

Potato tubers and potato crisps analyzed al laboratory scale contained no excessive amount of acrylamide. The study results showed useful information about AA prediction and can be used as a mitigation measure to prevent, predict and reduce the presence acrylamide in potato crisps.

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OPTIMIZATION OF OIL EXTRACTION FROM SOYBEAN USING AZEOTROPIC TERNARY SOLVENT MIXTURES AND CAKE ANALYSIS

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https://doi.org/10.34302/crpjfst/2023.15.4.12

Article history:
Received: 1 March 2023
Accepted: 2 December 2023

Keywords:
Azeotropic Mixture,
D-optimal Design,
Optimization,
Soybean Oil,
Ternary Solvent.

ABSTRACT

Soybean (*Glycine max*) sample was subjected to solvent extraction with an azeotropic ternary solvent mixture (5-10% water, 5-10% ethanol, and 80-85% ethyl acetate) optimised based on D-optimal Design (DOD) under the Mixture Methodology of the Design Expert (7.0.1). The azeotropic solvent mixture developed was 9.17%, 6.67%, and 84.17% of water, ethanol, and ethyl acetate, respectively, with a 15.56 % yield of soy oil. The extraction suited a Quadratic model and the Analysis of Variance (ANOVA) indicate a Correlation Coefficient (R^2) of 0.9921. The Refractive Index, Fatty acid, as well as Acid, Saponification, Iodine and Peroxide values of the Soy oil, are 1.454, 8.39, 16,3, 56.12, 15.17 and 27.00, respectively. Moisture, ash, fibre, lipid, crude protein and carbohydrate contents of the defatted soybean cake are 16.75, 4.85, 5.00, 2.60, 31.54 and 39.86 %, respectively. The optimised solvent mixtures demonstrated suitable performance for the safe extraction of oil from soybean

1.Introduction

Soybean is a leguminous plant seed, mainly cultivated for its oil and protein content (Lawson *et al.*, 2010). Oil from soybean have been obtained through various processes and techniques such as mechanical pressing, solvent extraction, supercritical fluid extraction (Koubaaa *et al.*, 2016; Rai *et al.*, 2016), compressed fluid extraction (Coelho *et al.*, 2016), microwave and ultrasound-assisted extraction (Araujo *et al.*, 2013) have been proposed for effective oil extraction from oilseeds. The solvent extraction process usually leads to lower turbidity and higher yield oil, compared to the mechanical process (Sulaiman et al., 2013), although the residual solvent after the extraction has attracted attention (Agu 2014).

Common solvents used for the extraction process include hexane and its isomers, petroleum ether, ethanol, butanol, and other organic solvents (Araromi *et al.*, 2017). Long and Abdelkader (2011) employed five solvents to extract lipids from *Nannochloropsis* microalgae. The solvent ratios were combined as hexane/cyclohexane (1:1), cyclohexane/2-propanol (2:1), hexane/2propanol (3:1), and cyclohexane/1- butanol (9:1), to be equivalent azeotropic mixture ratio, based on mole fractions established in the Azeotrope databank (Ponton, 2001). Scanty information exists in the literature on the use of an azeotropic mixture for

oil extraction for oilseeds particularly, soybean. This study is set to combine three solvents (water, ethanol, and ethyl acetate) at and near azeotropic conditions and then investigate the efficiency of the mixture in extracting oil from the soybean sample.

Various studies have been conducted on the optimization of the solvent extraction of edible and non-edible oils from various oilseeds. Sayyar *et al.,* (2009) optimized five factors for the extraction of oil from Jatropha seeds using n-hexane and petroleum ether. Mampouya *et al.*, (2013), optimised the Soxhlet extraction of oil from Safou Pulp using trichloroethane, chloroform, hexane, and petroleum ether as a solvent. Bokhari *et al.,* (2014) optimised selected parameters that affect the solvent extraction of crude rubber seed oil using response surface methodology (RSM). Dagostin *et al.,* (2015) studied the application of a mixture of alkyl esters and ethanol for Soybean (SB) oil extraction, while Glendara and Joenes (2015) optimised time and temperature that influenced the liquid extraction of SB oil using a binary mixture of ethanol and hexane. Adeyanju *et al.*, (2016) employed RSM to optimise the operating condition for the extraction of coconut oil. Our extensive literature search indicated that no work related to the use of an azeotropic solvent mixture for the extraction of soy oil from soybean has been conducted. This study specifically, employed D-Optimal Design under the Mixture Methodology of the Design Expert (7.0.1) to optimize the mixture of water, ethanol, and ethyl acetate solvents for the effective extraction of soy oil from soybean.

2.Materials and Method

2.1 Materials and Sample Preparation

The Soybean (SB) (*Glycine Max (L*) sample was obtained in the Market and authenticated at Forestry Research Institute of Nigeria (FRIN) Jericho, Ibadan, Nigeria. The SB sample was cleaned of chaff and other foreign materials and later oven-dried at 50 °C for 24 h to constant moisture content. The dried SB sample was dry-blended and sieved to uniform particle size (0.2 mm) (Araromi *et al.,* 2017). All the reagents used in this study were of analytical grade and obtained from designated suppliers.

2.2 Solvent Extraction

2.2.1. Soxhlet extraction of oil from SB

The Soxhlet apparatus was set up accordingly with much care to avoid leakages, then 300 mL of the solvent (water, ethyl acetate, and ethanol) mixture was mixed with 50 g of the SB sample and then heated to the azeotrope temperature (69.9 °C) of the mixed solvents for 4 h. The resulting solution was evaporated in a rotary evaporator to obtain pure Soy oil (Araromi *et al.,* 2017), which was cooled in a desiccator and then weighed. The oil yield was determined from Eqn. 1, based on the average of the three replicates of the experiment (Lawson *et al.,* 2010).

$$oil\ yield = \frac{weight\ of\ extracted\ oil}{weight\ of\ soybean\ fed\ in}\ X\ 100 \quad (1)$$

2.2.2. Optimization of ternary solvent mixture for extraction of Soy oil

The three solvent (water, ethanol, and ethyl acetate) mixture was optimized using D-optimal Design under the Mixture Methodology of the Design Expert (7.0.1) software. The component levels (5-10% water, 5-10% ethanol, and 80-85% ethyl acetate), (Table 1), based on mole fractions in the Azeotrope databank (Ponton, 2001) were fed into the software. The experimental runs generated by the software were subjected to the Soxhlet extraction process and the corresponding oil yield was documented. The data obtained were subjected to Analysis of Variance (ANOVA) using statistical tools embedded in the software. Three-dimensional plots and their respective contour plots were obtained based on the effects of the levels of the three components. The effects of the interaction of the three components on the response were studied. The significance of the model equations and their terms were evaluated using statistical tools such as coefficient of determination (R^2), Fisher value (F-value), probability (P-value), and residual (Mohammad *et al.,* 2014).

Table 1. Selected Factors and their Levels for the Central Composition Design (CCD)

Factors	Units	Levels	
		Low	High
Pressure (P)	N/m^2	1.0	2.50
Toasting time (T)	Min	30.00	60.00

The R^2 reveals the efficiency of the experiment, and thus, is expected to be very high (≈ 1), while the Adjusted R^2 (Adj R^2) and predicted R^2 (Pred R^2) were generated for the adjusted and predicted values by the software, respectively. Consequently, a suitable model for the optimization was characterized by the highest R^2 but not 'Aliased', lowest standard deviations, and the smallest differences between the generated Adj R^2 and Pred R^2. The Predicted Residual Error Sum of Squares (PRESS) measures the degree to which the model developed is likely to predict the responses in new experiments and, thus, it is desirable to have small values of PRESS (Montgomery, 2001).

2.3. Characterisation of Physicochemical Properties of Soy oil Samples

2.3.1 Acid value

The acid value and acidity of Soy oil were determined according to ISO standard 660. Alcohol (a mixture of 1/1(v/v) of 95% ethanol and diethyl ether) (25 mL) neutralized just before use with 0.1 M KOH solution in the presence of 3 drops of phenolphthalein was added to 0.5 mL of the Soy oil sample in a 250 mL conical flask. The flask was swirled for 2 mins, followed by the addition of 3 drops of indicator, and then the mixture was titrated against 0.1 mg/L solutions of the ethanolic potassium hydroxide until a permanent pink colour was attained (Amos-Tautua and Onigbinde (2013).

2.3.2. Peroxide value

The peroxide value of the Soy oil was determined using the ISO standard 3960. The soy oil (0.5 g) was dissolved in a solvent mixture of acetic acid and titrated against 0.05 M sodium thiosulphate using starch as an indicator (Amos-Tautua and Onigbinde (2013).

2.3.3. Iodine value

The iodine value (IV) was determined according to ISO standard 3961. The soy oil sample (0.2 g) was dispensed in a round neck bottle and mixed with chloroform (5 mL) and Wijj's reagent (8 mL), iodine trichloride (9 mL), and 10 g of iodine in chloroform (300 mL) /acetic (700 mL) solution. The bottle was shaken gently and placed in the dark for 1 h after which 7 mL of KI (100 g/1) and 75 mL of distilled water were added and titrated against 0.05 M sodium thiosulphate solution using starch as the indicator. A blank test was carried out simultaneously without the oil under the same conditions.

$$I.V = \frac{[(\text{Blank Titre} - \text{Sample Titre (mL)}] \times 0.01269}{mass\ of\ Sample\ ()g} \times 100 \quad (2)$$

2.3.4. Free Fatty Acid

The free fatty acid (FFA) content of the Soy oil extracted was determined using Eqn. 3 (Chai *et al.,* 2014) and this involves the use of the relationship that relates FFA to the acid value (AV) of an oil sample.

$$The\ free\ fatty\ acid\ (FFA) content = AV/\ 2 \quad (3)$$

2.3.5. Saponification value

The Saponification Value (SV) of the Soy oil sample was determined according to ISO standard 3961. The soy oil sample (1 mL) was poured into a conical flask and 25 mL of 0.1 M of ethanolic KOH was added to it. The mixture was boiled for 30 mins under reflux. Phenolphthalein (3 drops) was added to the warm mixture and titrated against 0.5 M HCl acid until the pink colour disappeared (endpoint). The same procedure was

administered to the blank sample and the SV was calculated from Eqn. 4 (Amos-Tautua and Onigbinde (2013).

$$\text{Saponification value } (mg/g) = \frac{(\text{BLI} - \text{EPI})}{\text{S}} \times \text{TF} \times \text{Cl} \times \text{K1} \quad (4)$$

Where EPI is Titration volume (mL), BLI is Blank level (25.029 mL), TF is Reagent (HCl) factor (1.006), Cl is concentration conversion coefficient (28.05 mg/mL), Kl is Unit conversion coefficient (1) and S is the Sample size (g).

2.3.6. Refractive Index

The refractive index of the extracted Soy oil samples with the azeotropic solvent was determined based on the relationship between refractive index and iodine value (IV) as proposed by Pekins (1995) (Eqn. 5).

$$\text{Refractive Index} = 1.45765 + 0.0001164 \times (\text{IV}) \quad (5)$$

2.4. Determination of Physicochemical Properties of Defatted Cake Samples

2.4.1. Moisture content

The cake sample (5 g) was weighed into pre-weighed aluminium drying dishes and dried to constant weight in an oven (MEMERT) at 50 ^{o}C for 24 h. (Lui *et al.,* 2013). The moisture content was determined and calculated as follows

$$Moisture\ content = \frac{M_1 - M_2}{M_1 - M_0} \times 100 \quad (6)$$

Where M_0 is the weight of the aluminium dish, M_1 is the weight of the fresh sample + dish, and M_2 is the weight of the dry sample + dish.

2.4.2. Ash content

The cake sample (5 g) was weighed into a porcelain crucible previously ignited and weighed. Each seed sample was charred by igniting the materials on a hot plate in a fume cupboard. The crucible was placed in a muffle furnace and maintained at 600 ^{o}C for 6 hr. The resulting materials were cooled in a desiccator and later re-weighed to determine their percentage ash content according to Eqn. 7 (Lui *et al.,* 2013).

$$\%\ Ash = \frac{(crucible\ weight + Ash) - (Empty\ crucible\ weight)}{Sample\ weight} \times 100\% \quad (7)$$

2.4.3. Crude fat

The cake sample (5 g) was placed in thimbles and plugged with cotton wool into a Soxtec System (HT2). The extraction cup was dried and weighed, and then 25 mL petroleum ether was added into each cup, which was inserted into the Soxtec system for 45 minutes in a rising position (Lui *et al.,* 2013). The percentage of fat in the sample was calculated according to Eqn. (8).

$$\%\ Fat = \frac{W_3 - W_2}{W_1} \times 100\% \quad (8)$$

Where $W_1$the weight of the sample, W_2 is the weight of the empty cup and W_3 is the weight of the sample and the cup.

2.4.4. Carbohydrate

The total percentage of carbohydrate content in the cake sample was determined by the different methods as recommended by (Lui *et al.,* 2013). This method involved the subtraction of the sum of crude protein, lipid, crude fibre, moisture, and ash constituent values from 100 (Eqn. 9). The value obtained is the percentage carbohydrate constituent of the sample.

$$\%\ Carbohydrate = 100 - (Moisture + Ash + Crude\ fiber + Crude\ protein + Fat) \quad (9)$$

2.4.5. Crude protein

The Kjeldahl nitrogen method was used to quantify the protein content of the cake sample. The sample (5 g) was introduced into the digestion flask and five Selenium tablets of Kjedahl catalyst were added to the sample in which 20 mL of concentrated acid was added and then digested for 8 h until a clear solution was obtained. The cooled digest was transferred into a 100 mL volumetric flask and made up to mark with distilled water.

The distillation apparatus set was rinsed for 10 min by boiling and 20 mL of 4% Boric acid was pipetted into a conical flask, then 5 drops of methyl red were added to each flask as an indicator. The sample was diluted with 75 mL of distilled water and 10 mL of the digest was made

alkaline with 20 mL of NaOH (20%) before distilling. The Boric acid solution was changed to green and the mixture was further distilled for 15 min, then the filtrate was titrated against 0.1N HCl (Lui *et al.*, 2013). The percentage of total nitrogen was calculated as:

$$\%\,Total\ Nitrogen = \frac{Tire \times Normality \times 0.014}{Sample\ weight} \times 100\% \quad (10)$$

$$Crude\ protein = \%Total\ Nitrogen \times Conversion\ fat \quad (11)$$

Normality = 0.14, Conversion Factor = 6.25

2.4.6. Crude Fiber

The crude fiber of the cake sample was determined using the method of (Lui *et al.*, 2013). The cake sample (5 g) was placed in a filter crucible, which was inserted into the hot extraction unit (Hot Extractor, Model-1017). A sufficient amount of pre-heated 0.128 M H_2SO_4 was added to the reagent in the heating system and few drops of ethanol were added through the valves. The mixture was digested for 30 min, then washed with boiling water and filtered to remove the acid content. The residue in the flask was boiled with the required amount of 0.223 M KOH for 30 mins and then filtered with subsequent washing in boiling water and acetone. The residual content was oven-dried at 105 °C and then ignited in the muffle furnace at 550 °C for 3 h. It was transferred to a desiccator and weighed as W_1, then burnt in a muffle furnace at 500 °C for 6 h., allowed to cool and reweighed as W_2. Then percent crude fibre was calculated from Eqn. 12.

$$Percentage\ Crude\ Fibre = \frac{W_1 - W_2}{W_0} \times 100 \quad (12)$$

3. Results and Discussion

3.1 Design Matrix Evaluation for Mixture Quadratic Model

The Std. Dev. obtained for the available models (Linear, Quadratic, Special Cubic, and Cubic), embedded in the software are 1.73, 0.31, 0.35, and 0.000, respectively, while the corresponding R^2 was 0.5644, 0.9921, 0.9924, 1.0000 (Table 2). Similarly, their Adj R^2 was 0.4400, 0.9821, 0.9771, and 1.0000, but only Linear, Quadratic, and Cubic had Pred R^2 values of 0.1679, and 0.8352, and 0.5954, respectively, while their PRESS was 39.97, 7.92 and 19.43, respectively. The cubic model demonstrated the highest R^2, lowest Std dev., and minimum deference (0.000) between its Adj R^2 (1.0000) and Pred R^2 (1.0000). However, the ed that it was 'aliased' and as a result may need to be discarded as a suitable model for the study (Aremu *et al*, 2019). The option of the quadratic model for the study was buttressed based on its relatively high R^2 (0.9921), a small difference (0.1469) between the Adj R^2 (0.9821) and Pred R^2 (0.8352), smallest PRESS value of 7.92, and low standard deviation (0.31) of the data obtained to the mean values (Montgomery, 2005).

Table 2. Model Summary Statistics for the Responses from Ternary Mixture developed

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	Adj R^2 and Pred R^2 Differences	PRESS
Linear	1.73	0.5644	0.4400	0.1679	0..2721	39.97
Quadratic*	0.31	0.9921	0.9821	0.8352	0.1469	7.92*
Special Cubic	0.35	0.9924	0.9771	0.5954	0.3817	19.43
Cubic^	0.000	1.0000	1.0000	ND	ND	ND

* Suggested, ^ Aliased, ND- Not Define

3.2. Responses from experimental data

Run 6 with 9.17%, 6.67%, and 84.17% (Water, Ethanol and Ethyl acetate) mixture, gave the highest Soy oil yield of 15.56 %, while Run 5 (7.50% Water, 10.00% Ethanol and 82.50% Ethyl acetate) gave the least Soy oil yield (9.15%), respectively (Table 3). The yield from this study is higher than 14.51% and 15.2% reported for the use of petroleum ether and ethanol by Amos-Tautua and Onigbinde (2013)

and Dagostin *et al.*, (2018), respectively, for the extraction of soy oil from soybean, but less than 18.8% and 21.6% obtained from the use of 5:95 and 10:90 ethanol: ethyl acetate mixtures by Dagostin *et al.*, (2018). However, the use of pure ethanol or a high proportion of ethanol in solvent mixtures for oil extraction is affected by issues of solubilization of the extracted oil (Baumler *et al.*, 2016). The diagnostic case studies of the soy oil yield response (Table 3) indicate that Runs 1, 2, 3, 7, 8, and 10 gave positive residual values while Runs 4, 5, 6, 8, and 9 gave negative residual values.

Table 3. Result of Response (Soybean oil yield) from Experimental data

Run	Components			Response
	A: Water (%)	B: Ethanol (%)	C: Ethyl acetate (%)	Soybean oil yield (%)
1	5.00	10.00	85.00	14.14
2	10.00	5.00	85.00	12.82
3	10.00	10.00	80.00	10.26
4	9.17	9.17	81.67	10.05
5	7.50	10.00	82.50	9.15
6	9.17	6.67	84.17	15.56
7	7.92	9.17	82.92	12.25
8	6.67	9.17	84.17	14.88
9	10.00	7.50	82.50	10.05
10	10.00	10.00	80.00	10.26

3.3 Analysis of Variance (ANOVA) and Regression statistics

The Soy oil yield has a Model F-value of 100, (Table 4), which implies a significant model, with only a 0.03 % chance of occurrence due to noise. The linear mixture components and model terms (AB, AC, and BC) are significant with p-values of 0.0002, 0.0007, and 0.0137, respectively, which is less than 0.05 ($p<0.05$). R^2 obtained for soy oil yield response was 0.9921 while the Adj R^2 value was 0.9821 (Table 4) and the closeness of these values implies that there is a good correlation between observed and predicted values in the model (Anbia and Amirmahmoodi, 2016; Khani *et al.*, 2016). The adequate precision is 25.882, which is greater than 4, thus indicating an adequate signal of the model, applications (Montgomery, 2005). The PRESS is 7.92, which shows the suitability of the model in predicting the responses in new experiments however, small values are desirable (Montgomery, 2005). The Coefficient of Variations (CV) obtained for this model is 2.59, which is less than 10 percent and suggests a high tendency of reproducibility of the model (Agarry and Ogunleye, 2012). Low CV and SD indicate the accuracy with which the experiment was conducted and high precision in predicting the Soy oil yield.

Table 4. Analysis of Variance (ANOVA) for Soybean oil yield response for the development of Ternary Mixture

Source	Sum of Squares	Df	Mean Square	F Value	p-value (Prob > F)
Model	47.65	5	9.53	100.00	0.0003*
Linear Mixture	27.11	2	13.56	142.24	0.0002*
AB	16.44	1	16.44	172.50	0.0002*
AC	8.29	1	8.29	87.04	0.0007*
BC	1.68	1	1.68	17.61	0.0137*

Residual	0.38	4	0.095		
Lack-of-Fit	0.38	3	0.13		
Pure Error	0.000	1	0.000		
Cor Total	48.03	9			

NA- Not Applicable, *Significant at 0.05< (prob>F) < 0.1

3.4. Model equations of responses for the development of Ternary Mixture

The coded and actual terms as generated by the software are represented in the positive and negative coefficients of a model quadratic equation (Eqn. 13) indicating the positive and negative effects of the independent variables on the selected responses (Alade *et al.,* 2012). The coefficients +22.94, +1.71, and +2.60 obtained for model terms A (water), B (Ethanol), and C (ethylacetate) indicate that soy oil yield was highly influenced by the three solvents used (Araromi *et al.,* 2017). Similarly, the coefficient obtained for the mixtures of water and ethanol has a positive coefficient of 1.29 and this suggests that the mixture of the two solvents has a stronger influence on soy oil extraction. The negative coefficients of AC (α_{13}= -0.48) and BC (α_{13}= -2.3) indicate that the combined components were not as effective as the mixtures of water and ethanol components (Aremu *et al.,* 2019). It has been suggested that mixed polarity solvent systems with alcohol usually gave better overall oil yields than purely hydrocarbon-based solvents for extractions (Long, and Abdelkader, 2011).

$$Soy\ oil\ yield = 22.94A + 1.71B + 2.60C + 1.29AB - 0.48AC - 0.23BC \quad (13)$$

Where A is the coded variable for water, B is the coded variable for Ethanol and C is the coded variable for Ethyl acetate. α_0 is the intercept term, α_1, and α_2, are the influences of A and B on the process while α_{12} is the combined effect of A and B on the process.

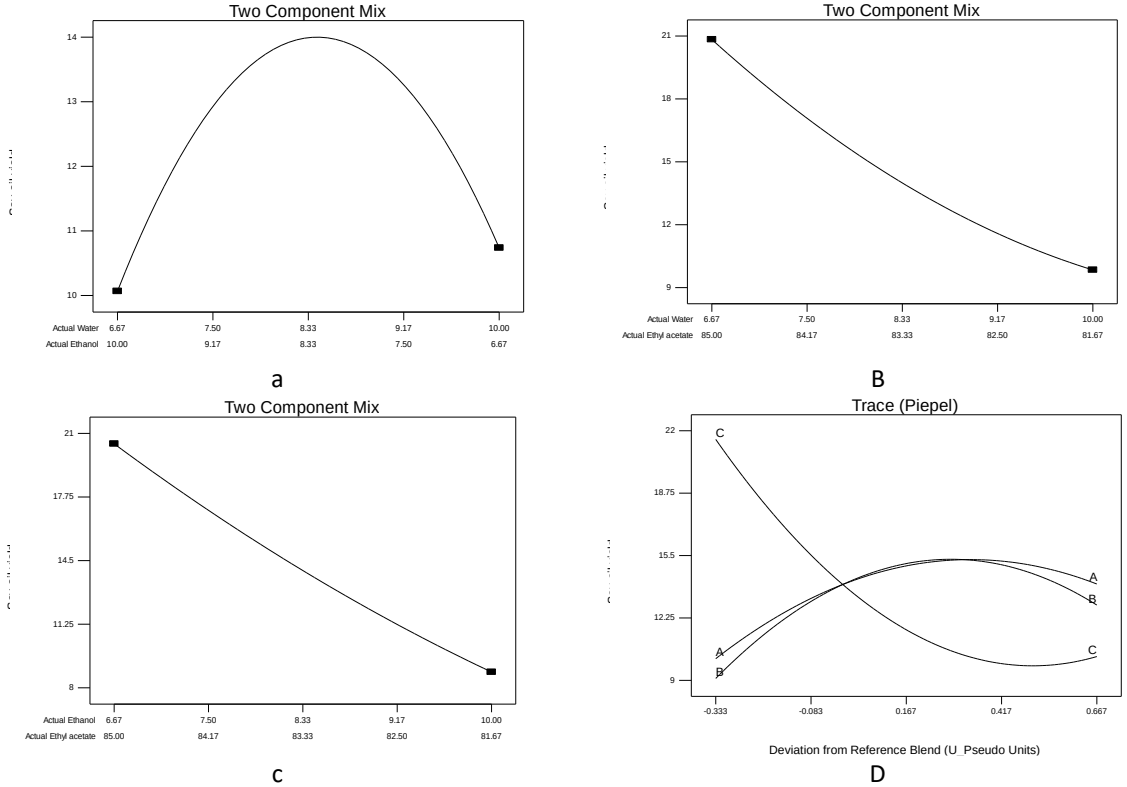

Figure 1. Plot of Two-Component Mix and Trace (Piepel) for the solvent mixtures

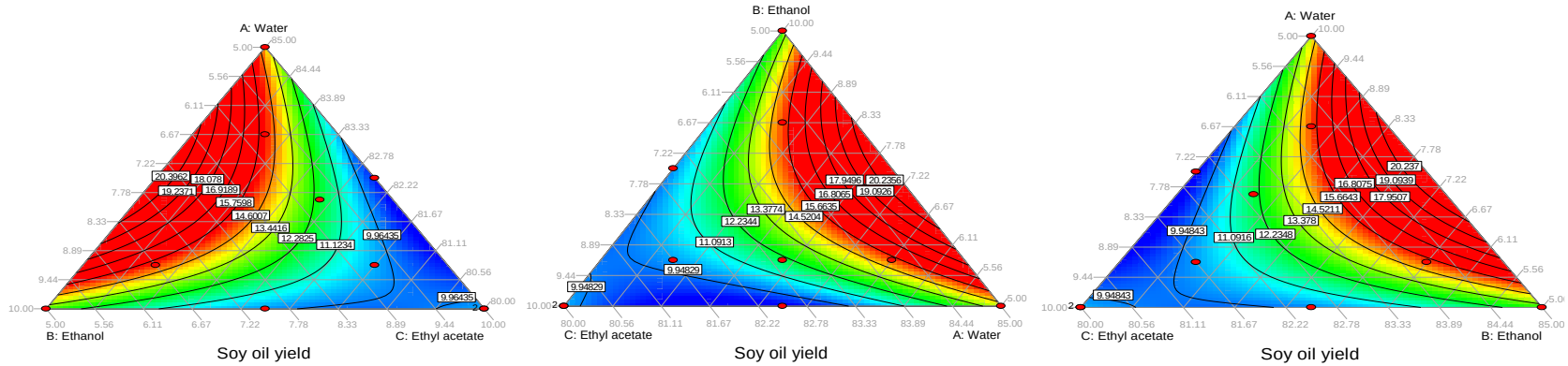

Figure 2. Contour plot of Soybean oil yield

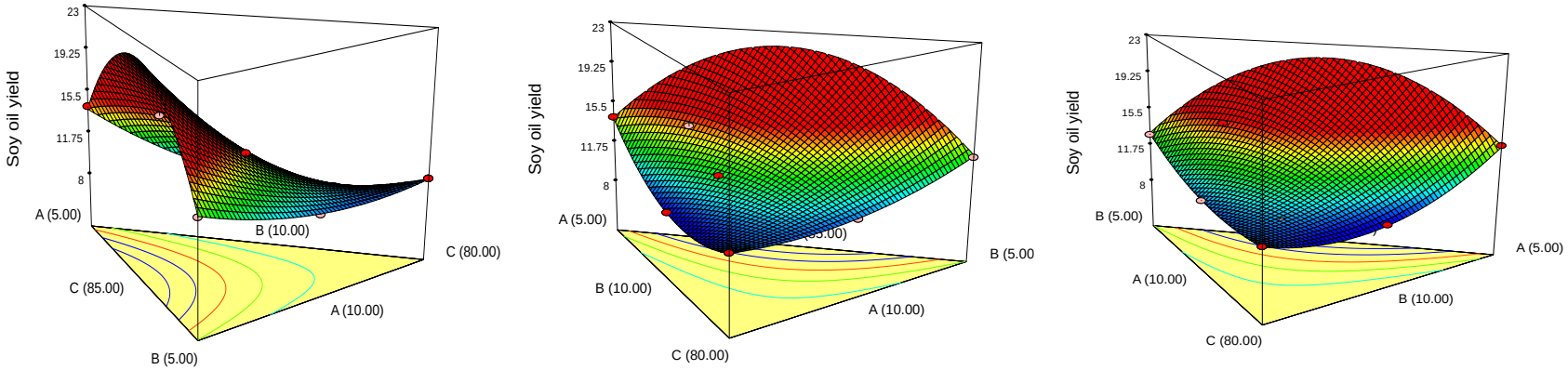

Figure 3. 3D plot of Soybean oil yield

3.5. Model graphs for the response to the development of Ternary Mixture

The interaction between water and ethanol components (Figure 1a) showed that soy oil extraction increased from 10 to 14% yield with increased volume of water and decrease the volume of ethanol until 8.33 % and 8.33% volume of the two components was reached at the maximum, at constant ethyl acetate volume (83.33 %) and then decreased as the volume of water increased to 10% while the volume of ethanol decreases to 6.67 %. This development suggests that an equal amount of water and ethanol may be effective in a ternary mixture involving ethyl acetate for the effective extraction of soy oil from its seed. The interaction between water and ethyl acetate components (Figure 1b) showed that soy oil extraction decreased from 21% to 9% with increased % volume (6.67 to 10) of water and decreased volume (85 to 81.67 %) of ethyl acetate, at constant ethanol volume (8.33 %). It could be observed that a ternary mixture involving a high volume of ethyl acetate and a low volume of water, in an azeotropic mixture with ethanol, is effective for the extraction of soy oil.

The interaction between ethanol and ethyl acetate components (Figure 1c) showed a similar trend observes in Figure 1b. Soy oil extraction decreased from 20% to 8% with increased volume (6.67 to 10 %) of ethanol and decreased volume (85 to 81.67 %) of ethyl acetate at constant water volume (8.33 %). The combined effect of this development is well illustrated in the trace (Piepel) diagram (Figure 1d) where curves A, B, and C represent the water, ethanol, and ethyl acetate components. The graphical illustration selected 8.33%, for each component.

The 3-D plots (Figure 2-3) indicate a quadratic model due to the curvy nature of the graph as also indicated in their contour plots (Alade *et al.,* 2012). Precisely, 3-D plots of the ternary azeotropic solvent mixture are expected to be characterized by a saddle curve as obtained in this study (Montgomery, 2009).

3.6. Numerical optimization studies

The SOY response selected was set to "maximize", while the solvent components [water (A), ethanol (B), and ethyl acetate (C) were all set within their percentage ranges (5-90%), (5-90%), and (80-85%). The numerical optimization of the data obtained was conducted using the software and the highest desirability is 1.000. The highest limit for SOY was 15.56 while the lowest limit was 9.15. Therefore, the optimum value suggested for water (A), ethanol (B) and ethyl acetate (C) components are 9.09 %, 5.91 %, and 85.00 %. The experimental value for soy oil yield was 15.56 while the numerical value was 17.928 from which the error difference gave 13.22 % (Table 5).

3.7. Physicochemical Properties of the Solvent Extraction Products

3.7.1. Physicochemical properties of the soy oil sample

The acidity of the soy oil extracted is 16.81 mgKOH/g which is slightly lower than the 19.21 mgKOH/g reported in soybean oil extracted with petroleum ether (Amos-Tautua and Onigbinde, 2013) (Table 6). The acid value obtained is higher than 0.37, 1.66, and 4.69 mgKOH/g of oil extracted from Cottonseed (CS), groundnut (GN), and melon seed (MS), but less than 65.50 mgKOH/g of maize seed oil (MZO) (Saxena *et al.,* 2011; Amos-Tautua and Onigbinde, 2013, Kadurumba *et al.,* 2018). The acid value is well related to the quality of fatty acid in the oil sample and the extent of storage on the oil quality. A high acid value suggests that the oil sample is susceptible to instability and rancidity over a long period (Aremu *et al.*, 2015; Kadurumba, *et al.*, 2018), and such oils are suitable for high cooking (Akintayo, 2004, Kadurumba *et al.,* 2018). The degree or extent of suitability of oil samples for consumption depends on their percentage of Free Fatty Acid (%FFA), and a lower level of % FFA indicates good quality. The % FFA value of the soy oil is 8.39%, which is relatively low, thus, suggesting the soy oil is suitable for human consumption, unlike MZO which has a relatively high % FFA (32.96%).

Table 6. Physicochemical Characteristics of Soy oil Extracted with Azeotropic Solvent mixture

Properties	**GNO**	**MZO**	**MSO**	**CSO**	**Soy Oil**
AV	4.69	65.50	1.66	0.37	16.81
FFA	2.33	32.96	ND	ND	8.39
SV	227.49	211.37	193.61	194.3	56.12
IV	38.65	47.25	112.53	106.8	15.17
PV	ND	ND	6.82	ND	27.00
RI	1.4622	1.4632	ND	1.4641	1.459

AV is Acid Value, FFA is Free Fatty Acid, SV is Saponification Value, IV is Iodine Value, PV is Peroxide Value, RI is Refractive Index. CSO is Cottonseed oil, GNO is Groundnut oil, MSO is Melon seed oil, MZO is Maize seed oil, and ND is Not Determined

Saponification value (SV) is an important physicochemical property of the oil and it indicates the extent of deterioration of oil during storage due to the oxidation effect (Aremu *et al.*, 2004, Kadurumba *et al.*, 2018). The SV (56.12 mmHg) of the soy oil is lower than 193.61, 194.5, 211.37, and 227.49 mgKOH/g were obtained for CSO, MSO, MZO, and GNO (Amos-Tautua and Onigbinde, 2013; Kadurumba *et al.*, 2018). The disparity between the SV reported in this study and the works of Amos-Tautua and Onigbinde, (2013) could only be linked to the solvent used, where the latter involved petroleum ether. The relatively low value of SV reported for soy oil indicates that the oil is less volatile and may not be suitable for the production of oil-based products such as ice cream, shampoo, and soap (Kadurumba *et al.*, 2018) nor applicable as drying oil (Araromi *et al.*, 2017).

The iodine value of soy oil is 15.17, which is lower than 38.65, 47.25, 106.8, and 112.53 reported for CSO, MZO, CSO, and MSO (Saxena *et al.*, 2011, Amos-Tautua and Onigbinde, 2013) and became more unsaturated. The low IV (15.17) of soy oil indicates that the oil is relatively saturated compared to others. The periodic value (PV) for the soy oil is 27.00 M/mol.kg which is higher than the 6.82 M/mol.kg obtained for MSO. Generally, refined oils are characterized by low PV while high PV indicates a high degree of oxidative rancidity of the oil sample. Furthermore, oil with high PV is highly depleted of antioxidant and thus require some level of antioxidant fortification to meet commercial grade (Kadurumba *et al.*, 2018). The refractive index (RI) of GNO, MZO, CSO, and soy oil are 1.4622, 1.4632, 1.4641, and 1.4069 respectively, which are within the ranges reported for various edible oils (Saxena *et al.*, 2011, Amos-Tautua and Onigbinde, 2013). The RI is an important oil characteristic that specifies the degree of conjugation of unsaturation as well as the length of the fatty acid chain and molecular weight of the oil sample (Amos-Tautua and Onigbinde, 2013).

3.7.2 Physicochemical Properties of Defatted Soybean Cake Sample

Moisture content (1.46 %) of the raw soybean sample (*Glycine Max (L*) used in this study is lower than 8.22 and 11.67 % reported for Gamasugen 1 (G1) and Gamasugen 2 (G2) varieties of soybean as well as sunflower seed (10.29 %) (Lisanti and Arwin, 2019) (Table 7). The high moisture content of seeds makes them prone to microbial attack and rapid deterioration, thus the low moisture content of the varieties of the soybean used in this study is very desirable (Buba *et al.*, 2015). The Ash content of the soybean sample is 16.75 %, which is higher than the 4.61 and 4.86 % of the G1 and G2 varieties of soybean respectively (Lisanti and Arwin, 2019). Percentage Ash content (%) is related to the amount of minerals present in the biomaterials.

Table 7. Physicochemical Characteristics of Soybean cake Defatted with Azeotropic Solvent mixture

Samples		Physicochemical Properties (%)				
	Moisture Content	Ash Content	Fibre Content	Lipid Content	Crude Protein	Carbohydrate Content
*Raw Soybean	1.456	16.75	4.64	20.30	33.87	22.99
*Defatted Soybean Cake	16.75	4.85	5.00	2.60	31.54	39.86
G1 Soybean	8.22	4.61	14.56	14.50	37.65	20.46
G2 Soybean	11.67	4.86	9.96	15.51	37.34	20.66
RUSSM	10.29	5.46	26.35	12.40	27.02	19.52
SESSM	8.44	4.96	18.4	6.45	45.31	17.77

G1 is Gamasugen 1, G2 is Gamasugen 2, RUSSM is Raw Undehulled Sunflower Seed Meal, and SESSM is Solvent Extracted Sunflower Seed Meal, * This study

The fiber content (4.64 %) of the soybean used is lower than the 14.56 and 9.96 % of G1 and G2 varieties of soybean. The lipid content of the three varieties ranged between 14.50 and 20.3 %, thus corroborating the submission of Rodrigues *et al.,* (2010) that categorized soybeans as low oil-containing biomass. The crude protein content (37.65 and 37.34 %) of the G1 and G2 soybean varieties is very close to the protein content (33.78 %) of the *Glycine Max (L*) used in this study. Soybean is one of the protein-rich oilseeds that has attracted wide cultivation in the world (Amos-Tutu and Onigbinde, 2013). The carbohydrate contents of the *G. Max (L*), as well as G1 and G2 soybean varieties, are 22.99, 20.46, and 20.66 %, respectively. These values are very close, it can therefore be suggested that variations in the proximate composition of different varieties of soybean may be due to the influence of environmental, genetic, and processing conditions (Grieshop and Fahey, 2001; Karr-Lilienthal *et al.,* 2004; Kumar *et al.,* 2006).

The trend of changes in the proximate compositions of the soybean cake after the azeotropic solvent extraction was monitored and documented in Table 7. This was compared with the changes observed in Sunflower seeds, subjected to the solvent extraction process. Essentially, there are differences between the proximate composition of the raw seed samples and the resulting (defatted) cake samples after the solvent extraction process. Distinctly, the difference in the lipid content before (20.30 % and 12.40 %) and after (2.00 % and 6.45 %) solvent extraction for the soybean and sunflower seeds, is very wide, thus indicating the impact of the solvent extraction. The two seed samples shared similar trends in their ash content after extraction. The increase in the moisture content of the soybean cake after extraction may be attributed to the water content in the azeotropic solvent mixture composition.

4.Conclusions

The composition of the azeotropic solvent mixture developed with the D-optimal Design under the Mixture Methodology was 9.17%, 6.67%, and 84.17% of water, ethanol, and ethyl acetate, respectively and was capable of extracting soy oil from its bean sample. The extraction process is best described by a Quadratic model and the physicochemical properties obtained indicate that the soy oil extracted is an oil sample fit for cooking.

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Acknowledgment

The authors appreciate the opportunity granted by the Bioenvironmental, Water and Engineering Research Group (BWERG) Laboratory and Food Science Lipid Laboratory, both in Ladoke Akintola University of Technology, Ogbomoso, Nigeria to undertake this study.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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

THERMOPHILIC ACTINOBACTERIA ISOLATED FROM TLEGHMA HOT SPRING: A POTENTIAL SOURCE OF THERMOSTABLE α-AMYLASE

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https://doi.org/10.34302/crpjfst/2023.15.4.13

Article history:
Received: 12 December 2022
Accepted: 20 November 2023

Keywords:
Actinobacteria;
Hot spring;
Streptomyces;
Thermostable amylase.

ABSTRACT

Thermostable α-amylase is a commercial enzyme that has found several biotechnological applications in recent years. This prompts researchers to check out the hot ecosystems the least explored to find producing microorganisms. Thermal waters are a poorly studied ecological niche compared to other ecosystems. Actinobacteria are known for their unique metabolic abilities to produce the most innovative bio-molecules. In this study, the isolation by conventional microbiological methods, allowed to obtain 13 thermophilic actinobacteria from a hot spring of TLEGHMA located in the eastern Algerian. All these isolates were characterized morphologically and by physiological methods. Molecular identification by sequencing of the rRNA16s gene, allowed to assign them to the genus *Streptomyces sp*. Among these isolates, 9 actinobacteria showed abilities to produce a thermostable α-amylase active at a temperature of 55°C. Two isolates named TA3 and TA4 are however, the most successful. They were assigned to *Streptomyces albidofalvus* and *Streptomyces cavourensis* respectively. they are able to produce a thermostable α-amylase at 55°C with an activity rate of 110.33U/ml by strain TA3 and 224U/ml by strain TA4 and with an optimum of activity in a pH equal to 9. These results show that these thermophilic *Streptomyces* from these hot waters, are a very important source of thermostable and alkalophilic α-amylase

1.Introduction

Hot springs are an environment with special physical, chemical and nutritional properties. These ecosystems are home to a large biodiversity of thermostable microorganisms (Chan et al., 2017; Msarah et al., 2018). This microbial population grows optimally at temperatures between 45-80°C (Alrumman et al., 2018). They also have the ability to acclimatize to many solvents and detergents and are resistant to acid and basic pH (Mohammad et al. 2017; Adiguzel et al., 2009). These microorganisms are an important source of several biotechnologically useful compounds, including antibiotics and enzymes. (Sayeh et al., 2010). These extreme ecosystems remain relatively unexploited compared to other ecological niches. Actinobacteria are an integral part of this microbial population (Zhaoqi et al., 2009; Medjemadj et al., 2020). These thermophilic bacteria are characterized by the presence of membrane lipids that contain more saturated, straight-chain fatty acids than their mesophilic analogues. This lipid specificity, allows these bacteria to grow at high temperatures by providing the right degree

of fluidity necessary for membrane function (Aditi and Nupur 2016). Research in the field of enzymes from thermophiles is more attractive than those using mesophilic microorganisms. The main reasons for this are their high stabilities and substrate specificities, The low risk of contamination by other bacteria in the fermentation process is greatly reduced due to the high temperature which stops the growth of mesophilic contaminants (Drejer et al., 2018). Several enzymes thermostable at high temperatures (50-65°C), have been discovered in recent years in various genera of actinobacteria, these are generally species belonging to the genus *Thermoactinomyces* that have been isolated from the sediments of thermal station (Aditi and Nupur. 2016). The works on the research of actinobacteria in the samples of thermal waters remain however very rare (Medjemadj et al., 2020; Bahamdain et al., 2020; Ruwini et al., 2022).

Amylase is an extracellular enzyme composed of three types: α, β and glucoamylase, which hydrolyzes mainly starch, dextrins and some small polymers into glucose units (Dash et al., 2015). Amylases are enzymes that have aroused the interest of industries for their wide applications especially in the food industry. This group of enzymes contributes to about 25% of the global enzyme market (Arikan, 2008; Kumar et al., 2014). The demand for α -amylase has increased due to multiple industrial applications because of its crucial role in starch hydrolysis processes used in the food, paper, brewing and textile industries (Hmidet et al., 2009).

Amylases are considered as food additives in the baking industry. The addition of a small amount of amylase to the flour provides simple sugars that are well assimilated by the yeast. This increases the production of carbon dioxide after fermentation and leads to an aerated bread (Pandey et al., 2000).

The use of alpha amylase "bread improver" has become a machine manufacturing practice in the bread industry, not only to improve bread quality but also to control the manufacturing process. This industry uses α-amylases for the regulation of diastatic activities of flours by hydrolysis of starch to maltose. This promotes the formation of soft crumb in baking and improves the texture of pastry cakes and cookies (Malhotra et al., 2002).

Sometimes, cane syrups can be disturbed by the presence of starch contaminations which also increase their viscosity and then affect the crystallization process. This drawback is eliminated by introducing a small amount of immobilized bacterial amylase at a temperature equal to or higher than 80°C (Reddy et al., 2003). In addition, α- amylase is used to facilitate sucrose extraction and refining operations from sugar beet or sugar cane to remove traces of starch interfering with purification. Nevertheless, fungal and bacterial α- amylases are widely used for the preparation of sweet syrups from corn starch and chocolate syrups (Martin et al., 2003).

According to (Tanyildizi et al. 2005), microbial amylases retain greater stability compared to animal and plant amylases.

The objective of this study is to isolate actinobacteria from the hot waters of a thermal spring located in eastern Algeria. It is also a question of searching for and identifying those thermophilic bacteria capable of producing the enzyme α-amylase. Finally, we plan to study some properties and the optimal conditions for the activity of this enzyme produced by the most efficient bacteria.

2. Materials and methods

2.1. Sampling site

Figure 1. Hammam Tleghma sampling site (Mila, Algeria) (© d-maps.com)

The thermal spring of Hammam Tleghma is located in the southeast of the wilaya of Mila

(Latitude: 36.1153, Longitude: 6.36417 36° 6′ 55″ North, 6° 21′ 51″ East) (Figure 1). The physicochemical characteristics of the thermal water of this station are grouped in Table 1.

Table 1.Physicochemical characteristics of the water of the thermal spring of Tleghma

Temperature	pH	Anions (mg/l)	Cations (mg/l)
58°C	7.16	Chlore: 190	Sodium: 120
		Bicarbonates: 274.5	Calcium:118.39
		Sulfates: 164	Potassium: 03
		Nitrates: 08	Magnesium: 31.54

2.2. Isolation of actinobacteria from the hot waters of Tleghma

Three selective isolation media for actinobacteria were used: AIA (Actinomycetes isolation agar), SCA (Starch casein agar) (Uzel, et al., 2009) and ISP5 (Shirling et Gottlieb, 1966).

These media were supplemented with an antifungal agent (fungizone) at a concentration of 50µg/ml. The plates were seeded and incubated at 30°C, 45°C, and 55°C for 20-30 days.

2.3. Phenotypic and physiological characterization of isolates

Fresh and Gram-stained microscopic observation was performed on all isolates. The slide culture technique described by Shirling and Gottlieb 1966, allows to describe the morphology of the aerial mycelium and that of the substrate of each isolate was also performed.

The scanning electronic microscopy (SEM) (ZEISS) was used to examine the morphology of spore chains of the isolates TA3 and TA4, it was performed in Scientific and Technical Research Center in Physico-Chemical Analysis in Algeria.

The production of melanoid pigments was tested on ISP7 medium (Shirling and Gottlieb, 1966). Growth of all isolates at different temperatures (4°C, 10°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C), at different concentrations of NaCl (5, 7 and 10g/l) and at different pH (3, 4, 5, 6, 7, 8, 9, 10,11) was then carried out on ISP2 medium.

2.4. Molecular identification

2.4.1. DNA extraction of Actinobacteria isolates

Genomic DNA was extracted from the isolates using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan), following the manufacturer's instructions. Extracted DNAs were eluted in 50µL of elution buffer. The quantity and purity of DNA were evaluated by a Nanodrop (Thermo Scientific 2000c, USA). DNAs with an A280 / A260 value close to 1.80 were considered pure. The extract was used immediately or stored at -20° C for further use. The genomic DNA was quantitated and assessed for integrity by agarose gel electrophoresis.

2.4.2. 16s PCR for identification

All PCR reactions were performed with the Gene Amp® PCR system (Applied Biosystems). PCR reactions were performed in 50 µL mixtures containing 10 PCR buffer, 1.5 mM MgCl2, 0.4 mMdNTP, 0.2 mM primer, 1.25 U of Taq DNA polymerase (Amplitaq Gold, Applied Biosystems) and 50 to 500 ng of genomic DNA template. Theprimers used for the amplification of the 16S rDNA were FC27 (5'-AGAGTTTGATCCTGGCTCAG-3) and RC1492 (5'-TACGGCTACCTTGTTACGACTT-3'). PCR conditions for 16S rDNA reactionswere 1 cycle of denaturation for 5 min at 94 °C; 30amplification cycles consisting of denaturation (94 °Cfor 30s), primer annealing (49 °C for 30 s), and primer extension (72 °C for 90s); and a final extension of 7min at 72 °C. The PCR products were sequenced.

The similarity of sequences was determined using BLAST

(http://www.ncbi.nlm.nih.Gov/Blast). Multiple sequences alignment used the Neighbour-Joining (N-J) method to determine the closeness of the isolates, and the phylogenetic tree results were viewed using the Mega version 11 program.

2.4.3. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of strains were deposited in NCBI GenBank database.

2.5. Amylase activity assay

Nutrient agar medium containing 1% soluble starch was inoculated with each actinobacteria isolate. After 07 days incubation at 55°C, the agar was covered with a solution of lugol. Hydrolysis is evidenced by the absence of coloration around the colonies on the contrary, the areas containing starch are colored in a brown color (Jeffrey, 2008). Isolates with a large clearing zone were used for α-amylase determination.

2.6. Amylolytic activity on liquid medium

2.6.1. inoculum Preparation

A suspension of each isolate was inoculated into nutrient broth with 1% starch and incubated at 55°C for 3 days until reaching an OD equal to 0.05 at 550 nm (Chaudhary and Prabhu, 2016).

2.6.2. Batch culture

1ml of the bacterial suspension is inoculated into a 100ml volume of Starch fermentation broth medium with the following composition (in g/l) (10g starch, 10g of nutrient broth, 15g agar) at pH 7. The cultures were then incubated under agitation (75 rpm) at 55°C for 5-6 days (Naif, et al.,2020).

2.6.3. Assay of α-amylase

A volume of 2 ml of the bacterial suspension of each batch cultured isolate is placed in an Eppendorf tube. This volume is then centrifuged at a speed of 3000 rpm for 20 min at a temperature of 4°C. A 0.5 ml volume of the supernatant is added with a 0.5 ml volume of a 0.1M phosphate buffer solution, containing starch at a concentration of 1g/l and adjusted to pH 7. The reaction mixture is then incubated at 55°C for 20 min. The reaction is then stopped by adding 1ml of DNSA reagent. The tubes are boiled for 5 minutes and then cooled to ambient temperature. The mixture is then completed to 5 ml by adding distilled water. The estimation of reducing sugar content is measured by reading the absorbance at a wavelength of 540 nm using a spectrophotometer type UV-1800A, Shimadzu, Japan (Bernfeld, 1955).

The results obtained are expressed as U/ml. One unit of amylase activity is defined as the amount of enzyme that produces one µmole of glucose (Ahmed et al., 2011).

2.7. Effect of temperature and pH on α-amylase activity

In this study, the variation of the two key parameters affecting the production of α-amylase was tested. These are temperature and pH. Starch fermentation broth liquid culture medium is inoculated with the bacterial suspension of the performing isolates and incubated at 4°C, 20°C, 30°C, 40°C, 55°C, 60°C and 70°C for 6 days. For pH, the same culture medium is adjusted to different pH using different buffers. We used acetate buffer (for pH 4), phosphate (for pH 7), Tris-amine methane buffer (for pH 9) and borate (for pH 10.2).

The incubation temperature is 30°C for all experiments. A volume of 1 ml is taken in Eppendorf from the 5 to 6 days old batch cultures.

The supernatant is recovered by centrifugation at a speed of 3000 rpm for 20 minutes. Then, the amylolytic activity is measured by the DNSA method with the variation of temperature and pH of the enzymatic reaction.

3.Results anddiscussions

3.1.Isolation and phenotypic characterization of actinobacteria isolates

The colonies obtained show the macros and micro-morphological characteristics of actinobacteria. After 30 days of incubation, all isolates show a typical macroscopic appearance of actinomycetes with different colors (white, green, gray) (Figure 2), (Table 2). This result is

confirmed by microscopic observation which shows the presence of filaments of different sizes and different fragmentations as well as the presence of spores in most isolates (Figure 3, 4and 5). All strains are Gram positive (Figure 3).A total of 13 isolates, 6 isolates from AIA medium, 2 from ISP5 medium and 5 from SCA medium, were selected after purification on ISP 2 medium. Generally, the isolation of actinobacteria pauses some difficulties, mainly because of their relatively long growth time. This feature allows time for other bacteria to contaminate the Petri dishes and occupy the environment especially by fungi and invasive bacteria (Williams et al., 1982; Crawford et al., 1993; Boudemagh and Bensouici, 2014). As such, we used three selective media for the isolation of these bacteria from the tested hot spring. These are Actinomycetes Isolation Agar (AIA) medium, containing sodium propionate and sodium caseinate which have an important antifungal role that prevent fungi from multiplying in the isolation medium. The presence of asparagine according to some researchers, favors the growth of actinobacteria over other microorganisms (Uzel et al., 2009, Akhagari et al., 2014). In the work of Medjemadj et al., 2020, this medium allowed the isolation of the greatest number of actinobacteria from thermal waters. The second medium used in this research is Starch Casein Agar (SCA), the presence of starch and casein in its composition, allows a selective growth of actinobacteria. This medium was successfully used in the work of Suzuki, 2001, where several rare genera such as *Actinomonospora*, *Actinopolyspara*, *Planomonospora* and *Planobispora*, could be isolated. *Streptomyces* and other genera were isolated (Medjemadj et al., 2020). Using ISP5 medium for selective isolation yielded only two actinobacterial isolates, despite its glycerol-rich composition, which is known to be favorable for actinobacteria (Jihani et al., 2012; Siddique et al., 2014).

3.2.Physiological characterisation of isolates

The results of the growth of the 13 isolates at different temperatures indicate that all 13 isolates show good growth between 30-55°C. However, the isolates (TA3 and TA4) are resistant to a temperature of 70°C.

According to the literature, thermophilic actinobacteria are generally classified into two groups. The first is represented by strictly thermophilic and moderately thermophilic actinobacteria, which grow between 37 and 65°C with an optimumbetween 55-60°C. The second category includes moderately thermophilic actinobacteria, which grow between 28-60°C with an optimum between 45-55°C (Jiang and Xu, 1993). Another group known as thermotolerant actinobacteria is also present. Its representatives can survive at temperatures up to 50°C (Lengeler et al., 1999).According to these two types of classifications, the isolates (TA3, TA4, TA5, TA6, TS1, TS2, TS3, TS4 and TS8) are moderately thermophilic actinobacteria. While the other isolates (TS4, TA1, TA10, TA12) are considered to be thermotolerant actinobacteria.

The actinobacteria isolated in our investigations show growth at temperatures ranging from 10 to 70°C, only two isolates (TA3 and TA4) in this group can survive at temperatures up to 70°C. This tolerance was considered by some researchers, is due to their molecular modification at the cellular and subcellular level, saturation of membrane lipids that reduces membrane fluidity at elevated temperatures, and the presence of histone-like in the DNA of hyperthermophiles that protects the DNA (Mrunmaya, 2013;Aditi Nupur, 2016).

The totality of the actinobacteria isolated from the thermal water, present a good growth in the pH interval going from 6 to 8. Among them, the isolates, TA1, TS3 and TS6 are acidotolerant bacteria, which can resist a pH equal to 4. However, the isolates (TA3, TA4, TA5, TA6, TA10, TA12 and TS8) are, according to the classification of Jiang and Xu, 1993, alkaline-tolerant and can resist a pH equal to 11 (Table 3). Our results are in perfect

agreement with other works where the majority of actinobacteria isolated from this kind of ecosystems, are able to live at temperatures ranging from mesophilic to thermophilic and at basic pH. The work of Mokrane, et al., 2016 succeeded in isolating and identifying a new strain *Thermoactinomyces khenchelensis* from the sediments of the thermal spring of khenchela in Algeria. This strain is able to grow in a temperature range of 37-55°C and a pH range of 7-9.Two strains *Nocardoides pakistanensis* and *Streptomyces caldifontis* isolated from Tatta Pani hot spring in Pakistan, are able to survive in a temperature range of 20-40°C and 18-40°C respectively at pH 6-9 (Archia, 2016). The *Saccharomonospora viridis SJ*-21 strain isolated from a hot spring in India is able to grow in temperatures between 35-60°C and pH between 7-10(Jani et al., 2012).

Regarding NaCl tolerance, 6 named isolates (TA1, TA3, TA4, TA5, TA6, and TA10) show good growth in 10g/l NaCl concentration. According to Larsen's 1986 classification, these actinobacteria are moderate halotolerants, whereas the other isolates (TA12, TS1, TS2, TS3, TS4, TS6, and TS8) are weak halotolerants. These results are in agreement with the studies of Perry and Staley in 1997which state that thermophilic actinomycetes are characterized by halophilia**.**

Table 2. Morphological characteristics of actinobacteria isolates

	Culture medium AIA						**Culture medium ISP5**		**Culture medium SCA**				
Caracteristics	**TA1**	**TA3**	**TA4**	**TA5**	**TA6**	**TA10**	**TA12**	**TS1**	**TS2**	**TS3**	**TS4**	**TS6**	**TS8**
Size (mm)	3	1.5	1	1.5	1	2	2	3	3	3	2	2	2
Color of MA	Whi	grey	Whi	grey	Whi	Whi	Whi	Gre	Gre	Gre	Whi	Whi	Whi
Color of MS	Whi	Bro	Whi	Bro	Whi	Whi	Whi	Bro	Bro	Whi	Whi	Yell	Whi
Melanoid pigments	-	-	+	-	-	-	-	-	-	-	-	-	-
Contour	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Reg.	Irrg.
Shape	Circ	Circ	Circ	Circ	Circ	Circ	Circ	Circ	Circ	Circ	Circ	Circ	Circ
Texture	Inla	Inla	Inla	Inla-pow	Inla-pow	Inla-pow	Inla	Inla-pow	Inla	Inla	Inla-pow	Inla-pow	Inla

Whi; White, Gre; Green, Bro; Brown, Yell; Yellow; Reg; Regular, Irrg; Irregular, Circ; Circular, Inla; Inlaid, Pow; Powdery. (+) : presence of melanoid pigments (-) : absence of melanoid pigments.

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

Figure 2. Macroscopic appearance of some isolates (a)TA6, (b) TA3, (c) TA4

(a) **(b)**

Figure 3. Microscopic appearance in the fresh state and after Gram staining of isolates (a) TA4 et (b) TA3

(a) **(b)**

Figure 4. Microscopic appearance by the slide technique (GX100).
(a) Aerial mycelium of TA6 isolate, (b) Substrate mycelium of TA6 isolate

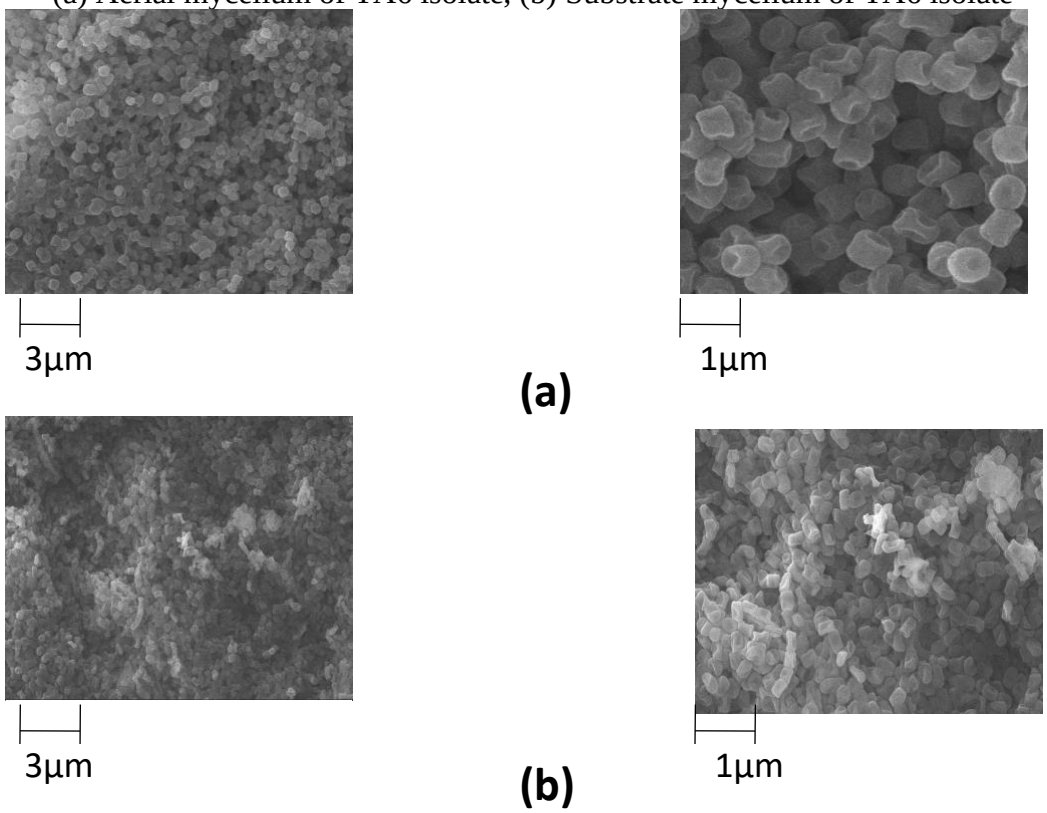


Figure 5. Scanning electron microscopy (SEM) (ZEISS) of two isolates.
(a) Isolate TA3. **(b)** Isolate TA4.

3.3.Molecular identification

Molecular identification indicates that all isolates belong to the genus *Streptomyces* (Table 4) (figure 6).This genus of actinobacteria has clear advantages in biotechnology (Hodgson, 2000; Manteca et al., 2008). The presence of *Streptomyces* in thermal springs is not uncommon, it has been proven in some works (Medjemadj et al., 2020; Akhgari et al. 2014; Zu et al., 1998). Also, the presence of actinobacteriain thermal springs has been demonstrated in some studies.

In the work of Medjemadj et al., 2020, three genera of actinobacteria were isolated from thermal waters of eastern Algeria. These are the genera *Rathayibacter, Streptomyces* and *Rhodococcus*. Arshia. 2016, successfully isolated a new species (*Nocardioides pakistanensis*) from the water samples of a hot spring located in Pakistan. *Planifilumy unnanense sp. Nov* is a new strain isolated from the hot spring in Yunnan Province, China. This thermophilic actinobacterium is able to grow in a temperature range of 50-75°C (Zhang, et al. 2007).From the sediments of a thermal spring in western Anatolia, Turkey, two actinobacteria: *Thermoactinomyces thalpophilus* and *T.saccharis*, which grow at 55°C, were isolated (Uzel et al., 2009).

The metagenomic approach in the work of Zhaoqi et al., 2009 showed a surprising diversity of culturable and non-culturable actinobacteria in three geographically distant hot springs.

It should be noted that in the literature, we found little research on the isolation of actinomycetes from thermal waters compared to those performed on sediments

Table3. Physiological characteristics of actinobacteria isolates

Characteristics	TA1	TA3	TA4	TA5	TA6	TA10	TA12	TS1	TS2	TS3	TS4	TS6	TS8
T°C range	20-55	10-70	20-70	20-60	10-65	20-55	20-55	20-60	10-60	20-65	20-55	20-65	20-65
Optimal growth	40	55-60	55-60	45-55	45	40	40	45	45	55	35	45	55
pH range	4-11	5-11	6-11	5-11	6-11	5-11	5-11	5-9	5-9	4-10	5-10	4-10	5-11
Optimal growth	7	9	10	8	8	7	7	6	7	6	7	6	8
NaCl (g/l)	5,7,10	5,7,10	5,7,10	5,7,10	5,7,10	5,7,10	5,7	5,7	5	5,7	5	5,7	5,7

Table 4. Molecular identification of actinobacteria isolated from the hot spring of Tleghma.

Isolate codes	Closest sequence match withBLAST (accession number)	Percentage of Similarity	Accession numbers
TA1	*Streptomyces rhizosphaericola* 1AS2c (NZ_SRZK01000437.1)	99.72¨%	OP456977
TA3	*Streptomyces albidoflavus* (NC_020990.1)	99,44%	MW301212
TA4	*Streptomyces cavourensis* strain 1AS2a (NZ_CP024957.1)	99,32%	MW301210
TA5	*Streptomyces albidoflavus* (NC_020990.1)	99.53%	OP003989
TA6	*Streptomyces rhizosphaericola* strain 1AS2c (NZ_SRZK01000437.1)	98.03%	OP003990
TA10	*Streptomyces albidoflavus* (NC_020990.1)	99.64%	OP003991
TA12	*Streptomyces rhizosphaericola* strain 1AS2c (NZ_SRZK01000437.1)	99.77%	OP003992
TS1	*Streptomyces griseoflavus* strain JCM 4479 (NZ_BMUC01000046.1)	98.51%	OP480010
TS2	*Streptomyces azureus*strain ATCC 14921 (NZ_DF968281.1)	99.14%	OP480011
TS3	*Streptomyces torulosis* strain NRRLB-3889 (NZ_LIRK01000400.1)	99.02%	OP003986
TS4	*Streptomtcesmelanogensis*strain JCM4398 (NZ_BMTS01000080.1)	99.39%	OP003987
TS6	*Streptomyces calvus* strain DSM 41452 (NZ_CP022310.10)	99.06%	OP480012
TS8	*Streptomtcesaurantiogriseus*strain JCM4346 (NZ_BMSX01000069.1)	98.39%	OP003988

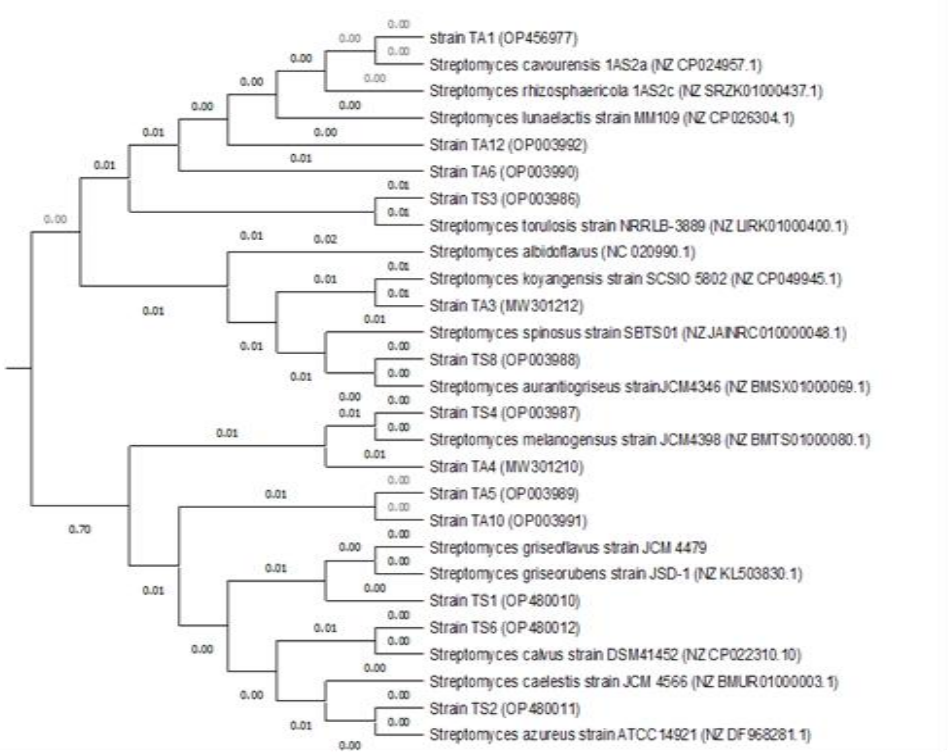


Figure 6. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences amplified from the hot spring isolates, showing the nearest neighbours of the isolated strains. GenBank accession numbers are given in parentheses.

3.4. Enzyme activity at 55°C

The amylolytic activity is calculated and shown in Table 5 and Figure 7. The results show that 9 isolates (TA3, TA4, TA5, TA6, TA10, TA12, TS3, TS4 and TS8) among the 13 isolated, are able to produce α-amylase in the temperature 55°C. Isolates TA3 and TA4 gave however, better enzymatic activity (Figure 7 and 8).

The hydrolysis of starch by enzymes of microbial origin has found wide applications in different industrial fields and has replaced tedious and very expensive chemical methods (Vidyalakshmi et al., 2009).

Among a wide range of microbial species capable of secreting α-amylase, bacteria are the most efficient. In addition, genetic engineering studies are easier to perform with bacteria and they are also very suitable for the production of recombinant enzymes (Nielsen et al., 2000)

Actinobacteria and especially the genus *Streptomyces* are characterized by the production of several enzymes (Hang et al., 1996). These bacteria are better candidates in the industrial field because of their ability to survive in harsh physicochemical conditions, such as temperature and pH (Al-Dhabi et al., 2016; Krishnasamy, 2017).

Thermostable α-amylase is widely used in biotechnology, as the main technological steps of starch processing, such as saccharification, gelatinization and liquefaction, all require high temperature. Therefore, thermostable α-amylase has a definite application in industrial sector (Gazali., 2018).

3.4.1. Effect of temperature and pH on α-amylase activity of TA3 and TA4 isolates

pH and temperature are two important parameters that control α-amylase production (Pandey et al., 2000).

In this study, the 13 isolates obtained were tested for their abilities of thermostable α-amylase production at a temperature of 55°C. The results show that the two isolates TA3 and

TA4 identified as *Streptomyces albidoflavus* and *Streptomyces cavourensis*, present the highest amylolytic activity equal to 110.33 and 224 U/ml respectively (figure 9 (a)) . The isolates (TA5, TA6, TA10, TA12, TS3, TS4 and TS8), also show amylolytic activity but less important ranging from 12-48 U/ml.Concerning the maximum production of α-amylase tested at temperature 30°C, it was observed at pH 9 for isolates TA3 and TA4, with values equal to 105U/ml and 208U/ml respectively (figure 9. (b)).

These important results are in perfect agreement with those of Gommez and Steiner, 2004 who showed that thermo-enzymes are generally not only thermostable, but they are also active at extreme pH.

According to the literature, thermophilic actinobacteria capable of producing thermostable α-amylase have been widely searched mainly in desert and semi-desert soils throughout the world. A thermostable α-amylase was obtained by a strain named *Streptomyces sp. SLBA-08* isolated from a semi-arid soil was reported (Edilla et al. in 2012). In other similar works, the *Streptomyces fragilis DA7-7* strain isolated from a desert soil in Riyadh province in Saudi Arabia, shows a very high amylolytic activity equal to 923.12 U/ml at a temperature of 28°C, which decreases with increasing temperature. Our results are more interesting because our two strains show high enzymatic activity at an optimal temperature of 55°C. (Krishnasamy., 2017).

In a more recent study, *Streptomyces sp. Al-Dhabi* strain isolated from the soil of Jazanen Saudi Arabia region is able to produce a thermostable α-amylase with maximum activity equal to 124± 12.1 U/ml in a temperature of 40°C, and at pH equal to 8 (Naif et al., 2020).

Studies that focus on the production of thermostable α-amylase by thermophilic *Streptomyces sp*. isolated from hot springs are very rare. This study is the first report on the production of this enzyme from thermal waters in Algeria. According to our knowledge, the few works done on the role of *Streptomyces* in the production of thermostable α-amylase are those of Sabita et al. In 1999, who state that the *Streptomyces megaspores* strain SD12 isolated from the thermal spring of Occidental Maharashtra, is able to show an optimal amylolytic activity equal to 52,534 U/ml in a pH of 6 and a temperature of 60°C. Chaudhary and Prabhu in 2016 also found amylolytic activity in two isolates of the thermophilic *Streptomyces* genus isolated from a hot spring located in Vajreshwari, India.

Table 5. Amylolytic activity at 55°C of actinobacteria isolates

Isolates	Amylolytic activity(U/ml)
TA1	-
TA3	110.33
TA4	224
TA5	20
TA6	12
TA10	16.5
TA12	23.5
TS1	-
TS2	-
TS3	13
TS4	48
TS6	-
TS8	15

(-) : Absence of enzymatic activity

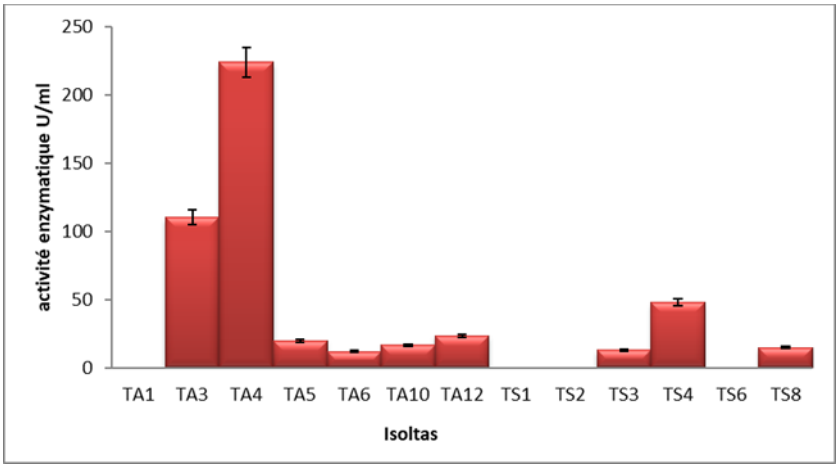
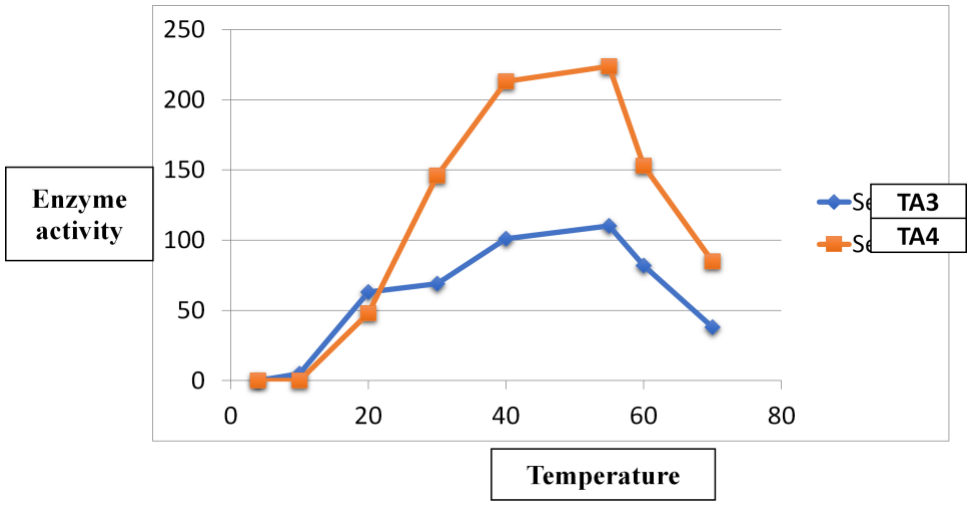
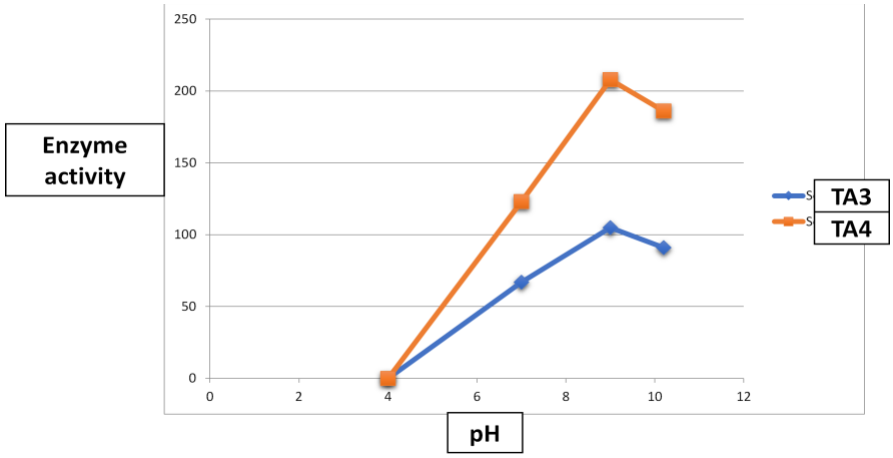


Figure 7. Enzymatic activity at 55°C of α-amylase produced by selected isolates

Figure 8. Starch hydrolysis zones of isolates (a) : TA3, (b) : TA5, (c) : TS4, (d) : TA4 isolates



(a)



(b)

Figure 9. Effect of temperature (a) and pH (b) on the activity of amylase produced by TA3 and TA4 isolats

4. Conclusions

In this study, we reveal that thermal waters are populated by thermophilic and thermotolerant actinobacteria. This result is very important and agrees with recent works, which affirm the presence of these bacteria in thermal waters. Molecular identificationby r16sDNA sequencing showed that all isolates belong to the genus *Streptomyces*. Two successful isolates assigned to *Streptomyces albidoflavus* and *Streptomyces cavourensis*, are capable of producing thermostable α-amylase with an optimum of 55°C. These two strains also present an alkalophilic character by producing the desired enzyme at a pH equal to 9. According to our knowledge, it is for the first time that these bacteria producing this enzyme, are isolated from hot springs in Algeria. These ecosystems constitute an inexhaustible reservoir of very promising bacteria from the biotechnological point of view.

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Acknowledgment

We thank Professor Mahmoud Kitouni who allowed us to carry out part of this work in the laboratory of Microbiological Engineering and Applications, Constantine 1- frères Mentouri University, Chaabat Erssas Campus, Ain El BeyRoad, Constantine 25000, Algeria.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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EFFECT OF BLOCK FREEZE CONCENTRATION PROCESS ON ACEROLA JUICE (*MALPIGHIA EMARGINATA*)

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https://doi.org/10.34302/crpjfst/2023.15.4.14

Article history:
Received: 8 May 2023
Accepted: 1 December 2023

Keywords:
Concentration;
Phenolic compounds;
Antioxidants.

ABSTRACT

Acerola is a fruit rich in vitamin C, in addition to having high levels of anthocyanins and carotenoids, which are antioxidant pigments that give the fruit its red color. Thus, the objective of this work was to concentrate bioactive compounds, by the method of block freeze concentration, using acerola juice in natura. Fresh juice and freeze concentration fractions (concentrate and ice) were evaluated for pH, acidity, soluble solids, total solids, quantification of total phenolic compounds and antioxidant activity. From the results obtained, it was observed that the method of freeze block concentration in blocks resulted in the concentration of soluble and total solids and acidity. In addition, it significantly concentrated the phenolic compounds, keeping the process efficiency above 39%. Regarding the antioxidant activity, the values were significantly higher in the obtained concentrates than in the initial juice. The highest antioxidant potential found was for the juice retained in the last step of the process, with activity about 1.7 times greater than the initial juice. As the freeze concentration stages progressed, an increase in the concentration factors in the total solids content was observed, with an average increase of approximately 131% in the third stage. Thus, the results obtained in this work suggest that the method of block freeze concentration applied to acerola juice, provided a product with greater antioxidant activity and concentration of phenolic compounds, which shows that this is a viable method for the concentration of bioactive compounds. from acerola.

1.Introduction

Acerola (*Malpighia emarginata*) is native to the Antilles and is cultivated in tropical and subtropical climate regions, like northern South America and Central America, and mainly including Brazil, which has the greatest plantations in the world (Barros *et al.*, 2020). Is a fruit known for its high content of ascorbic acid (in the range of 1500–4500 mg/100 g) and other important bioactive compounds, like phenolic compounds, including benzoic acid derivatives, phenylpropanoids, flavonoids and anthocyanins, and of total carotenoids (Nascimento *et al.*, 2018). The fruit is used in the industry for the production of pulp, juices, and jellies and also has a great potential for use in the production of food supplements that enhance the immune response of the body due to the presence of antioxidant compounds and high vitamin C content (Belwal *et al.*, 2018).

The antioxidant activity of red fruits, such as acerola, is due to the chemical structure of anthocyanins. Anthocyanins are glycosylated polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium, containing two benzoyl rings separated by a heterocyclic ring. Different anthocyanins are characterized by differences in: number and degree of methylation of hydroxyl groups; nature, number and position of sugar moieties attached to the phenolic molecule

(aglycone); nature and number of aliphatic or aromatic acids attached to the sugars (Santini and Huyke, 1993). Besides the colorant property, the anthocyanins have been found to exhibit potential therapeutic effect as antiinflammatory, radiation-protective, chemoprotective, vasoprotective, inhibition of LDL oxidation and decrease the risks of cardiovascular diseases (Wang *et al.*, 1997; Seeram and Nair, 2002).

In the food industry, thermal processing is a widely used method to extend the shelf life of foods. However, it has some drawbacks, such as the degradation of heat-sensitive nutrients and changes in food color (Patras *et al.*, 2010; Van Boekel *et al.*, 2010; Sui *et al.*, 2016).

Non-thermal technologies, like freeze concentrantion are widely used in the food industry for their ability to preserve product quality by preventing the thermal degradation of temperature-sensitive compounds (Chemat *et al*., 2017). The block freeze concentration (BFC) consists of crystallizing part of the water from a liquid to separate it from the concentrated solution. Cryoconcentration can be understood as dehydration, allowing to preserve to the maximum the organoleptic and nutritional characteristics of the liquid food (Safiei and Shaikh Alaudin, 2021; Wu *et al.*, 2017).

This technology uses low temperatures during the process, avoiding undesirable chemical, physical and biological changes that occur in other types of processing (Gunathilake *et al*., 2014).

Block freeze concentration method is reported as promising and effective in obtaining food products with high nutritional value and preservation of sensorial properties, such as fruit juices, coffee and whey (Meneses *et al.*, 2021; Haas *et al.*, 2022; Almeida *et al.*, 2023; Benedetti *et al.*, 2015).

Based on these aspects, this study seeks to evaluate the concentration of the bioactive compounds in the acerola juice, obtained from block cryoconcentration by gravitational method.

2. Materials and methods

2.1. Materials

Fresh acerolas were purchased from a local supplier (Mato Grosso do Sul, Brazil), crushed in a fruit processor and filtered to separate out the seeds and skin from the juice, avoid the presence of solids in the juice that might interfere with the freeze concentration process. Acerola juice was kept in a freezer at -18 ± 2 ºC (Biplex CRM45 Consul, Brazil) until the cryoconcentration process.

2.2. Block freeze concentration process

The freezing conditions were performed by the method of Aider and Ounis (2012), with modifications. The method is based on total freezing of the solution followed by partial thawing through simple gravitational separation, it is possible to obtain two fractions: concentrate (C) and ice (G). A schematic representation of the three process stages, sample division, and sample masses are shown in Fig. 1. The initial weight of 2 L of acerola juice was frozen in polypropylene pots at -18 ± 2 ºC in static freezer.

The cryoconcentration apparatus consisted of a mesh stainless steel screen, a plastic funnel, a tripod stand, a 500 mL beaker flask, and a semi-analytical balance (AUX 320 Shimadzu) (Fig. 1). At each stage, the frozen samples were removed from the pots and placed on top of the stainless-steel screen maintained in contact with the funnel, and partial defrosting was allowed using only gravitational force.

Thawing was conducted inside of a refrigerator, with a controlled temperature of 10±2 ºC, until the fraction thawed collected in the beaker reached 50% of the initial frozen sample. The liquid that was thawed consisted of the concentrate of the first step C1. The frozen sample that remained in the stainless-steel screen was taken as the ice fraction I1. The C1 concentrate was again frozen at -18 ± 2 ºC for 24h, used as a feeding solution for the second stage. In the second stage, 50% of the frozen solution was thawed, collected and refrozen as a feeding solution for the third stage. Samples of concentrate (C1, C2, C3) and ice were (I1, I2, I3) taken from each step for further analysis and

stored at -18 ± 2 °C for analysis. All cryoconcentrates and their ice were evaluated to determine concentration performance, physicochemical and phenolic composition and antioxidant capacity.

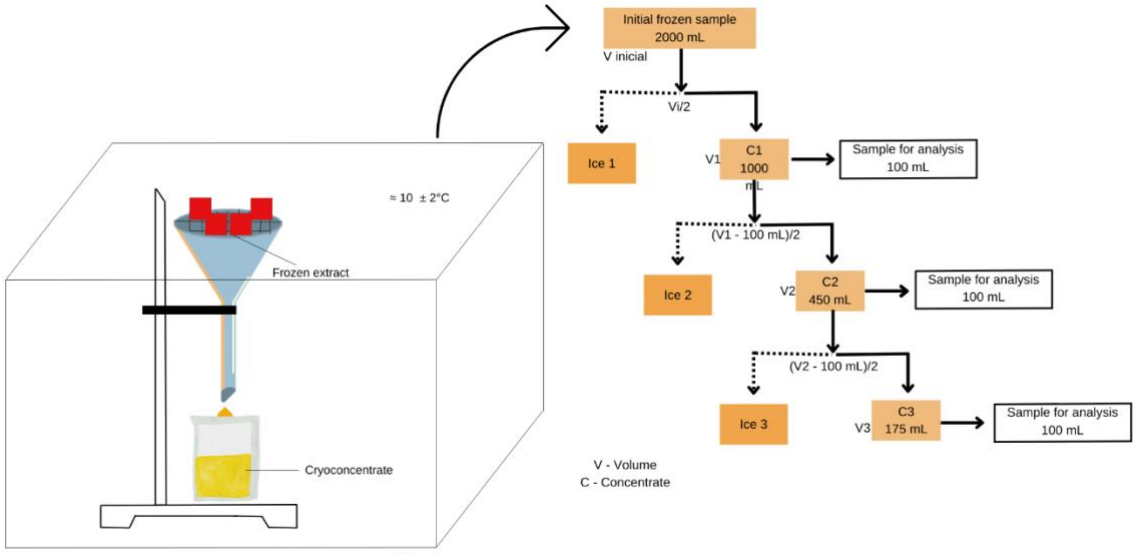

Figure 1. General diagram of BFC process by gravitational method of the acerola juice.

2.3. Physicochemical analysis

The physicochemical analysis was carried with orange juice, concentrated orange juice, and ice fraction samples. The pH was determined with digital pH meter DM 22 (Digimed, São Paulo, Brasil), and total soluble solids (Brix) were measured at 20 ◦C (±1 ◦C) on a refractomer model PAL-1 ATAGO). The titratable acidity and total dry matter content were also determined, according to AOAC (2005). All analyzes were performed in triplicate.

2.4. Block freeze concentration performance

The concentration factor (CF) at each freeze concentration stage was determined as a function of the increase in the concentration of the solution in relation to the quantity of total dry matter content in the initial acerola juice, as proposed by Aider and Ounis (2012). The total dry matter content was determined by measuring the mass loss after drying at 105 °C for 24 hours, and expressed as dry matter content/total mass (g.100 g^{-1}) (AOAC, 2005). The CF was calculated as the ratio between DMn is the total dry matter content (g) of the concentrated fluid in each freeze concentration stage and DM0 is the total dry matter content (g) of the initial acerola juice. The results were expressed in %.

As described by Belén *et al.* (2012), the efficiency of the freeze concentration process (PE) (%) was determined based on the increase in the content of total phenolics compounds (TP) of the concentrated fluid in relation to the IC remaining in the ice, as calculated through the following equation:

$$PE(\%)=\frac{TPC_n-TPI_n}{TPC_n} \quad (1)$$

TPC_n and TPI_n are the concentration of total phenolic compounds in concentrated juice and ice fractions.

2.5. Determination of total phenolic compounds

Total phenolic compounds polyphenols were determined spectrophotometrically on a UV-1600 (Pró-Análise, São Paulo, Brazil) spectrophotometer using the Folin-Ciocalteu assay, according to Singleton and Rossi (1965). The absorbance of samples was measured at 765 nm and results expressed as gallic acid equivalents (mg GAE.100 mL^{-1} juice). All analyzes were performed in triplicate.

2.6. Determination of antioxidant activity

The determination of antioxidant activity was performed by the DPPH method (1,1-diphenyl-2-picryl-hydrazyl) according to Rufino *et al*. (2007) with some modifications. The absorbance reading was performed at 515 nm in a spectrophotometer (model UV-1600, Pró-Análise, São Paulo, Brazil). Results were expressed in TEAC (antioxidant activity equivalent in Trolox) as µ MolTrolox. g^{-1} sample. All analyzes were performed in triplicate.

2.7. Statistical analysis

In this study, all data were presented as mean ± standard deviation (SD). Tukey's least significant difference test (LSD), with a significance level of 5%, was used to compare the means. All analyzes were performed using the software Statistica (v. 7.0, TIBCO Software Inc., Palo Alto, CA, USA).

3.Results and discussions

3.1. Physicochemical analysis

The increase in the soluble solids content in the natura juice concentrate and in the ice, in each stage of the cryoconcentration process, is shown in Figure 2.

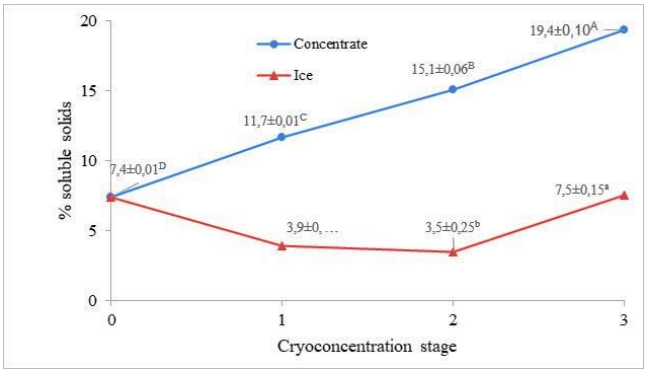

Figure 2. Soluble solids content of concentrated fractions and residual ice as a function of cryoconcentration steps for fresh juice.

Data are expressed as mean ± SD (n = 3) of the soluble solids content in the concentrated samples and ice in each. Different superscript lowercase letters indicate a significant difference ($p < 0.05$) between feed and concentrated fluid at each stage. Different superscript capital letters indicate a significant difference ($p < 0.05$) between food and ice at each stage.

The concentration of soluble solids in the concentrates increased significantly ($p < 0.05$) in all steps, when compared to the initial juice. The total soluble solids content of acerola juice increased from 7.4 ºBrix to 19.4 ºBrix in the concentrate of the third stage, representing about 2.6 times of the initial value. In the concentration of orange juice, Haas et al. (2022) obtained significantly from 17.37 to 38.07 ◦Brix in juice concentrates. Meneses et al. (2021) achieved an increse from 4% (w/w) to 14.1% (w/w) in the three stages of green tea concentrantion. Petzold *et al.* al. (2015) achieved cryoconcentration in blueberry and pineapple juices, which resulted, after 3 repetitions, in an increase from 13ºBrix to 33ºBrix in both juices, that is, a concentration of 2.5 times.

Figure 3 shows the total solids content in each stage of cryoconcentration for acerola juice. There was an increase in solids content ($p < 0.05$) in all stages of concentrate.

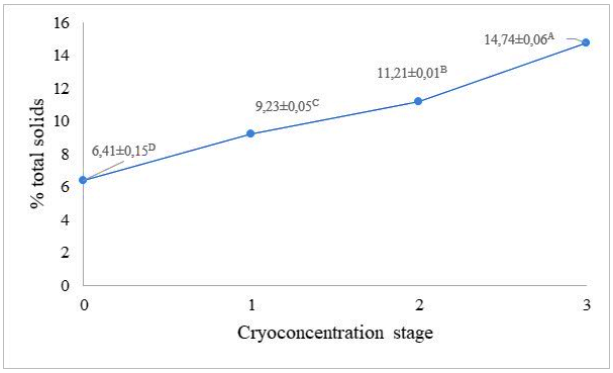

Figure 3. Total solid content in concentrated fractions of cryoconcentration.
Different superscript capital letters indicate a significant difference ($p < 0.05$) between juice and concentrate at each stage.

The concentrated juice presented increase of the total solids content, in the concentrate of each stage of the cryoconcentration process. The concentration factor increased ($p < 0.05$) in all stages when compared to the initial juice. The concentration factor increased ($p < 0.05$) in all stages when compared to the initial acerola juice. Values of 143.97 ± 0.04% were obtained in the first stage, 121.44 ± 0.01% in the second stage, 131.47 ± 0.04% in the third stage. This same behavior of the concentration factors found for fresh juice and concentrates corroborates Adorno *et al.* (2016), for strawberry juice, who reported a significant increase in the total solids content in the concentrates obtained in the four stages of cryoconcentration in blocks.

The same was observed by Haas *et al.* (2022), for orange juice and by Almeida *et al.* (2023), by concentrating *Morinda citrifolia L.* tea.

Table 1 presents the results of the analysis of pH and titratable acidity of the concentrate and of the ice obtained during the three stages of cryoconcentration of acerola juice.

3.2. Total phenolic content

The results of the content of total phenolic compounds in acerola juice and the concentrates from cryoconcentration are shown in Table 2. Analyzing the effect of cryoconcentration in each stage, an increase in all three is observed when compared to the initial juice. There was also an increase ($p < 0.05$) in the content of phenolic compounds present in the juice ice fractions. In the second stage, the CFT content was reduced, but in the third stage there was a significant increase ($p<0.05$) when compared to the previous stage. This behavior of the CFT content in the ice fractions can be explained, according to Aider *et al.* (2007), by the fact that the content of phenolic compounds behaves this way due to the formation of hydrogen bonds, since it has the ability to bind to a large number of water molecules. With the increase of phenolic compounds in the solution, the interstitial water becomes less available for freezing, as a result, during the process of separating the concentrated fluid from the ice, the frozen phase retains greater amounts of phenolic compounds.

The highest efficiency was observed in the second stage of cryoconcentration for fresh juice, with a value of 70.49±0.20%.

Table 1. Physicochemical composition of initial juice, cryoconcentrated acerola juice and ice fractions obtained by block freeze concentration

Samples	Physicochemical parameters	
	pH	Titratable acidity
IJ	$3,66^{b}$±0,02	$0,90^{d}$±0,01
C1	$4,15^{a}$±0,15	$1,54^{c}$±0,03
C2	$3,90^{ab}$±0,15	$1,68^{b}$±0,01
C3	$3,65^{b}$±0,15	$2,19^{a}$±0,01
I1	$3,87^{A}$±0,16	$0,05^{C}$±0,01
I2	$3,53^{A}$±0,25	$0,03^{B}$±0,01
I3	$3,53^{A}$±0,15	$0,09^{A}$±0,01

Results are expressed as mean ± standard deviation (n = 3). Different lowercase letters indicate statistical difference ($p \leq 0.05$) between initial orange juice and cryoconcentrated juices (C1, C2 and C3). Different uppercase letters indicate statistical difference between ice fractions (IF1, IF2 and IF3) at every stage. OJ, initial juice; I1, ice fraction of stage 1; I2, ice fraction of stage 2; I3, ice fraction of stage 3; C1, cryoconcentrated orange juice of stage 1; C2, cryoconcentrated orange juice of stage 2; C3, cryoconcentrated orange juice of stage 3.
Titratable acidity expressed in citric acid (g/100 mL).

Table 2. Total phenolic content for the cryoconcentrated and ice fractions and process efficiency of the three-stage cryoconcentration of orange juice

Stage		TPC (mg GAE.100 mL^{-1})	Eficiency (E%)
Acerola juice		$0,524^{Ab}$±0,01	
Stage 1	C1	$0,578^{ab}$±0,02	49,21±0,10
	I1	$0,293^{BC}$±0,07	
Stage 2	C2	$0,594^{ab}$±0,10	70,49±0,20
	I2	$0,175^{C}$±0,01	
Stage 3	C3	$0,662^{a}$±0,03	39,21±0,70
	I3	$0,403^{BC}$±0,10	

Data are expressed as mean ± SD (n = 3) of the total phenolic content in the concentrated samples and ice in each cryoconcentration stage. Different superscript lowercase letters indicate a significant difference ($p < 0.05$) between the initial juice and concentrates of each cryoconcentration stage. Different uppercase letters indicate a significant difference between the initial juice and the ice of each cryoconcentration stage.

The results found corroborate those reported by Adorno *et al.* (2016) for strawberry juice, Nunes *et al.* (2015) for aqueous extract of yerba mate and Benedetti *et al.* (2015) for tofu whey concentration. However, they differ from those reported by Boaventura *et al.* (2013) for aqueous extract of yerba mate and Belén *et al.* (2012) for wastewater from tofu production. According to these authors, the greater efficiency of cryoconcentration is usually presented in the first stage, with a decline occurring with the evolution of the stages due to the increase in the retention of solids in the ice. The results found suggest that a lower retention of CFT in ice promotes greater efficiency in the process.

3.3. Antioxidant activity assays

Table 3 presents the DPPH results for the antioxidant capacity of fresh juice and concentrates, expressed in µmol TEAC. mL-1. A significant increase ($p<0.05$) in the antioxidant activity of all concentrates (C1, C2, C3) can be observed when compared to fresh juice. Concentrate C3 had an increase of approximately 1.71 times in antioxidant potential. Haas *et al.* (2022) and Almeida *et al.* (2023) observed an increase in antioxidant activity in the concentrated fractions of cryoconcentration, by the DPPH method, for orange juice and *Morinda citrifolia* L. tea., respectively. Higuera (2013) and Moreno *et al.* (2014) also reported an increase in antioxidant activity (determined by the DPPH method) of coffee aqueous extract concentrates and coffee extract, respectively, corroborating the present study. The main bioactive compounds in acerola are vitamin C and carotenoids. Among the carotenoids, anthocyanins stand out, which have a suitable chemical structure to act as an antioxidant, as they can donate hydrogens or electrons to free radicals. The greater antioxidant activity is related to the presence of hydroxyl groups in the 3' and 4' positions of ring B, which confer high stability to the formed radicals (Cao *et al.*, 1997). The free hydroxyl groups in positions 3 and 5, together with the carbonyl group in position 4' are electron donors (Rice-Evans *et al.*, 1996).

Therefore, anthocyanins are important free radical scavengers. Also due to their chemical structure, anthocyanins can act as singlet oxygen deactivators.

Table 3. Antioxidant activity via DPPH in initial juice and cryoconcentrated fractions obtained by block freeze concentration

Samples	DPPH method (µmol TROLOX. g^{-1})
Acerola juice	$16,42^{b}$±2,12
C1	$23,72^{c}$±1,04
C2	$22,68^{c}$±0,61
C3	$28,12^{a}$±1,69

Data are expressed as mean ± SD (n = 3) of the total phenolic content in the concentrated samples and ice in each cryoconcentration stage. Different superscript lowercase letters indicate a significant difference ($p < 0.05$) between the initial juice and concentrates of each cryoconcentration stage.

4. Conclusions

This study showed that it is possible to increase the content of bioactive compounds, represented by the content of phenolic compounds and antioxidant activity, by the block cryoconcentration method, for in natura acerola juice. Cryoconcentration proved to be an alternative to preserve the nutritional quality of acerola juice and promoted an increase in the concentration factor in relation to the total solids content, with an average increase of approximately 144% in the first stage, 121% in the second stage and 131 % in the third step.Concentrated fluids showed an increase in the content of compounds in all stages of cryoconcentration, especially in the final stage (C3). In addition, there was 260% in the soluble solids content and 240% in the acidity of the concentrated acerola juice (C3).The antioxidant capacity measured by the DPPH method increased significantly about 1.7 times when compared to the initial juice, with the highest concentration found in C3.The results of this study indicate that the method of cryoconcentration in blocks, applied to acerola juice, increased the content of total and soluble solids, the antioxidant activity and the content of total phenolic compounds, proving to be a viable method for the concentration of compounds biological agents and an important technology for the concentration of acerola juice.

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Acknowledgment

The current manuscript was carried out with the UEMS support, Institutional scientific initiation program (PIBIC/UEMS). The authors would like to thank National Council for Technological and Scientific Development (CNPq) for the scholarship granted. We also confirm that the authors have no conflict of interest.

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FORMULATION AND EVALUATION OF UV RESISTANT PROPERTY OF THE HERBAL CREAM USING CRUCIFEROUS VEGETABLES LEAVES

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https://doi.org/10.34302/crpjfst/2023.15.4.15

Article history:
Received: 29 November 2022
Accepted: 2 December 2023

Keywords:
UV resistance ;
Herbal cream;
Brassicaceous family;
GC-MS;
Antioxidant activity;

ABSTRACT

The demand for cosmeceuticals is rapidly expanding. The expansion is due to the availability of new ingredients, the financial rewards for developing successful products, consumer formulation needs maintenance of quality standards. the quality of a formulation should satisfy the consumer's need in terms of its performance. Herbal creams have several advantages over conventional chemical-based creams. Herbs and herbal preparations have a high potential due to their antioxidant activity, and anti-inflammatory activity primarily. The present study aims to prepare a UV resistant herbal cream containing the leaf extracts of brassicaceous vegetables (cauliflower, cabbage). The objective of this study was to develop sunscreen cream formulations with high sun protection factor (spf) and satisfied characteristics. This study focuses on uv protection from the sun and discusses potential herbal candidates with antioxidant properties that can serve as a strong barrier in cosmeceuticals to protect skin against harmful UV rays. GC-MS result provides the presence of various bioactive compounds and the identified 19 bioactive compounds such as N-hexadecanoic acid, dodecanoic acid, cyclohexanone, 4-H-Pyran-4-one, oleic acid etc. Mainly due to their antioxidant activity, these compounds have been suggested for potential use in cosmetics and the pharmaceutical industry, and this review aims to summarize current knowledge on the natural sources and biological activity of the substances.

1. Introduction

UV radiation is part of natural energy. It must be produced by the sun. On the electromagnetic spectrum, UV light the shorter wavelengths than visible light. There are 3 general types of UV rays. UVa, UVb and UVc. Each of these has a different wavelength. Two types of UV radiation are confirm to the risk for skin cancer (Amandine *et al*., 2019). Although UVc possesses the highest energy, it has the potential greatest for biological damage and are filtered effectively by the ozone layer. Therefore, it is not considered to be a factor in solar exposure of human beings and is not of biological relevance. Amount of UVA and UVB which reaching is affected by latitude, altitude, season, cloudiness, time of the day, and ozone layer on the earth's surface. The highest irradiance at the equator and of the higher altitudes. On the earth's surface, the ratio of UVB to UVA is 1: 20. The response to UVB irradiation on human skin leads to erythema, edema, and pigment darken continued by the thickening of the epidermis and dermis, delayed tanning and affects the synthesis of vitamin D.

The main cruciferous vegetable species is the *Brassica. Oleracea* (cabbage) which

includes vegetable type forms and forage forms such as kale, cabbage, broccoli, Brussels sprouts, cauliflower and others; Phenolic compounds have received considerable attention for being potentially protective factors against cancer and heart diseases, Flavonols are the most widespread of the flavonoids. Quercetin, kaempferol and isorhamnetin, these are main flavonols in *Brassica* crops. These are most found as O-glycosides. All these compounds are found in *Brassica* family. Flavonoids protect the plants against UV rays, which, to some extent, results from the fact that they can act as a screen absorbing, Besides UV absorption, flavonoid compounds may also transfer the photo energy to or from other molecules via sensitization. The role of flavonoids in response to UV radiation is mostly due to the scavenging of ROS generated during irradiation. It is reported that the ethanolic leaf extract of *Brassica oleracea* L. var capitata contains the highest amount of phenolic compounds and exhibited the greatest anti-oxidant activity, the various activities such as antioxidant, phenolic flavonoid content all leads in the factor of protection against UV rays.

Herbal extracts and oils have complex conformations, which results in the exposition of dissimilar effects, such as antioxidant, sun blocking, anti-inflammatory, and immunomodulatory. The efficacy of extracts in improving the skin advent and handling of various skin diseases is well understood. Plants due to their antioxidants likely are known as an attractive choice to be used in sunscreen designs for the prevention of skin impairment due to solar radiation. Because of the damaging effects of UV radiation, sunscreen formulations are developed to protect the skin against the harmful effects of UV radiation by either forming a protective barrier on the skin's surface or by absorbing the harmful rays. The UV filters mostly need oily base for their dissolution, so the sunscreen formulations commonly use oily base for solubilizing the UV filters. It was reported that oil-in-water or water- in-oil system were most common for sunscreen formulation. But the oily vehicle of emulsion system can leave the skin greasy which may not be suitable for acne prone skin. Emulgel, a formulation comprising emulsion incorporated in gel base can overcome earlier problem by making the formulation more water-based and less greasy (Anandan *et al*.,2018). Sunscreen it refers to the photoprotecting agent for UV protection. It is used to aid the tody's defense mechanisms to protect against harmful UV radiation from the sun. Hence after extraction of quercetin should be done to make an effective UV protection herbal cream. The sunscreen agents used currently in sunscreens for UV protection are not efficient against reactive oxygen species (ROS). Therefore, these formulations contain antioxidants to neutralize these ROS (Bhattacharya & Sherje,2020).

2. Materials and methods

2.1.Materials

30 gm of powder was extracted by Soxhlet apparatus with methanol (300ml). Extraction with different solvents like petroleum ether, ethyl acetate, methanol, distilled water was done using the soxhlet apparatus. Briefly, for every 300 mL of the each solvent, 30 g of the crushed plant leaves powder was used for soxhlet extraction. After extraction for 3 consecutive days, the crude liquids were evaporated. After evaporation extracts were preserved at 37°C. Then the extracts are dissolved in distilled water for phytochemical analysis (Ashish Aswal *et al*.,2013).

2.2 Extractive value determination

Extractive values are used for the determination of exhausted or adulterated drugs. The value of the crude drug limits the quality as well as the concentration of the drug. Thus, petroleum ether, ethyl acetate, methanol and water soluble extractive values were determined. The obtained extracts were concentrated to dryness by keeping filtrate for complete evaporation of solvent. The value in percentage was calculated by using the formula and recorded (Assunta Raiola et al.,2017).

Extractive value (%) = Weight of dried extract/ Weight of plant material X 100

(1)

2.3. Sun protection factor analysis

The in vitro sun protection factor was determined by the ultraviolet-visible spectrophotometry method described. Spectrophotometric readings were obtained for each extract (100 µg•mL−1) at 290–320 nm and SPF values were determined using Equation

$$SPF = CF \times \Sigma\ 290\ 320 \times EE(\lambda) \times I(\lambda) \times Abs(\lambda) \quad \textbf{(2)}$$

where SPF stands for sun protection factor: CF for correction factor; EE(λ) is the erythemogenic effect of wavelength radiation (λ) nm (Ahmady *et al*,2020).

2.4. Antimicrobial activity

Agar well diffusion method:

The agar well diffusion method was employed for the determination of antimicrobial activity. To brief, wells are made in Nutrient agar plates using cork borer (7 mm diameter) and the inoculums containing 100µl of microbial strains was spread on the plates with the help of glass spreader in an aseptic condition. 20µl,40µl ,60,(T1) and 20µl, 40µl, 60µl (T2) of *Brassica oleracea* extracts; 30mg of standard antibiotics was filled in wells with the help of sterile micropipettes separately. The plates were incubated at 37o c for 24 hours at room temperature. The diameter for the zone of inhibition was measured in millimeter (mm) (Brian *et al*.,2009).

2.5. Secondary metabolite analysis of plant extracts

2.5.1. GC-MS Analaysis

The Clarus 680 GC was used in the study of employed a fused silica column, packed with Elite-5MS (5% biphenyl and 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components are separated using Helium at a constant flow of 1 ml/min. The injector temperature is set at 260°C during the chromatographic run. The 1µL of extract sample injected is into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min and 300 °C, where it was held for 6 min. The mass detector conditions was transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

2.6. Antioxidant assay of selected plant extracts

2.6.1. Ferric Reducing Antioxidant Power (Frap) Assay:

The assay was based upon the methodology of Benzie and Strain(1996). The FRAP reagent consists of 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$ and 250 mM sodium acetate buffer (pH 3.6). FRAP reagent was freshly prepared by mixing TPTZ solution, $FeCl_3$ solution and acetate buffer in a ratio 1:1:10. A 100 µl of extract solution I added with 900 µl of FRAP reagent. After the mixture stood at 37 °C for 4 minutes, the absorbance at 593 nm was determined against the blank. Ascorbic acid was used as standard. (Ayyappadasan *et al*., 2017), (Rubavathi *et al*., 2020)

$$\%Activity = AControl - Asample\ /AControl \times 100 \quad \textbf{(3)}$$

2.6.2. DPPH ASSAY(2, 2 Diphenyl-1-Picrylhydrazyl Radical Scavenging Assay)

The free radical scavenging capacity of extracts was determined by using DPPH. The DPPH solution (0.006% w/v) is prepared in 95% of methanol. Different concentrations of the test sample which is to be examined for antioxidant activity is prepared (20-100µg/ml). Different concentration of test sample of extracts were mixed with 5 µl of DPPH solution in dark. Ascorbic acid is strong which is a antioxidizing agent is taken as standard. 3 ml of different concentration of standard solution of ascorbic acid was mixed with 5 µl of DPPH solution in dark. The prepared

solution of ascorbic acid and plant extracts samples was incubated for half an hour and then absorbance is taken at 517 nm. Methanol serves as a blank and the experiment was expressed as the inhibition percentage of free radical by the sample and was calculated (Daniela *et al*.,2020)(Rubavathi and ramya, 2016).

$$\%\text{inhibition} = A_{Control} - A_{sample} / A_{Control} \times 100 \quad \textbf{(4)}$$

2.6.3. H_2O_2 Assay

The ability of the extract to scavenge hydrogen peroxide was determined. A solution of hydrogen peroxide (2mmol/l) was prepared in the phosphate buffer (pH 7.4). Extracts (1–10 µg/ml) were added to hydrogen peroxide solution (0.6 ml). The absorbance of the hydrogen peroxide at 230 nm was determined after 10 mins contrary to a blank solution containing phosphate buffer without hydrogen peroxide, compared with ascorbic acid, the reference compound (Ahmady *et al*,2020).

H_2O_2 activity(%) = (Abs control – Abs sample/Abs control) *100 **(5)**

Where, Abs (control): Absorbance of the control

Abs (test): Absorbance of the extracts/standard.

2.6.4. Phosphomolybdate Assay

Total antioxidant activity (TAC) of REE and RME was determined using ascorbic acid as the standard. Phosphomolybdate reagent was prepared by mixing 100 ml of 28 mM sodium phosphate, 100 ml of 0.6 M sulphuric acid and 100 ml of 4 mM ammonium molybdate solutions. To 3 ml of phosphomolybdate reagent, 300 µl of different concentrations (50-250 µg/ml) of extract solutions were added and incubated in the dark room for 90 min at 95°. The absorbance was measured spectrophotometrically at 765 nm (Désiré-Ndayazi *et al*.,2017).

$$\text{TAC \%scavenging} = (A_0 – A_1/A_0) \times 100, \quad \textbf{(6)}$$

where, A_0 is the absorbance of the control after 10 min and A_1 is the absorbance of the sample at 10 min. The concentration of extract at which 50% inhibition is observed (IC_{50}) is calculated in µg/ml.

2.7 Anti Inflammatory Activity

A concentration series of 50, 100 and 250 µg/ml of FELE of P. guajava and Diclofenac sodium was taken as the test sample and the reference drug respectively. The test was performed using six wells for each sample. The absorbances were measured at 255 nm using a multi-mode micro plate reader. The control represents 100% protein denaturation. The test procedure was repeated 6 times. The results were compared with the reference drug. The percentage of inhibition was calculated using the following formula (Fouad *et al*.,2013).

$$\textbf{\% Inhibition} = 100 \text{ X } (V_t/V_c - 1) \quad \textbf{(7)}$$

where, V_t = absorbance of the test sample V_c = absorbance of the control The plant extract concentration for 50% inhibition (IC_{50}) was determined by the dose-response curve.

2.8. Preparation of Herbal Cream

Oil in water (O/W) emulsion-based cream (semisolid formulation) was formulated. The emulsifier (stearic acid) and other oil-soluble components (Cetyl alcohol, almond oil) are dissolved in the oil phase (Part A) and heated to 75° C. The preservatives and other water-soluble components such as Methyl paraban, Propyl paraban, Triethanolamine, Propylene glycol, ethanol extract of Aloe vera, Cucumis sativus and Daucus carota were dissolved in the aqueous phase (Part B) and heated to 75° C. After heating, the aqueous phase was added in portions to the oil phase with continuous stirring until cooling of emulsifier took place (Francisco *et al*.,2011).

3. Results and discussions

3.1.Phytochemical analysis of *Brassica oleracea*

The phytochemical compounds present in petroleum ether, ethyl acetate, methanol and water extracts of *Brassica oleracea* leaves were shown in the table 1 as given below. Alkaloids were absent in all extracts. Flavonoids were present in all extracts which may indicate the presence of antioxidant, anticancer, antimicrobial activity, and nutraceutical applications. Terpenoids were present in water and methanolic extracts which have aromatic qualities and play a role in herbal remedies. Tannins are present in all extracts. Saponin was absent in all extracts which may help to bind proteins. Similar observations were reported by so and metabolites present in the *Brassica oleracea* extracts were compared to the report by (Geetha Surendran *et al*.,2019). Carbohydrates, phenols, proteins and amino acids were present in all extracts.

Table 1. Phytochemical analysis of *Brassica oleracea* variety capitata and *Brassica oleracea*var.botrytis

Compound	*Brassica oleracea*var.capitata				*Brassica oleracea*var.botrytis			
	PE extract	Ethyl acetate extract	CH_4 extract	H_2O extract	PE extract	Ethyl acetate extract	CH_4 extract	H_2O extract
Carbohydrates	+	+	+	+	+	+	+	+
Amino acids	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Flavonoids	+	-	+	+	+	-	+	+
Alkaloids	-	-	-	-	-	-	-	-
Phenols	+	+	+	+	+	+	+	+
Terpenoids	-	-	+	+	-	-	+	+
Saponins	-	-	-	-	-	-	-	-
glycosides	+	+	+	+	+	+	+	+
Proteins	+	+	+	+	+	+	+	+

3.2. Antimicrobial activity

The *B.oleracea* extracts of water, petroleum ether, methanol, ethyl acetate were tested against *E.coli*, *S.aureus*, *K.pneumoniae* and *P.vulgaris* were represented in the Table 2. Chloramphenicol was used as a standard for evaluating the antimicrobial activity of the plant extracts. The Ethyl acetate extract of cabbage showed the maximum zone of inhibition against *E.coli* at 0.7cm and the Water extract of cabbage showed the maximum zone of inhibition against *S.aureus* at 0.7 cm. The methanolic extract of *Brassica oleracea* (cauliflower) variety showed the highest antimicrobial activity against *P.vulgaris* with 0.9 cm. other extracts of *Brassica oleracea* showed no zone of inhibition. In comparison to other extracts, the standard values of both the species *P.vulgaris* and *S. aureus* showed the highest zone of inhibition in comparison with the other organism that is taken into consideration. Methanol extract showed the highest antimicrobial activity (Ayshwarya,and Sudharameshwari, 2015))

3.3. Sun protection factor analysis

SPF is a number given to sunscreen formulation to determine efficacy and is useful when applied at about 2mg/cm^2. In the present work different extracts (petroleum ether, ethyl acetate, methanol and water) of *Brassica oleracea* var.capitata (cabbage) and vari. Botrytis (cauliflower) was estimated the sun protection factor. Compared to other extracts the methanolic extracts of *Brassica oleracea* var.capita showed a high sun protection factor

was indicated in the Table 3. SPF value for sunscreen above - is considered as good sunscreen activity. From both varieties of *Brassica oleracea* the methanolic extracts of cabbage and cauliflower have high sun protection value than other extracts. Both methanolic extracts of *B.oleracea* have the maximum sun protection factor. The SPF analysis was done in triplicates. In three times, methanolic extracts showed high sun protection value. The absorbance values are taken in UV spectrophotometer at 290-320 nm. When the standard value is taken to consider it also shows high SPF for methanolic extracts rather than other extracts. On conclusion gives the methanolic extracts of *B.oleracea* can be used as a source of UV protection formulation.

Table 2. Antimicrobial activity of the *Brassica oleracea* extract

	Cauliflower				**Cabbage**			
Organism	Petroleum ether	Ethyl acetate	Methanol extract	Water extract	Petroleum ether	Ethyl acetate	Methanol extract	Water extract
E.coli	**0.5**	**0.3**	**0.6**	**0.4**	**0.5**	**0.7**	**0.6**	**0.3**
P.vulgaris	**0.2**	**0.4**	**0.9**	**0.6**	**0.1**	**0.12**	**0.1**	**0.1**
S.aureus	**0.1**	**0.1**	**0.2**	**0.3**	**0.2**	**0.4**	**0.6**	**0.7**
K.pneumonia	**0.15**	**0.1**	**0.5**	**0.4**	**0.1**	**0.2**	**0.2**	**0.1**

Table 3. Sun protection factor analysis of 8 extracts of *B.oleracea* cabbage and cauliflower

Name of the extracts	**Wavelength absorbance (nm)**							**Sun protection factor (SPF)**
	290	**295**	**300**	**305**	**310**	**315**	**320**	
EE*I	**0.015**	**0.082**	**0.287**	**0.328**	**0.186**	**0.084**	**0.018**	
Petroleum ether extract of cabbage	1.150	1.162	1.186	1.230	1.271	1.300	1.329	86
Ethyl acetate extract of cabbage	1.140	1.149	1.175	1.207	1.227	1.241	1.261	84
Methanolic extract of cabbage	1.154	1.170	1.196	1.238	1.280	1.312	1.324	87
Aqueous extract of cabbage	1.141	1.150	1.185	1.225	1.270	1.299	1.320	85
Petroleum ether extract of cauliflower	1.150	1.152	1.153	1.154	1.147	1.125	1.031	79
Ethyl acetate extract of cauliflower	1.146	1.151	1.152	1.148	1.139	1.102	0.983	78
Methanolic extract of cauliflower	1.143	1.158	1.182	1.211	1.247	1.275	1.311	85
Aqueous extract of cauliflower	1.139	1.138	1.135	1.118	1.092	1.064	1.081	77

3.4. Antioxidant activity

3.4.1 DPPH ASSAY

The antioxidant activity of *Brassica oleracea was* assessed based on the free radical scavenging effect of the stable free radical DPPH as per the method of (Anandan *et al*., 2018). DPPH assay was carried out for the methanolic extracts *Brassica oleracea* (cauliflower) and *Brassica oleracea* (cabbage) (Fig:3.1).The methanolic extract of *Brassica oleracea* (cauliflower) shows 40 % of inhibition at (40µg/ml) concentration. The methanolic extract of *Brassica oleracea* (cabbage) shows 30% of inhibition at (40µg/ml) concentration (Figure 1). The standard shows 30% inhibition in *Brassica oleracea*(cauliflower) whereas in 25% in *Brassica oleracea* (cabbage) which is moreover similar to the test standard. The standard value is compared with the existing inhibiting value of the compound and found to be 32% at 40 µg/ml concentration (Guriya *et al*.,2015).

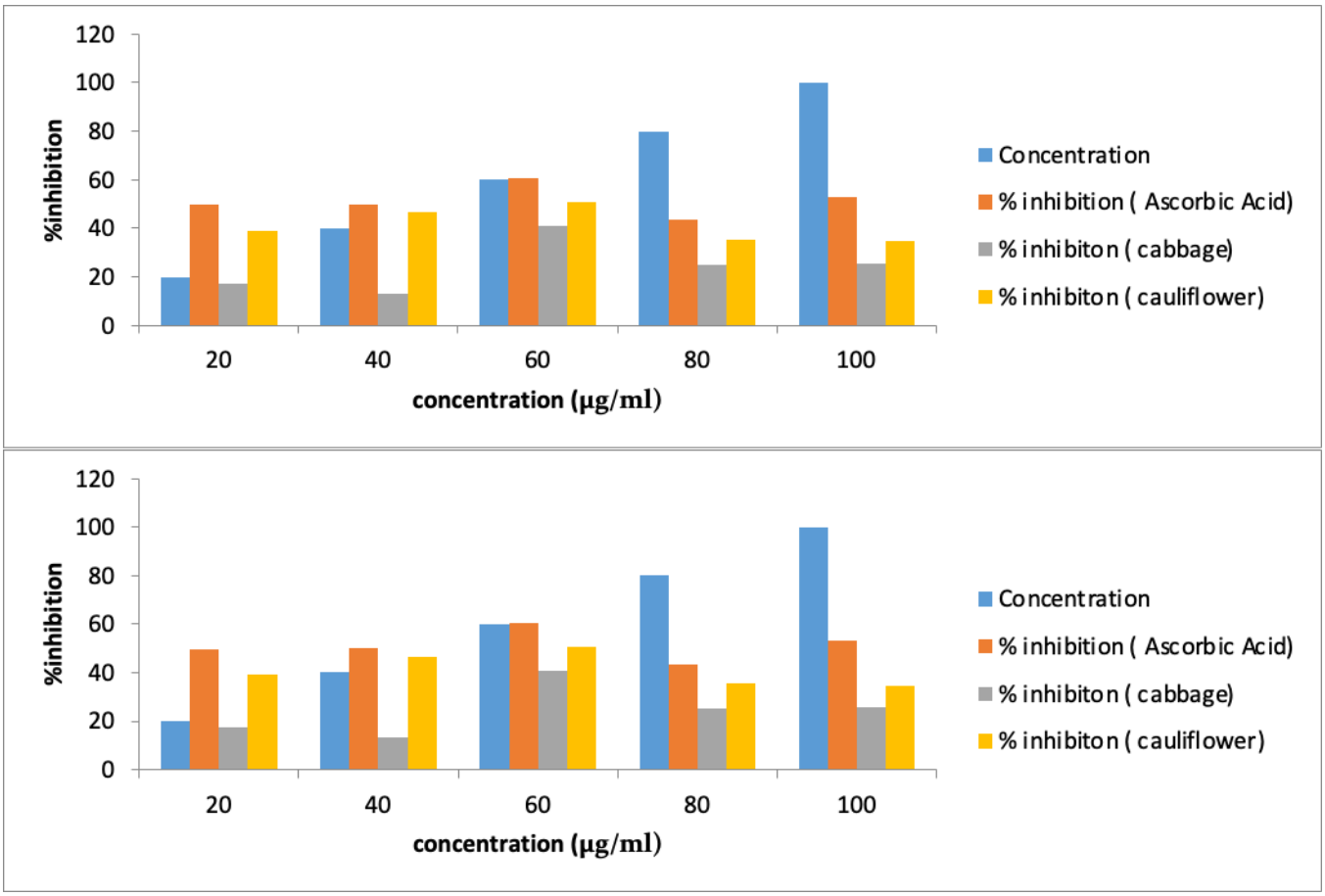


Figure 1. Determination of DPPH assay of *B.oleracea* extracts

3.4.2. Frap Assay

The antioxidant activity of the *Brassica oleracea* (variety capitata and variety botrytis) was done by FRAP assay method .FRAP assay was carried out for the methanolic extracts *Brassica oleracea* (cauliflower) and *Brassica oleracea*. The antioxidant activity of the brassica oleracea (variety capitata and variety botrytis) was done by FRAP assay method (Francisco *et.al*.,2011).FRAP assay was carried out for the methanolic extract of *Brassica oleracea* (cauliflower) shows 40 % inhibition at (100 µg/ml) concentration was described in Figure 2. The methanolic extract of *Brassica oleracea* (cabbage) shows 40 % of inhibition at 80µg/ml concentration in the standard kept for

Brassica oleracea (cauliflower) and *Brassica oleracea* (cabbage) is 35 % of inhibition at (100 µg/ml) concentration and *Brassica oleracea* (cabbage) shows 35 % of inhibition at 80µg/ml concentration (Guriya *et al*.,2015). The standard value is compared with the existing inhibiting value of the compound and found to be little increase in the inhibition activity at 80µg/ml concentration and the standard value is about 40% and at 100 µg/ml the standard activity of inhibition is significant increase about 60% (Guriya *et al*.,2015).

3.4.3. H_2O_2 Assay

H_2O_2 assay was carried out for the methanolic extracts *Brassica oleracea* (cauliflower) and *Brassica oleracea* (cabbage). The methanolic extract of *Brassica oleracea* (cauliflower) shows 61.51 % inhibition at (60µg/ml) concentration. The methanolic extract of *Brassica oleracea* (cabbage) shows 58.55% of inhibition at (60µg/ml) concentration (Figure 3). The standard value is compared with the existing inhibiting value of the compound and found to be a significantly similar activity of inhibition at 60µg/ml concentration and the standard value is about 60% (Guriya *et al*., 2015).

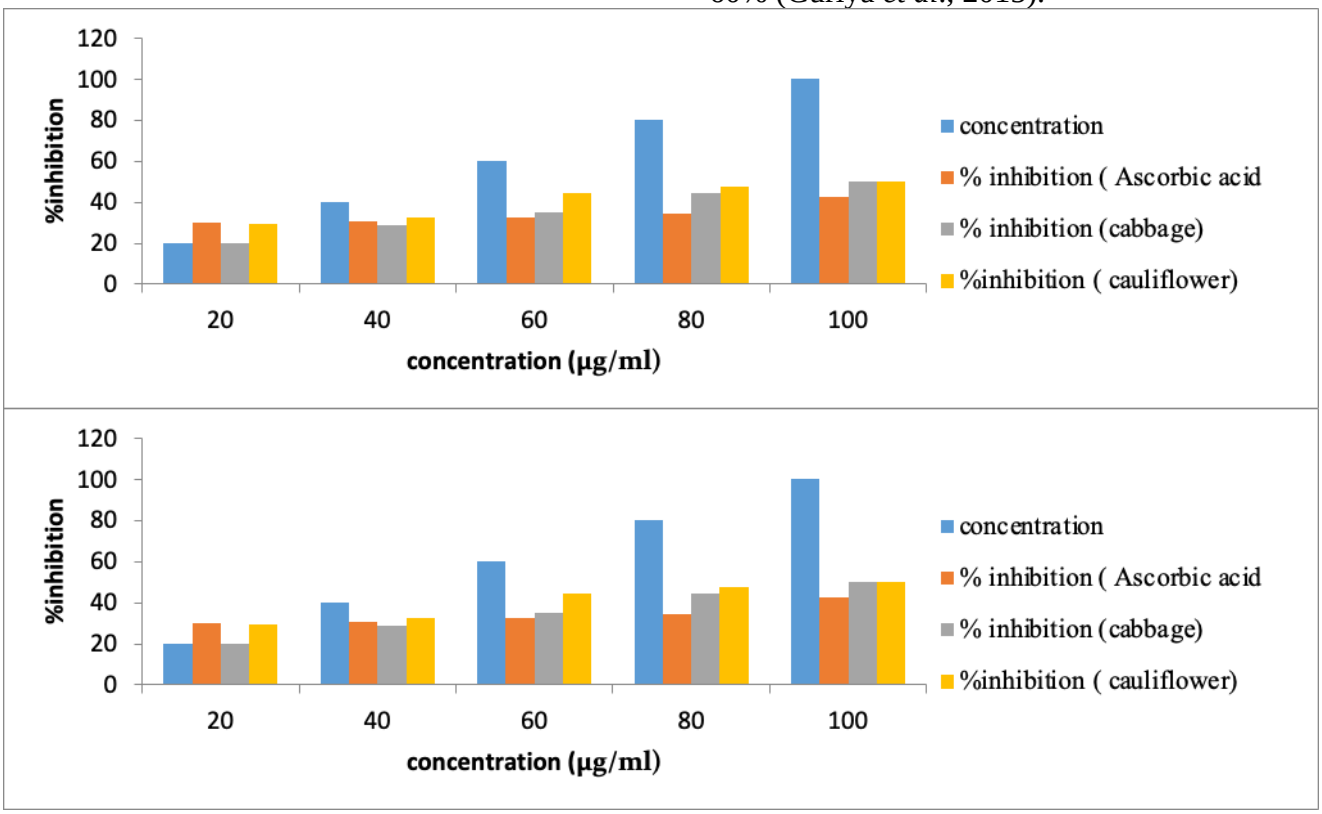
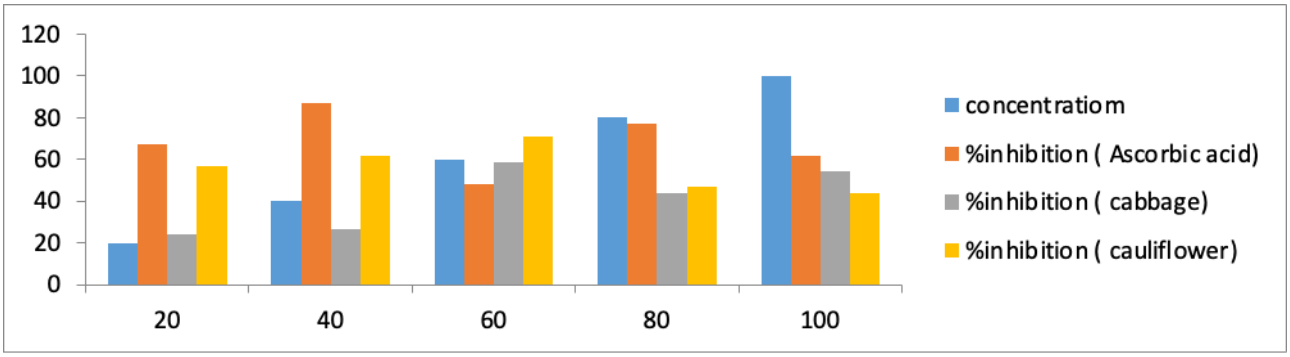


Figure 2. Determination of FRAP assay of *B.oleracea* extracts



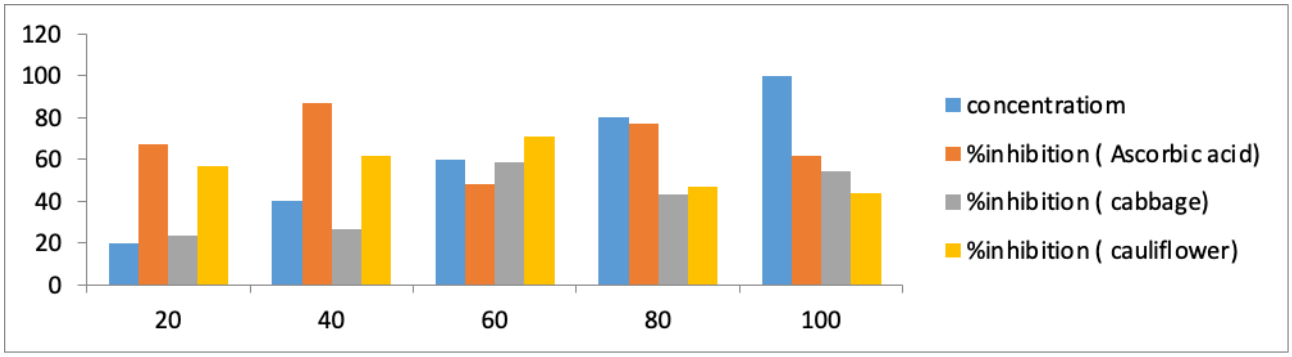


Figure 3. Determination of H_2O_2 assay

3.4.4. Phosphate molybdenum assay

The antioxidant activity of the *Brassica oleracea* was done by phosphate molybdenum assay (Ahmady *et al*,2020). Cauliflower and cabbage show highest antioxidant activity. PM assay was carried out for the methanolic extracts *Brassica oleracea* (cauliflower) and *Brassica oleracea* (cabbage) as represented as Figure 4. The methanolic extract of *Brassica oleracea* (cauliflower) shows 70.76% of inhibition at (60µg/ml) concentration. The methanolic extract of *Brassica oleracea* (cabbage) shows 61.79 % of inhibition at (60µg/ml) concentration. The standard value is compared with the existing inhibiting value of the compound and found to be significantly decreased activity of inhibition at 60µg/ml concentration and the standard value is about 55.5% (Hasan *et al*.,2016), in the standard value too the methanolic extract shows greater range of anti oxidant activity which is same of the test compounds.

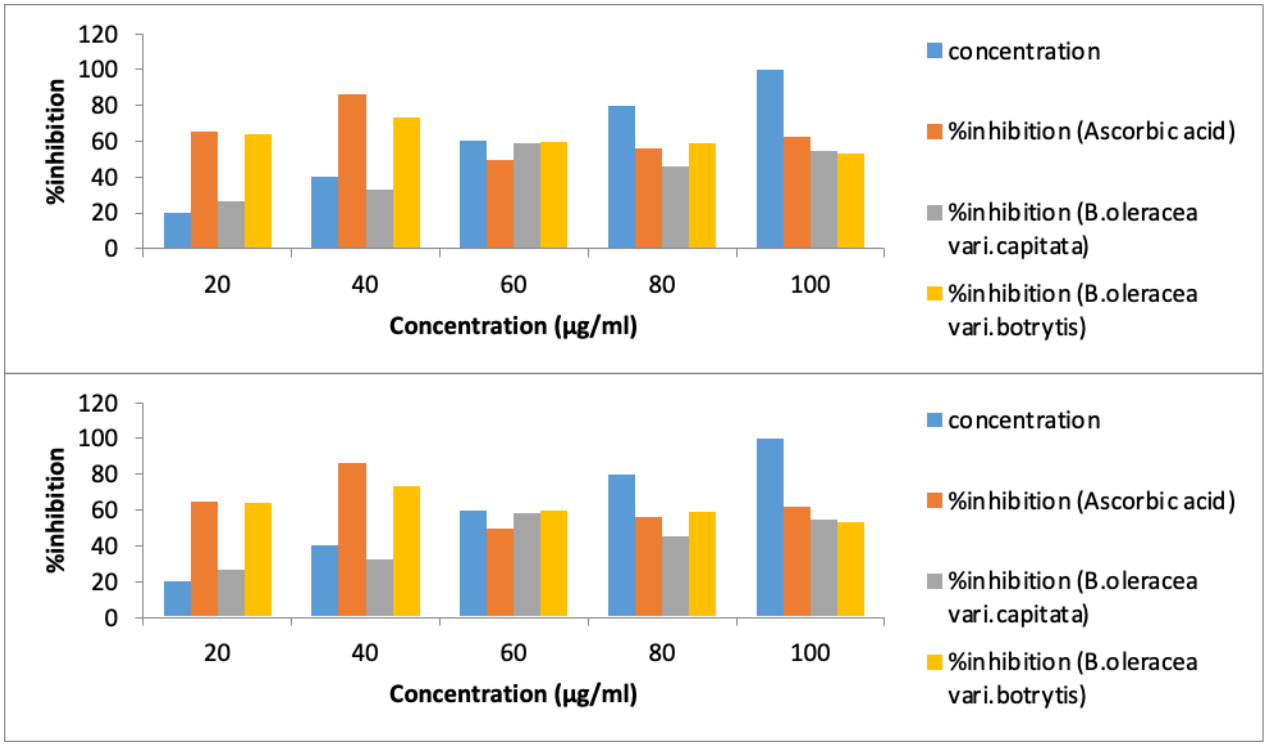


Figure 4. Determination of phosphate molybdenum assay

3.5. Anti inflammatory activity

Anti inflammatory activity was done by bovine serum albumin method (Ahmady *et al*,2020). Anti inflammatory was carried out for the methanolic extracts *Brassica oleracea* (cauliflower) and *Brassica oleracea* (cabbage)

(Figure 4). The methanolic extract of *Brassica oleracea* (cauliflower) shows 70 % of inhibition at (60µg/ml) concentration. The methanolic extract of *Brassica oleracea* (cabbage) shows 53.9% of inhibition at (100µg/ml) concentration. The standard value is compared with the existing inhibiting value of the compound and found to be significantly increased activity of inhibition at 100µg/ml concentration and the standard value is about 56.3% and the activity is decreased to 45% at 60µg/ml concentration (Guriya *et al*.,2015).

3.6. GC-MS ANALYSIS

Gas chromatography-mass spectrometry (GC-MS) was used to identify the secondary metabolites of the plant extracts. GC-MS analysis was done in IIT Bombay ,Powai. We have identified 19 compounds in *Brassica oleracea* variety botrytis.

From the Table 4. GC-MS analysis of *B.oleracea* was tabulated for all compounds and several compounds shows a greater results when compared to the other compounds. The compounds such as n-Hexadecanoic acid, Dodecanoic acid, Cyclohexanone, 4H-Pyran-4one, 2,3-dihydro-3,5,-dihydroxy-6-methyl-, 3-Deoxy-d-mannoic lactone, 1,2,3,-Propanetriol, 1,5-{Hydroxymethyl-2{5H}-furanone, 4H-Pyran-4-one, 2,5-Dimethyl-4-hydroxy-3{2h}-furanone showed show a great results in the range on the area percentage. Notably, the compound 1,2,3,-Propanetriol shows the highest reading on the area percentage. Few other compounds like n-hexadecanoic acid, v2-hexadecen-1-ol, alpha-tocopherol ,phytyl palmitate and pentalene, extracted from B. nigra (Sharma et al. 2018).

Table 4. GC-MS analysis of *B.oleracea* variety botrytis extracts

S.No	Identified compounds *(B.oleracea variety botrytis)*	Molecular formula	RT1 (min)	Area %	% content	[M]+
1.	Hexadecanoic acid	$C_{19}H_{38}O_4$	26.66	8312025.59	1719.83389	330
2.	Octanoic acid, 2-dimethylaminoethyl ester	$C_{12}H_{25}NO_2$	23.45	3107998.49	4599.52067	215
3.	17-Octadecynoic acid	$C_{18}H_{32}O_2$	21.08	1278959.70	11177.296	280
4.	9H-Pyrido[3,4]indole	$C_{11}H_8N_2$	19.29	4226087.02	3382.63345	168
5.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	18.52	15761614.16	906.969499	256
6.	Z-{13,14-Epoxy}tetradec-11-en-1-ol acetate	$C_{16}H_{28}O_3$	18.26	593790.98	24074.6387	268
7.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	17.43	2441084.30	5856.12848	296
8.	3`,5'Dimethoxyacetophenone	$C_{10}H_{12}O_3$	13.27	8981171.898	1591.69688	180
9.	Dodecanoic acid	$C_{12}H_{24}O_3$	12.9	19057230.51	750.124909	216
10.	Oleic Acid	$C_{18}O_{34}O_2$	10.94	657500.61	21741.886	282
11.	2-Cyclohexylpiperidine	$C_{11}H_{21}N$	10.48	8338312.66	1714.412	167
12.	Cyclohexanone	$C_{10}H_{14}O$	9.38	15191626.77	940.998849	150
13.	Tridecanonic acid	$C_{15}H_{30}O_3$	9.09	2269100.36	6299.98723	258
14.	2-Furancarboxaldehyde	$C_6H_6O_3$	7.99	14817080.70	964.785411	126
15.	1-Gala-1-ido-octonic lactone	$C_8H_{14}O_8$	7.25	2968822.42	481.512667	238
16.	4H-Pyran-4one, 2,3-dihydro-3,5,-dihydroxy-6-methyl-	$C_6H_8O_4$	6.58	16097077.59	888.068236	144
17.	CIS-2-Ethyl-2-hexan-1-0l	$C_8H_{16}O$	5.56	4021458.22	3554.75614	128
18.	Benzreneacetaldehyde	C_8H_8O	4.95	6449394.38	2216.53	120

19.	1,3-Propanediamine,N'-{ethylcarbonimidoyl}-N,N-dimethyl-	$C_9H_{17}N_3$	4.31	9661655.91	1479.59143	155
20.	Hexadecanoic acid	$C_{19}H_{38}O_4$	26.64	3521989.17	10169.1574	330
21.	Oleic acid	$C_{18}H_{34}O_2$	21.08	10491030.06	3413.93191	282
22.	n- Hexadecanoic acid	$C_{16}H_{32}O_2$	18.48	13468488.53	2659.21913	256
23.	Desulfoglucobrassicin	$C_{16}H_{20}N_2O_6S$	16.80	2585139.29	138.5444	368
24.	3-Deoxy-d-mannoic lactone	$C_6H_{10}0_5$	16.05	20909323	1712.90397	162
25.	3-Deoxy-d-mannoic lactone	$C_6H_{10}0_5$	15.81	25614292.95	1398.26863	162
26.	1,2-{3,4-Dimethoxyphenyl}-6-methyl-3,4-chromanediol#	$C_{18}H_{20}O_5$	13.27	8062767.55	4442.10528	316
27.	d-mannose	$C_6H_{12}O_6$	13.02	3229147.28	11091.3685	180
28.	3-Hydroxylauric acid	$C_{12}H_{24}O_3$	12.56	5070227.17	7063.91668	216
29.	Thioxan-3-one,oxime	C_5H_9NOS	10.90	3439724.10	10412.3648	131
30.	N-Nitroso-2,4,4-trimethyloxazolidine	$C_6H_{12}N_2O_2$	9.70	11474402.29	3121.3532	144
31.	2-Methyl-9-beta-d-ribofuransylhypoxanthine	$C_{11}H_{14}N_4O_5$	9.36	10398241.79	3444.39599	282
32.	1,2,3,-Propanetriol	$C_5H_{10}O_4$	8.64	121668318.17	294.371511	134
33.	2-Furancarboxaldehyde	$C_6H_6O_3$	8.29	11907757.84	3007.7587	126
34.	1,5-{Hydroxymethyl-2{5H}-furanone	$C_5H_6O_3$	7.74	53763650.57	716.388524	114
35.	2(3H)-Furanone	$C_4H_60_3$	7.51	2497618.42	14339.9256	102
36.	4H-Pyran-4-one	$C_6H_6O_4$	7.40	1397618.432	25268.4578	142
37.	4H-Pyran-4-one	$C_6H_6O_4$	6.75	53763650.57	666.16872	142
38.	9-Oxa-bicyclo{3,3,1}nonane-1,4-diol	$C_8H_{14}O_3$	6.28	2497618.42	14339.9256	158
39.	2,5-Dimethyl-4-hydroxy-3{2h}-furanone	$C_6H_8O_3$	5.59	13976408.45	25268.4578	128
40.	2,5-Dimethyl-4-hydroxy-3{2h}-furanone	$C_6H_8O_3$	5.29	10562986.24	3390.67585	128
41..	5-Butyldihydro-2{3H}thiophenone	$C_8H_{14}OS$	5.05	5615685.05	6377.79042	158
42.	2{Hexamethyleneimino}ethanol	$C_8H_{17}NO$	4.32	19899424.73	1799.83378	143

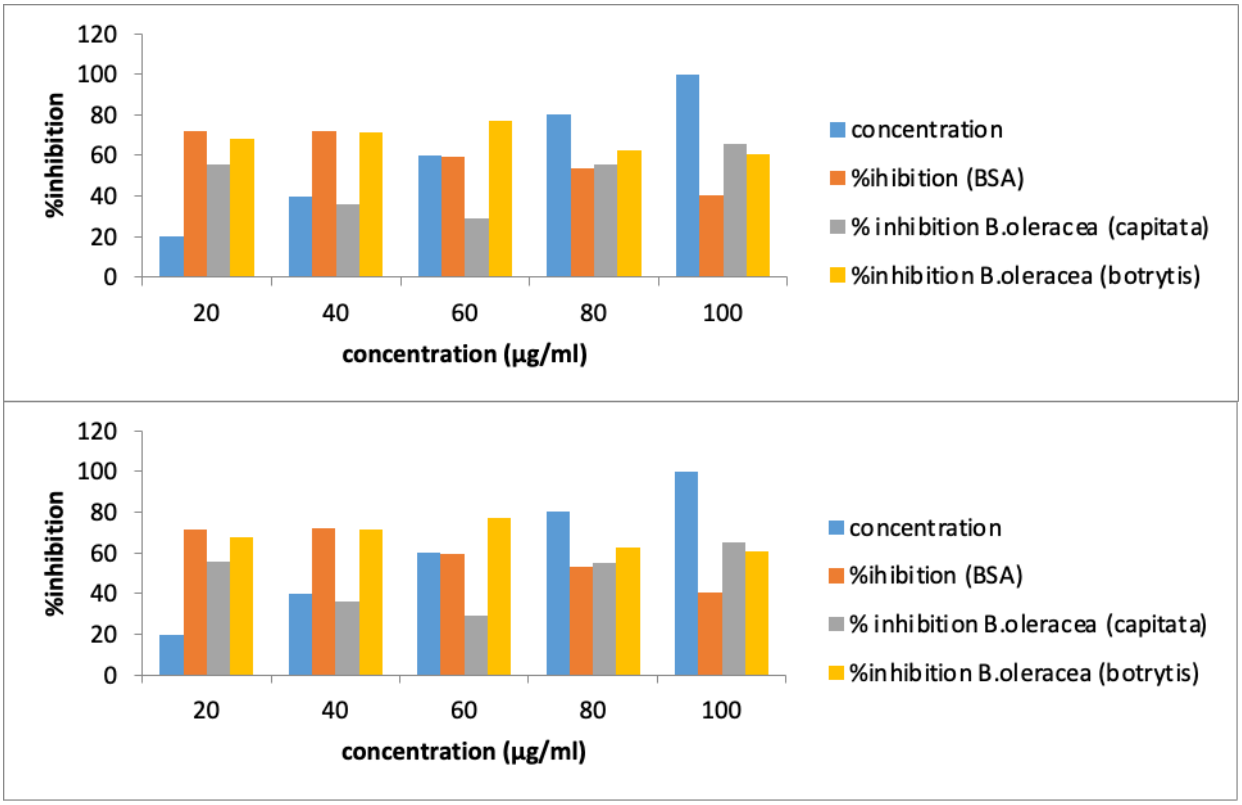


Figure 5. Determination of anti inflammatory activity by BSA method.

Figure 6. Formulation of UV-resistant herbal cream

3.7. Formulation of ultraviolet resistant herbal cream

UV resistant herbal cream, refers to the cream which act as a UV protecting agent. Hence after the extraction of flavonoid compounds the cream was formulated using (Ahmady *et al*,2020). The main ingredients will be Shea butter, aloe vera, coconut oil, almond oil along with bees wax. UV resistant herbal cream was represented as Figure 6.

Content		**Percentage**
Coconut Oil	-	10
Almond Oil	-	10
Water	-	25
Methanolic Extract (Cabbage)	-	7.5
Methanolic Extract (Cauliflower)	-	7.5
Beeswax	-	20
Shea Butter	-	20

4. Conclusions

The present study focused on collection of Brassicaceous plant leaves (*B.oleracea* variety.*capitata*,variety *botrytis*) to extract the sinapate ester derivatives and flavonoid compounds in low production cost to give an herbal cream containing UV resistant properties for persons who are affected by Ultraviolet

rays. The leaves were collected and extracted the UV-resistant compounds using different solvents like petroleum ether, ethyl acetate, Methanol and water. The extracts were dried using Petri dishes and stored at room temperature. Then the extractive value can be calculated. Estimation of Antibacterial activity, The Ethyl acetate extract of cabbage showed the maximum zone of inhibition against *E.coli* with 0.7 cm, and the Water extract of cabbage showed the maximum zone of inhibition against S.aureus with 0.7 cm.. The methanolic extract of *Brassica oleracea* (cauliflower) variety showed the highest antimicrobial activity against *P.vulgaris* with 0.9 cm. other extracts of brassica oleracea showed no zone of inhibition. The Methanol extract showed the highest antimicrobial activity. And sun protection factor analysis was carried out for the four different extracts. The compounds such as n-Hexadecanoic acid, Dodecanoic acid, Cyclohexanone, 4H-Pyran-4one, 2,3-dihydro-3,5,-dihydroxy-6-methyl-, 3-Deoxy-d-mannoic lactone, 1,2,3,-Propanetriol, 1,5-{Hydroxymethyl-2{5H}-furanone, 4H-Pyran-4-one, 2,5-Dimethyl-4-hydroxy-3{2h}-furanone showed show great results in the range on the area percentage in GC-MS analysis. The highest sun protection factor showed in methanolic extracts. Evaluating the antioxidant activity by DPPH, FRAP, H_2O_2 and phosphate molybdenum methods. Then the UV resistant compound has rich in the antioxidant property. The anti inflammatory activity also carried out by bovine serum albumin method. The maximum inhibition was obtained in both *Brassica oleracea* variety capitata (cabbage), and *Brassica oleracea* variety botrytis (cauliflower). Nearly 1g of extracts are produced in an extraction process. The cream without extract and the cream with extract pH can be calculated using pH meter. The without-extract cream pH is 4.2. Finally, the physicochemical properties of the cream were analyzed.

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Acknowledgment

The authors would like to acknowledge the Management, CEO, Principal and Department of Biotechnology, K.S. Rangasamy College of Technology, Tiruchengode for providing necessary facilities and support. We also acknowledge the support offered by DST, DST FIST No: 368 (SR/FST/College–35/2014)] and DBT-STAR College Scheme (BT/HRD/11/09/2018) for their support for the instrumentation.

CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

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ASSESSMENT OF PERSISTENT ORGANOCHLORINATED PESTICIDES RESIDUES IN COCOA BEANS FROM SELECTED COCOA FARMS IN EKITI STATE, NIGERIA

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https://doi.org/10.34302/crpjfst/2023.15.4.16

Article history:
Received: 29 November 2022
Accepted: 1 December 2023

Keywords:
Organochlorine;
Cocoa beans;
Pesticides residues;
Gas chromatograph.

ABSTRACT

With the importance of cocoa beans and its products, it is imperative to know whether the levels of pesticides residues are kept below the recommended levels to minimize the risk to human health. This study investigates the incidence and levels of organochlorinated pesticide residues in cocoa beans from eighteen selected cocoa farms in Ekiti State, Nigeria. Method 3550C of USEPA was employed to extract the pesticides from the samples, while a Gas Chromatography coupled with Electron Capture Detector (GC-ECD) was used for pesticides identification and quantification after careful extraction and clean-up on silica gel. The mean OCPs concentrations ranged from 0.0003 µg/g (α-BHC) to 0.163 µg/g (endosulfan sulphate) with α-BHC as the most frequently found pesticide residue. The concentration levels of lindane, α-BHC, p,p'-DDT, p,p'-DDD, heptachlor, heptachlor-epoxide and endosulfan II were below the maximum residual limit (MRL) in food as reported by European Union, while 0.92%, 2.78%, and 4.6% of endosulfan I, (β-BHC and δ-BHC) and (aldrin and dieldrin) respectively were above the MRL. The detectable levels of OCPs in the beans make it inevitable to conduct regular monitoring so as to ensure that the levels remains below prescribed limits by national and international standards.

1. Introduction

Cocoa farmers in Nigeria have actively used organochlorine pesticides (OCPs) for decades without minding the possible residues on the soils and farm products (Ibigbami *et al.*, 2017). Fourteen of the thirty-six Nigerian state grow cocoa, with the main producing States in the South-western part of the country, where most production areas located in Ondo, Osun, Ekiti, Ogun, Edo and Oyo States. The size of cultivated areas vary across states but an average cocoa farmer in Nigeria has a farm size of 2.5 ha (Oguntade, 2003), while the average delivered per farmer is less than 5 bags per season. Cocoa is the leading cash crop foreign exchange earner in Nigeria contributing over 26% of the Gross Domestic Product (GDP) of the non-oil export in Nigeria, and 19% contribution to the world market (UNCTAD, 2004; Faturoti *et al*., 2012). Pesticides are often employed by farmers because of pests and diseases. Cocoa mirids has been recognized as one of the most serious pest since 1908 due to their devastating effect (Dungeon, 1910). The most common species of mirids in West African countries are *Distantiella theobroma* and *Sahlbergella singularis*. Mirids damage alone, if left unattended to for three years and can reduce yields by as much as 75%.

Pesticides such as copper sulphate, benzene hexachloride, Aldrex 40, diazinon, chlorpyrifos, fenittrothion, Gammalin 20 (lindane), DDT among others have been used by Nigerian cocoa farmers (Asogwa and Dongo 2009; Oyekunle *et al*., 2017, ICCO, 2010),).

Organochlorine pesticides are a class of toxic chemicals containing carbon, hydrogen and chlorine; it is composed of five broad groups namely: dichlorodiphenyltrichloroethane (DDT) and analogues (e.g. diclofor, methoxychlor); the hexachlorocyclohexane or bezene hexachloride and their isomers (e.g. lindane, the ϒ-isomer); the cyclodienes e.g. chlordane, heptachlor, aldrin, dieldrin, endrin, endosulfan; the chlordecones: kelevan, mirex and the toxaphenes (Pope *et al*., 1994). Organochlorines (OCs) pesticides are toxic to biological organisms due to their high lipophilic properties (Lopez *et al*., 2010), resistance to chemical and biological degradation (Afful *et al*., 2010); adsorb on particulate matter due to low water solubility (Yang *et al*., 2005); ability to bioaccumulate and biomagnify (Zhou *et al*., 2006, Malik *et al*., 2009).

The use of OCPs for treatment of pests and diseases by cocoa farmers over the years would no doubt have led to the accumulation of their residues in the cocoa fruits.

Residues of OCPs have been reported in cocoa beans from Nigeria by Oyekunle *et al.* (2017), Aikpokpodion *et al.* (2012a, 2012b), while Owusu-Ansah *et al.* (2012), Frimpong *et al.* (2012), Okoffo *et al*. (2016) reported the levels in Ghana cocoa beans. With the little information on pesticides contamination of cocoa beans from Nigeria, hence, it is important to constantly monitor the residue concentrations of OCPs in cocoa beans given the potential health risk of pesticides. Therefore, the study aim in determining the occurrence and levels of persistent organochlorine pesticides in cocoa beans from Ekiti State to ascertain if their concentrations meet the prescribed limits by international standards.

2. Materials and methods

2.1. Study area

Ekiti is located between latitude 7^{o} 25^{1} – 8^{o} 2^{1} N and longitude 5^{o} 00^{1} – 6^{o} 00^{1} E in the rainforest belt of Southwestern Nigeria and lies south of Kwara and Kogi States, east of Osun State and bounded by Ondo State in the east and south. Figure 1 shows the map of Ekiti State indicating the Local Government Areas where the samples were collected. The State is mainly an upland zone rising over 250 m above sea level. It lies in an area underlain by metamorphic rock and dotted with rugged hills.

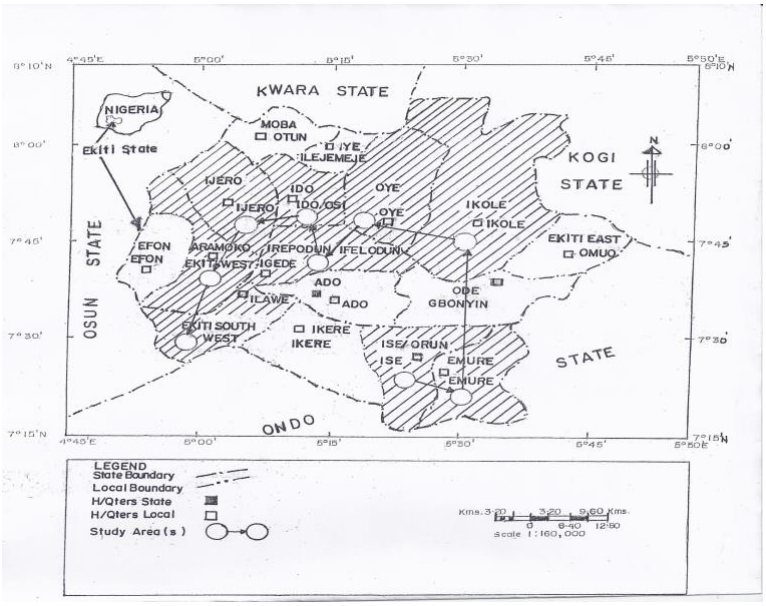

Figure 1. Map of Ekiti State showing the position of the study areas

Source: Min. of Lands, Urban and Regional Planning, Ado-Ekiti/Dept. of Geography and Planning Science, Cartographic Unit, Ekiti State University (EKSU), Ado-Ekiti.

Ekiti State enjoys tropical climate with two distinct seasons: rainy (April - October) and dry (November - March) seasons with a temperature range of 21ºC and 28ºC, and often with high humidity. Ekiti State is buoyant in both agricultural (with cocoa as its leading cash crop) and forest resources (notably timber).

Food crops like yam, cassava, and grains (such as rice and maize) are grown in large quantities. Other notable crops like kolanut and varieties of fruits are also cultivated in commercial quantities. Ekiti State covers about 6,353 km^2 land area.

This study covers eighteen cocoa-growing communities in Ekiti State: Ijero (IJR), Aramoko (ARA), Ido-Ile (IDE), Ikoro (IKO), Igede (IGD), Esure (ESR), Ise (ISE), Emure I (EMR), Emure II (EMU), Orun (ORN), Igbara-Odo (IGR), Ilawe (ILW), Ilupeju (ILJ), Ire (IRE), Ayedun (AYD), Ipawo (IPW), Usi (USI) and Ifaki (IFK).

2.2. Sample collection and preparation

Three cocoa trees were randomly selected where two ripe pods were harvested from each of the cocoa trees and combined to make composite samples. Six samples were collected in each farm making a total of 108 cocoa beans samples. The beans were allowed to ferment for 3-5 days before being sun dried and dehulled, grind in agate mortar and later blended with Excella Mixer Blender to fine particles. The samples were then packed in glass sample bottles prior to further analysis.

2.3. Chemicals and reagent used

The reagents used were of spectra purity. They include GC grade n-hexane, acetone and diethyl ether, silica gel 60 F_{254} and anhydrous sodium sulphate.

2.4. Extraction procedure and clean-up

The extraction of OCPs residues from the cocoa beans samples was carried out using the EPA 3550C method (USEPA 2000). For each sample, About 20 g was mixed with 20 g of anhydrous Na_2SO_4 in a pre-cleaned 250 mL conical flask. A 50 mL solvent mixture of acetone and n-hexane (1:1 v/v) was added to the solid mixture. This was followed by sonication in a high frequency ultrasonic bath for 10-15 minutes before allowing to settle on standing.

The extract was then decanted into round bottom flask. The extraction process was repeated twice with the same starting beans-Na_2SO_4 mixture. The sequential extract were combined and concentrated to 2mL using a rotary evaporator. It was re-dissolved in 5 mL n-hexane and later concentrated to 2 mL in a rotary evaporator at 40ºC.

The clean-up involved the use of a column of about 15 cm (length) x 1cm (internal diameter) packed with activated silica gel (2 g) and anhydrous Na_2SO_4 (1 g) on top of the silica gel (adsorbent). The column was conditioned with 15 mL n-hexane prior to clean-up. The extract was introduced into the column and eluted with 20 mL of n-hexane and diethyl ether (1:1 v/v). The eluate was concentrated by drying on the rotary evaporator and recovered into 2 mL n-hexane. The extract was transferred into glass GC vials for subsequent GC analysis. The OCPs in the extracts were determined by a Gas Chromatograph (GC) coupled with electron capture detector (ECD).

2.5. Gas chromatographic condition

The gas chromatography conditions for the analysis of organochlorine were as follows: GC model: Agilent 7890A Autosampler; the carrier gas flow rate was 4.0 mL/min; injector temperature: Split injection: 20:1; carrier gas: nitrogen; inlet temperature: 250 ºC; column type: HP5 MS; column dimension: (30 m x 0.25 µm x 0.32 mm; oven program: initial temperature at 80 ºC for 1 minute, first ramping 10 º C/min for 10 min (180 ºC); maintained for 3 min; second ramping at 10 ºC/min for 12 min (300 ºC); maintained for 2 min; detector: electron capture detector (ECD); detector temperature: 300 ºC; hydrogen pressure: 22 psi; compressed air: 35 psi. The total run time was 28 minutes.

2.6. Identification, quantification and quality control

The limits of detection (LODs) were determined at a signal to noise ratio (S/N) of 3

for each pesticides. A detectable ion should produce a signal that is at least three times the baseline noise, that is, signal-to-noise ratio = 3 and the limit of quantification (LOQ) was based on the signal to noise ratio of 10 as shown in Table 1.

Table 1. Limit of detection (LOD) and limit of quantification (LOQ) (mg/l) for the detection of OCPs

OCPs	**LOD**	**LOQ**
α-BHC	0.007	0.025
β-BHC	0.007	0.023
Lindane	0.006	0.020
δ-BHC	0.007	0.023
Chlorothalonil	0.009	0.029
Heptachlor	0.005	0.016
Aldrin	0.005	0.017
Heptachlor-epoxide	0.006	0.019
Endosulfan I	0.007	0.023
Dieldrin	0.007	0.025
Endosulfan II	0.007	0.022
p,p'-DDD	0.006	0.021
Endosulfan sulphate	0.007	0.022
p,p'-DDT	0.009	0.030

To determine the validity of the methodology, a standard addition method was employed where a known amount of pesticides was added to the samples and then analysed for the total amount of OCPs. The samples were spiked with mixed OCPs standard solutions (1, 2, 5 µg/l). The spiked samples were allowed to stand for some hours and then extracted, clean-up and analysed as described above. Recovery and precision (expressed as relative standard deviation) were calculated for three replicated samples and the data are presented in Table 2. The percent recovered ranged from 81.2% (dieldrin) to 96.9% (β-BHC).

Table 2. Mean percent recovery for the OCPs

OCPs	**Percent recovered**
α-BHC	93.6 ± 1.9
β-BHC	96.9 ± 0.7
Lindane	91.0 ± 3.1
δ-BHC	89.8 ± 2.3
Chlorothalonil	90.9 ± 1.4
Heptachlor	87.4 ± 2.9
Aldrin	90.2 ± 2.5
Heptachlor-epoxide	88.8 ± 4.3
Endosulfan I	89.7 ± 2.7
Dieldrin	81.2 ± 4.4

Endosulfan II	87.7 ± 4.6
p,p'-DDD	91.4 ± 0.9
Endosulfan sulphate	91.6 ± 1.1
p,p'-DDT	89.5 ± 6.8

The mean percent recovery were within the 70 - 110% acceptable range for recovery by EU guidelines for evaluating accuracy and precision of a method (EU 1999), and thus shows that the procedure employed in this method are reproducible, efficient and reliable for OCPs analysis. Standard solutions of OCPs were run in GC-(ECD) under set chromatographic conditions and mean peak areas were plotted against concentrations to obtain calibration curves of individual pesticides. Under the set chromatographic conditions, standard calibration curve was prepared for each OCP. The signatory retention time for each OCP was used as confirmatory indicator. Linearity was determined by plotting calibration curve with standard solution in n-hexane containing four different concentrations (0.1, 0.25, 0.5, 1.0 ng/µL).

2.7. Data analysis

Data generated in the study were subjected to statistical analysis to test for spatial variations with analysis of variance (ANOVA) using SPSS 15.0 package. One level of confidence limit ($p = 0.05$) was considered in the interpretation of the statistical results.

3. Results and Discussion

The study investigated three broad classes of OCPs, namely: chlorinated benzenes, dichlrodiphenylethanes and cyclodienes in cocoa beans from Ekiti State cocoa farms.

The mean concentrations of chlorinated benzenes are shown in Table 3. About 90.7%, 81.5%, 80.6% and 76.8% of the samples contained α-BHC, β-BHC, δ-BHC and lindane respectively with mean concentration of 0.0003(Ayedun) - 0.0134 µg/g (Igbara-Odo), 0.0057 (Esure) - 0.135 µg/g (Ijero), 0.0033 (Ayedun) - 0.143 µg/g (Usi) and 0.0005 (Ayedun) - 0.0205 µg/g (Ilupeju) respectively. Statistical analysis showed significant variation ($p < 0.05$) in BHCs among the sampled farms.

The mean BHCs reported in this study were lower than those reported by Oyekunle et al. (2017) from cocoa stores at Ondo (0.64 ϒ-BHC - 1.13 β-BHC µg/g) and Ile-Ife (0.41 α-BHC - 0.86 δ-BHC µg/g). The mean concentration of lindane fell within the range reported by Frimpong *et al.* (2012) and Okoffo *et al.* (2012), while those reported by Apau and Dodoo (2010) from central region of Ghana (0.411 mg/kg) were higher. Previous studies showed that HCB was a trace contaminant in several pesticides containing chlorine such as lindane, technical HCH, pentachloronitrobenzene (PCNB), pentachlorophenol (PCP), pentachlorophenol-Na (PCP-Na), attrazine, simazine, picloram, chlorothalonil, dimethyl tetrachloroterephalate (DCPA) (USEPA, 1998; Benzon, 1999; Pacyna *et al*., 2003; Barber *et al.*, 2005).

The occurrence of the BHC isomers in the cocoa beans could be as a result of the contaminant in several pesticides containing chlorine. The result indicated that HCB residues were also likely from the historical application of technical HCH and lindane. The level of lindane recorded in this study were below maximum residue limits (MRLs) set by European Union (1.0 mg/kg) in food and cocoa fruits. About 2.78% of the total samples were above FAO/WHO food standards for β-BHC and δ-BHC (0.2 µg/g) in food (Codex Alimentarius Maximum Residue Limits (Codex, 2004).

Table 3. Mean concentration (µg/g) of chlorinated benzene in the cocoa beans samples

Sampling farm	**α-BHC**	**β-BHC**	**Lindane**	**δ-BHC**	**TOCP**
ARA	0.0082±0.010	0.0185±0.009	0.0049±0.051	0.0207±0.027	0.0604±0.055
AYD	0.0003±0.004	0.0077±0.014	0.0005±0.001	0.0033±0.003	0.0128±0.019
EMR	0.0025±0.009	0.0303±0.024	0.0037±0.001	0.0083±0.007	0.0449±0.033
EMU	0.0006±0.007	0.0175±0.028	0.0013±0.002	0.0076±0.007	0.0271±0.038
ESR	0.0019±0.003	0.0057±0.008	0.0027±0.004	0.0079±0.009	0.0178±0.024
IDE	0.0072±0.008	0.0088±0.114	0.0111±0.014	0.0242±0.025	0.130±0.161
IFK	0.0019±0.002	0.0355±0.036	0.0040±0.004	0.0619±0.070	0.103±0.112
IGD	0.0056±0.009	0.0169±0.020	0.0904± 0.012	0.0203±0.027	0.0519±0.068
IGR	0.0134±0.005	0.093±0.083	0.0160±0.014	0.0392±0.029	0.1630±0.132
IJR	0.0013±0.002	0.1350±0.042	0.0052±0.007	0.0196±0.022	0.0721±0.064
IKO	0.0042±0.005	0.0289±0.020	0.0124±0.016	0.0230±0.021	0.0685±0.063
ILJ	0.0121±0.017	0.0667±0.137	0.0205±0.027	0.0171±0.020	0.116±0.201
ILW	0.0065±0.004	0.0507±0.059	0.0092±0.004	0.0209±0.007	0.0873±0.729
IPW	0.0127±0.008	0.0916±0.067	0.0151±0.080	0.0514±0.038	0.171±0.121
IRE	0.0108±0.007	0.0635±0.028	0.0116±0.013	0.0532±0.023	0.139±0.707
ISE	0.0026±0.003	0.0501±0.063	0.0028±0.003	0.0101±0.009	0.0656±0.078
ORN	0.0024±0.005	0.0420±0.071	0.0036±0.007	0.0154±0.014	0.0634±0.096
USI	0.0067±0.005	0.1060±0.116	0.0073±0.008	0.1430±0.193	0.2630±0.032

Table 4. Mean concentration (µg/g) of dichlorodiphenylethanes in the cocoa beans samples

Sampling farm	***p,p'*-DDD**	***p,p'*-DDT**	**TOCP**
ARA	0.0535±0.027	0.0382±0.025	0.0917±0.052
AYD	0.0008±0.001	0.0016±0.003	0.0024±0.004
EMR	0.0092±0.011	0.0163±0.021	0.0255±0.031
EMU	0.0016±0.002	0.0031±0.006	0.0047±0.008
ESR	0.0078±0.012	0.0618±0.069	0.0697±0.082
IDE	0.1920±0.031	0.0636±0.068	0.0829±0.099
IFK	0.0077±0.006	0.0191±0.025	0.0268±0.031
IGD	0.0082±0.012	0.0041±0.006	0.0124±0.019
IGR	0.0195±0.013	0.0271±0.012	0.0466±0.025
IJR	0.0067±0.008	0.0118±0.019	0.0176±0.119
IKO	0.0179±0.020	0.0036±0.060	0.0544±0.077
ILJ	0.0197±0.029	0.0132±0.014	0.0329±0.042
ILW	0.0219±0.022	0.0484±0.032	0.0704±0.056
IPW	0.0417±0.029	0.0268±0.021	0.0685±0.051
IRE	0.0405±0.049	0.0297±0.030	0.0702±0.078
ISE	0.0004±0.100	0.0076±0.009	0.0080±0.009
ORN	0.0045±0.073	0.0062±0.007	0.0107±0.015
USI	0.0102±0.078	0.0226±0.035	0.0328±0.043

Table 4 showed the mean concentrations (mg/kg) of dichlrodiphenylethanes in the cocoa beans samples. About 71.2% and 72.2% of the samples contained *p,p'*-DDT and *p,p'*-DDD with mean concentration ranged of 0.0016 (Ayedun) - 0.0636 µg/g (Ido-Ile) and 0.0008 (Ayedun) - 0.0535 µg/g (Aramoko) respectively. Significant variation was recorded in *p,p*'-DDD, while *p,p*'-DDT showed no significant variation ($p > 0.05$) among the sampling farms. The high incidence of DDT in some of the samples could be as a result of continuous applications in spite of the ban on its usage.Studies in Africa have found large amount of DDT residue in breast milk, fish, soil and cow's milk (Ejobi *et al*., 1996, 1998; Nyangababo *et al*., 2005; Ogwok 2009). DDT concentrations in cocoa beans have been reported by Aikpokpodion *et al*. (2012a) in Ondo, Cross river and Ogun States, Nigeria. Their results showed that 70% of the cocoa beans analysed from Ondo State had DDT residue, while 10% from Cross River and Ogun States had DDT residue. The mean concentration (57.8 ± 81.5 and 82.2 ± 54.4 µg/g) reported by Oyekunle *et al.* (2017) for *p,p*'-DDT in Ile-Ife and Ondo were higher than those reported in this study, while those reported by Frimpong *et al.* (2012) from Ghana were within the same range with the present study. The level of *p,p*'-DDT and its metabolite (*p,p*'-DDD) from all the study areas were below EU set standards of 0.5 µg/g for DDT (EU, 2005). The occurrence of DDT in the farms confirmed the usage in Ekiti State in the past, as most of the sampled farms were over 50 years. DDT and its metabolites can magnify through the food chain due to their chemical and physical nature. They are lipophilic and are stored mainly in body fat. Report of Darko and Acquaah (2007) confirmed the lipophilic nature of DDT and its metabolites that were concentrated in beef fat than the tissue (meat). The detection of DDT in cow milk at high concentration was also an indication of the ability of the pesticide to associate with animal tissue (Clerknevik, 2000). Through their persistence and lipohilicity, pesticide may concentrate in the adaptive tissues and in the blood serum of human. This was confirmed by the findings of Sosan *et al.* (2008) who reported the detection of organochlorine residue in the serum of 42 out of 76 cocoa farmers in South-western Nigeria.

The mean concentrations (µg/g) of cyclodienes in the samples are shown in Table 5. Many of the cyclodienes species analysed are breakdown products of the parent pesticide. Seven cyclodienes pesticides were evaluated in the study. About 90.7% of the beans samples contained dieldrin; 76.8% had endosulfan II; 75.9% contained endosulfan I and heptachlor; aldrin was found in 75%; endosulfan sulphate in 71.2%; while 73.1% contained heptachlor-epoxide.

Table 5. Mean concentration (µg/g) of cyclodienes in the cocoa beans samples

Sampling farm	Heptachlor	Heptachlor-epoxide	Aldrin	Dieldrin	Endosulfan I	Endosulfan II	Endosulfan-sulphate	TOCP
ARA	0.0207±0.027	0.0125±0.012	0.0098±0.010	0.0210±0.024	0.0372±0.047	0.0165±0.030	0.1630±0.115	0.281±0.266
AYD	0.0032±0.04	0.0005±0.001	0.0013 ±0.002	0.0002±0.006	0.0024±0.038	0.0012±0.002	0.0038±0.007	0.0127±0.021
EMR	0.0086±0.006	0.0031±0.002	0.0043±0.003	0.0175±0.026	0.0081±0.012	0.0116±0.011	0.044±0.117	0.147±0.117
EMU	0.0064±0.09	0.0013±0.002	0.0026±0.003	0.0034±0.005	0.0023±0.003	0.0024±0.003	0.0763±0.015	0.026±0.042
ESR	0.0089±0.014	0.0153±0.0031	0.0051±0.007	0.0182±0.027	0.0094±0.013	0.0022±0.004	0.0014±0.002	0.0606±0.097
IDE	0.0279±0.030	0.0129±0.003	0.0061±0.004	0.0233±0.023	0.0119±0.011	0.0174±0.013	0.1550±0.208	0.174±0.097
IFK	0.0090±0.008	0.0026±0.003	0.0055±0.004	0.0039±0.004	0.0083±0.007	0.0033±0.004	0.0923±0.133	0.125±0.164
IGD	0.0282±0.039	0.0126±0.018	0.0155±0.021	0.0107±0.018	0.0291±0.004	0.0201±0.022	0.0072±0.011	0.123±0.172
IGR	0.0633±0.035	0.0276±0.023	0.0422±0.035	0.0197±0.013	0.0430±0.034	0.0413±0.027	0.0811±0.047	0.318±0.214
IJR	0.0084±0.009	0.0132±0.015	0.0092±0.012	0.0080±0.009	0.0059±0.007	0.0306±0.011	0.0151±0.022	0.112±0.117
IKO	0.007±0.008	0.0103±0.011	0.0057±0.008	0.0106±0.008	0.0249±0.028	0.0155±0.012	0.1080±0.199	0.183±0.278
ILJ	0.0353±0.048	0.0104±0.016	0.0209±0.029	0.0073±0.014	0.0319±0.031	0.0121±0.019	0.0205±0.038	0.138±0.195
ILW	0.0215±0.010	0.0076±0.062	0.0117±0.006	0.0114±0.005	0.0320±0.007	0.0327±0.045	0.1570±0.148	0.274±0.229
IPW	0.0021±0.004	0.0243±0.013	0.0139±0.005	0.0231±0.013	0.0329±0.024	0.0259±0.031	0.0392±0.031	0.171±0.121
IRE	0.0152±0.009	0.0289±0.025	0.0138±0.022	0.0642±0.057	0.0101±0.016	0.0616±0.010	0.0866±0.096	0.280±0.332
ISE	0.0094±0.012	0.0057±0.008	0.0050±0.006	0.0045±0.007	0.0253±0.022	0.0151±0.011	0.0301±0.054	0.0914±0.120
ORN	0.0140±0.022	0.0045±0.008	0.0067±0.008	0.0025±0.006	0.0051±0.006	0.0075±0.010	0.0175±0.028	0.0279±0.092
USI	0.0257±0.032	0.0096±0.010	0.0365±0.010	0.0083±0.007	0.0205±0.024	0.0199±0.021	0.0538±0.047	0.174±0.168

Analysis of variance showed no significant variation ($p > 0.05$) in endosulfan II, dieldrin and heptachlor-epoxide, while heptachlor, aldrin, endosulfan I, endosulfan sulphate and TOCP showed significant variation among the sampled farms. The mean concentration of heptachlor and heptachlor-epoxide in the beans samples ranged from 0.0032 (Ayedun) - 0.0633 µg/g (Igbara-Odo) and 0.0002 (Ayedun) - 0.0289 µg/g (Ire) respectively. Samples from Ipawo, Ire, Esure, Ijero and Ikoro showed higher level of heptachlor-epoxide than heptachlor (the parent compound). The heptachlor and is metabolite level obtained in this study were lower in most cases than those reported by Oyekunle et al. (2017) where 0.63 – 2.27 µg/g and 0.59 – 3.96 µg/g in Ondo and 0.02 – 3.96 µg/g and ND – 6.13 µg/g in Ile-Ife were reported for heptachlor and heptachlor-epoxide. The level of the heptachlor-epoxide in the beans samples was directly related to the heptachlor application on the farms since heptachlor-epoxide is a metabolite of heptachlor. None of the beans samples exceeded the EU MRL of 0.2 µg/g for heptachlor and heptachlor-epoxide in food.

The mean aldrin concentration ranged from 0.0013 (Ayedun) - 0.0422 µg/g (Igbara-Odo), while that of dieldrin ranged from 0.0002 (Ayedun) - 0.0642 µg/g (Ire). The mean concentration of aldrin (0.13 – 10.2 µg/g) and dieldrin (ND – 14.5 µg/g) reported by Oyekunle *et al.* (2017) were comparatively similar in some cases, while most showed higher level than those reported in this study.

About 4.6% of the beans samples showed concentrations above FAO/WHO (0.05 µg/g) for aldrin and dieldrin in food. The mean concentration (µg/g) of endosulfan I, endosulfan II and endosulfan sulphate ranged from 0.0023 (Emure I) - 0.0430 µg/g (Igbara-Odo), 0.0012 (Ayedun) - 0.0616 µg/g (Ire) and 0.0014 (Esure) - 0.163 µg/g (Aramoko), respectively. The notable differences in the amounts of these pesticides were perhaps due to the farming systems adopted by the cocoa farmers, the age of the farms, the active ingredient concentration as prepared by the farmers among other factors. Due to few reports on endosulfan in cocoa beans, few comparable data are available. It was reported that 28 out of 45 (64%) of the cocoa beans samples in Ghana had endosulfan I, while endosulfan II was detected in 23 out of 45 (52%) with concentration range of ND – 0.10 mg/kg (Frimpong *et al.*, 2012). This was comparable to some samples reported in this study. Aikpokpodion *et al.* (2012) reported ND – 0.74 mg/kg for endosulfan 1 with an average value of 0.55 µg/g in Ondo State, the value which is comparatively higher than the average value of 0.0012 - 0.163 µg/g for samples collected in this study. Endosulfan I and endosulfan II were lower in most cases than what Oyekunle *et al.* (2017) reported for Ondo and Ile-Ife, while endosulfan sulphate was comparable in some farms from the study areas.

About 0.72% and 15.7% of the samples exceeded the maximum residue limit (0.1 µg/g) set by the (Codex, 2004; EU, 2005) for endosulfan and endosulfan sulphate, while none of the endosulfan II exceeded the EU-MRLs standards in food. The level of the enodsulfans obtained in the samples were mostly within the range reported by Scholten *et al.* (2010) in 139 cocoa beans obtained from fifteen cocoa producing countries. The high level of endosulfan in some cases suggests extensive usage of endosulfan in Ekiti cocoa farms. The cultivation activities of cocoa farmers, persistent frequent and indiscriminate applications of pesticide as reported by Aikpokpodion *et al.* (2012) were said to been responsible for the higher level of endosulfans in some Nigeria farms.

4. Conclusions

Residues of OCPs were found in cocoa beans of the selected cocoa farms with α-BHC as the most frequently found pesticide residue. The concentration levels of lindane, α-BHC, *p,p*'-DDT, *p,p*'-DDD, heptachlor, heptachlor-epoxide and endosulfan II were all below the maximum residual limit (MRL) in food, while 0.92%, 2.78%, 4.6% and 15.7% of endosulfan I, (β-BHC and δ-BHC), (aldrin and dieldrin) and endosulfan sulphate respectively were above the MRL. The high proportion of samples with detectable amounts of the pesticide residues

points to previous use in the studied areas. The study therefore, recommends the need for continuous survey and monitoring of pesticides residue in cocoa farms and products in order to ensure that the residual levels remain below prescribed limits by the national and international standards. There is also need for an intense awareness among farmers on reasons why they should stop the use of banned pesticides on cocoa and other food products.

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Acknowledgements

The authors wish to acknowledge the technical assistance rendered by the Chemical Laboratory of the National Food and Drug Administration and Control (NAFDAC), Oshodi, Lagos, Nigeria.

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

IDENTIFICATION OF PHENOLIC COMPOUNDS AND CHANGES IN THEIR CONTENT DURING PROCESSES OF WHITE WINES

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https://doi.org/10.34302/crpjfst/2023.15.4.17

Article history:
Received: 6 June 2022
Accepted: 17 September 2023

Keywords:
Phenolic compound;
White wine;
Vinification;
Vitis vinifera.

ABSTRACT

Gaziantep (in Turkey) is one of the oldest cities in the World, in which the history of winemaking and viticulture began in ancient ages. It is home to wild white grapes *Vitis vinifera* Dökülgen and Paf. Three young white wines were produced from these two grapes. Ten phenolics and some chemical characteristics were quantitatively detected in the white wines and musts depending on white wine processes. Phenolic compounds significantly ($p<0.05$) increased during the fermentation process. After aging, (+)-catechin and procyanidin B2 contents of white wines were ranged from 1.12 to 1.35 and from 60.69 to 69.02 mg/L respectively. After aging, the quercetin, rutin and myricetin contents of white wines-1, 2 and 3 were ranged from 0.47 to 0.59, from 0.28 to 0.32 and from 0.11 to 13 mg/L respectively. White wines represented with abundant flavanols, tyrosol and chlorogenic acid produced from Dökülgen and Paf mixture with 7:3 ration. It was found that there is a significant difference between hydroxycinnamic acid and hydroxybenzoic acid content of white wines. Dökülgen and Paf white grapes contribute higher amount of phenolic characteristics, better acidity and sugar to white wine while Dökülgen grape contributed more sugar.

1. Introduction

The phenolic compounds in wines change according to the grape type, ripeness of the grapes, the irrigation and fertilization of the soil, viticulture and the climatic conditions of the region, fermentation (yeast flora, pH and temperature), and wine production techniques (Erkmen and Bozoglu, 2016). The Southeast region of Anatolia is one of the most successful wine regions with its excellent climate, soil and geographical features suitable for viticulture. In this region, 65 kinds of grapes were grown. Dökülgen, Paf, Kabarcık, Rumi, Dımışkı, and Muhammediye are major white grapes (Celik et al., 2005; Erkmen, 2005). Regional differences affect the development of the vine, ripening of grapes, the composition and sensory properties of grapes and wine. The region conditions are important factors that determine the quality and style of wine (Bekar and Bayram, 2016). Phenolic compounds play important roles in the quality of the wine. There has been no report on the phenolic characterization of white wines produced from Southeast region grapes as well as the southeast region of Turkey. This research has been carried out to indicate young white wine production from Gaziantep grapes, to reveal the importance of grapes in the white wine production, to indicate suitability of white grapes for wine production, to indicate regional process condition on white wine production and to indicate the availability of grapes for white wine production with specified

phenolic characteristics. The evolution of ten phenolic compounds were conducted during production steps of young white wines produced from Dökülgen and Paf white grapes. Brix, pH, alcohol and free SO_2 changes during white wine processes were also studied.

2. Materials and Methods

Two white grapes (vernacular "Dökülgen and Paf") from *Vitis vinifera* subsp. *sylvvestris* L. cultivated in Southeast region of Turkey were harvested from vineyards in September 2018 at the appropriate maturity in 20 kg plastic crates and transported to the winery in the Food Engineering Department (Gaziantep University, Gaziantep, Turkey). Dry yeast *Saccharomyces cerevisiae* (LALVIN ICI-D47), potassium metabisulfite (PMB, $K_2S_2O_5$), yeast nutrient (VitaStart) and disinfectant (Bioxeco-5) were obtained from Vinomarket (İzmir, Turkey). HPLC-grade chemicals and standard phenolic compounds were supplied from Sigma-Aldrich (Interlab, Adana, Turkey).

2.1. White wine production

Three white wines were produced from Dökülgen and Paf grapes. All wines were processed in the same way according to the process scheme given in Fig. 1. White wine production steps used in this manuscript were explained by Ceyhan (2019). Wines were filled into dark green colored bottles (75 ml) and the bottles were capped with cork using cork stopper closing machine (Atlantis Cam Ambalaj Ltd. Şti., İzmir, Turkey). The bottles were aged in the horizontal position for 3 months in the darkroom at 20°C.

2.2. Analysis

About 150 ml of the samples were removed in duplicate during fermentation (after 3, 5 and 12 days), after resting (7 days), after maturation (45 days) and after aging (3 months). Samples were also removed from musts. The Brix, alcohol, pH and phenolic compounds analysis were made from must and white wine samples. *S. cerevisiae* and yeast counts, and pH analysis were also carried out from white wine samples according to the standard methods (Erkmen, 2022). Water-soluble dry matter of samples was determined by the refractometer at 20°C. Electronic ebulliometer (Bulteh 2000, Stara Za gora, Bulgaria) was used for the alcohol analysis with the calcoholometric method (OIV, 2019). Free sulfur dioxide (SO_2) analyses were performed by the calorimetic method (Aktan and Kalkan, 2000).

Phenolic compounds analyze. The water used in the analysis was obtained from a Milli-Q water purification system (Millipore; Bedford, MA, USA). All solvents used were previously filtered through 0.45 μm membrane filter (Millipore) and degassed before use. Phenolic standard solutions. For all standards (gallic acid, (+)-catechin hydrate, routine hydrate, procyanidin B2, p-coumaric acid, chlorogenic acid, resveratrol, tyrosol, myricetin, and quercetin), stock solutions were prepared by dissolving the phenolic compounds at four different concentrations with the methanol-water solution (50:50 v/v). The phenolic standards were determined by HPLC (Gomez-Alonso et al., 2007; Burin et al., 2011) and the results were given in mg/L.

Chromatographic analysis was performed using a Shimadzu LC-20AB (Shimadzu Corporation, Kyoto, Japan) high-performance liquid chromatography (HPLC) equipped with a vacuum degasser (DGU-20A5), quaternary-pump LC-10AT, UV detector (SPD-20A), SIL- autosampler (20A HT) and VP column furnace (CTO-10AS). The LCsolution (v.1.25; 2002-2009 Shimadzu Corporation) was used to control the gradient settings, UV and data acquisition. The separation was performed using a C18 analytical column of 4.6 mm x 250 mm, 5 μm particle size (GL Sciences, Kyoto, Japan). A C18 guard column of 4.6 mm x 12.5 mm, 5 μm particle size (GL Sciences, Kyoto, Japan) was used to prevent contamination of the analytic column from any non-soluble residues coming from the samples. Peak areas were determined at 280 and 320 nm wavelengths for all phenolic compounds.

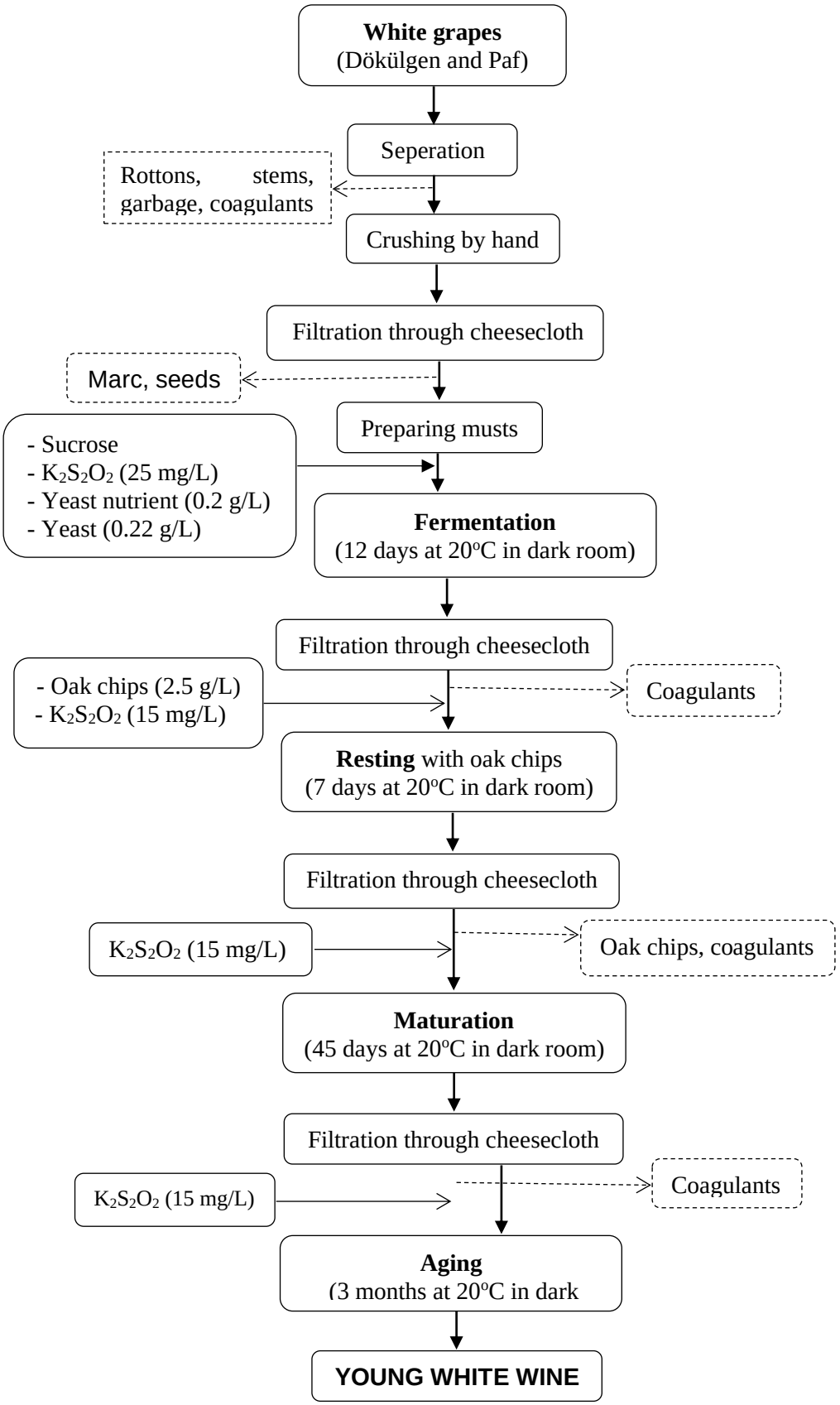

Figure 1. General White wine production flow chart

The samples were centrifuged for 5 min at 5000 rpm. Approximately 2 mL of the resulting solution was removed with a 0.45 μm PTFE syringe filter (Millipore) and added into 1.5 mL colored vial. Samples were analyzed as soon as possible using HPLC. In the quantitative analysis of phenolic compounds, the modified HPLC methods were used (Gomez-Alonso et al., 2007; Burin et al., 2011). Two solvent gradient elutions were used in the study. Solvent A is the acetic acid-water solution (2:98 v/v) and solvent B is the methanol-water solution (50:50 v/v). The injection volume was adjusted to 25 μL, the flow rate to 1 mL/min and the temperature to 30±1°C. The samples were injected in duplicate. HPLC gradient program solvent flow concentration was used in the analysis.

Phenolic compounds in white wine and must samples were identified through comparison of their retention times and UV spectra with those obtained by injection of the standard solution under the same conditions. Peak area at maximum absorbance was used for the quantification of phenolic compounds using the internal standard curve. A standard curve for each phenolic compound was constructed separately by plotting peak area (y-axis) versus the concentration of the phenolic compound (x-axis). The standard curve was fitted by linear least-squares regression (r^2 0.98). Values were reported as mg/L. The analytical method was a reproducible value of ≥92% for phenolic compounds.

2.3. Statistical analysis

The wine production was repeated three times. At each repeat, parallel white wines were prepared. Samples were taken in parallel at each step of wine production, and the results of analyzes were given as the mean ± standard deviation values of the three repeats. The wines were compared depending on process time and wine types by analysis of variance with ANOVA test using SPSS v.22 (IBM SPSS Corporation, Chicago, IL, USA) with a 95 % confidence level (a confidence interval of $\alpha = 0.95$).

3. Results and Discussion

At the beginning of fermentation, the initial numbers of *S. cerevisiae* were 6.37, 6.34 and 6.26 log cfu/ml in musts-1, 2 and 3 respectively. After 3 days of fermentation, yeast counts of white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 7.49, 7.48 and 7.57 log cfu/mL respectively. *S. cerevisiae* was decreased during settling, maturation and aging periods. After aging, the final survived numbers of *S. cerevisiae* were 2.13, 2.11 and 2.09 log cfu/mL for white wines-1, 2 and 3 respectively.

3.1. Changes in pH and brix values

pH values of musts-1, 2 and 3 were determined as 3.71, 3.63 and 3.59 respectively (Table 1). After fermentation and resting, the pH values of white wines were decreased. After aging, pH values of white wines-1, 2 and 3 were slightly decreased to 3.47, 3.44 and 3.39. During maturation, there was a slight increase in the pH values of white wines due to the precipitation of tartaric acid in wine as potassium bitartrate and the cleavage of malic acid to lactic acid by lactic acid bacteria. The most suitable pH value in terms of quality of the wine (such as color, microbial, chemical and oxidative stability) should be in the range of 2.7-3.8 (TFC, 2008).

After fermentation, Brix values of white wines-1, 2 and 3 were significantly ($p<0.05$) decreased to 8.22, 8.79 and 8.64 % respectively (Table 1). During resting, maturation, and aging, Brix values of white wines were slightly decreased. After aging, Brix values of white wines-1, 2 and 3 were decreased to 7.46, 7.53 and 7.18 % respectively. There are significant ($p<0.05$) differences among the Brix values of white wines. The European Union Commission Regulation has indicated that dry wines with moderate acidity may contain no more than 9 g/L of residual sugar (Jordao et al., 2015).

Changes in alcohol and free SO_2 values. During fermentation, alcohol content of wines-1, 2 and 3 were increased to 13.15, 13.36 and 13.39% respectively (Table 2). After resting, alcohol values of wines were slightly increased and slightly decreased during maturation and aging. The final alcohol contents of white wines-

1, 2 and 3 were 13.19, 13.36 and 13.65 % respectively. Decreases in alcohol amounts may be due to the evaporation of alcohol during processes and oxidation of ethyl alcohol. According to the Turkish Food Codex (TFC, 2008), regulation for wine, the amount of alcohol by volume of wine must be at least 9% and the maximum 15%. Alcohol strengthens of wine provides warmth, sweetness, durability, and taste to the wine.

Table 1. Changes of pH and Brix during white wine production*

	pH			**Brix (%)**		
Day	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3
Must	3.71 ± 0.01^{aA}	3.63 ± 0.02^{aB}	3.59 ± 0.03^{aC}	21.55 ± 0.05^{aA}	22.12 ± 0.03^{aB}	22.60 ± 0.02^{aC}
3	3.27 ± 0.01^{bA}	3.23 ± 0.01^{bB}	3.24 ± 0.02^{bB}	18.50 ± 0.03^{bA}	19.38 ± 0.03^{bB}	19.68 ± 0.02^{bC}
12	3.38 ± 0.02^{cAB}	3.36 ± 0.02^{cB}	3.39 ± 0.01^{cA}	8.22 ± 0.01^{cA}	8.79 ± 2.71^{cA}	8.64 ± 0.02^{cA}
19	3.33 ± 0.02^{dA}	3.35 ± 0.02^{cB}	3.37 ± 0.02^{cAB}	7.06 ± 0.02^{cdA}	7.50 ± 0.02^{dB}	7.25 ± 0.04^{cC}
64	3.49 ± 0.03^{eA}	3.47 ± 0.02^{dB}	3.56 ± 0.02^{dA}	7.21 ± 0.03^{cA}	7.40 ± 0.02^{dA}	7.03 ± 0.02^{cA}
154	3.47 ± 0.01^{eA}	3.44 ± 0.01^{eB}	3.39 ± 0.02^{eC}	7.46 ± 0.02^{cdA}	7.53 ± 0.03^{eB}	7.18 ± 0.02^{cC}

*Values are the mean±SD (n=3). In the columns, different small letters represent significant differences among pH and brix during processes. In the rows, different capitalized letters represent significant pH and brix differences among wines. They were determined by the least significant difference test at $p<0.05$.

Table 2. Changes of alcohol and free SO_2 during white wine production*

	Alcohol (%)			**Free SO_2 (mg/L)**		
Day	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3
3	4.65 ± 0.05^{aA}	4.71 ± 0.01^{aB}	4.56 ± 0.02^{aA}	-	-	-
12	13.15 ± 0.05^{bA}	13.36 ± 0.02^{bB}	13.39 ± 0.03^{bB}	13.50 ± 1.53^{aA}	12.50 ± 0.58^{aA}	12.00 ± 1.00^{aA}
19	13.69 ± 0.02^{cA}	13.70 ± 0.02^{cB}	13.89 ± 0.04^{cA}	23.00 ± 1.00^{bA}	20.00 ± 1.00^{bB}	18.50 ± 1.53^{bB}
64	13.40 ± 0.02^{dA}	13.43 ± 0.03^{cB}	13.86 ± 0.02^{dB}	28.00 ± 1.00^{cA}	26.00 ± 1.00^{cAB}	23.11 ± 1.53^{cC}
154	13.19 ± 0.01^{bA}	13.36 ± 0.03^{dB}	13.65 ± 0.02^{bC}	24.26 ± 0.58^{bA}	23.16 ± 1.73^{dAB}	22.12 ± 1.53^{cB}

*Standartd deviations indicated in Table 1 subscript.

SO_2 has widely used chemicals in preventing the growth of undesirable microorganisms. At the end of fermentation, free SO_2 amounts of wines-1, 2 and 3 were 13.50, 12.50 and 12.0 mg/L respectively (Table 2). After aging, the free SO_2 amounts in white wines-1, 2 and 3 were 24.26, 23.16 and 22.12 mg/L respectively. Free SO_2 positively affects the aging of the wine and prevents the formation of free aldehyde. According to TFC (2009), maximum permissible free SO_2 should not exceed 30 mg/L in wine.

3.2. Changes in flavanols

The changes of flavonols contents during processing steps of white wines were given in Table 3. At the end of fermentation, (+)-catechin and procyanidin B2 contents of the white wines-1, 2 and 3 were significantly increased to 1.12, 1.17 and 1.52, and 63.05, 64.03 and 70.28 mg/L respectively. After fermentation, amounts of procyanidin B2 and (+)-catechin in white wines were 3-4 and 19-28 times greater, respectively, than musts procyanidin B2 and (+)-catechin. After aging, (+)-catechin and procyanidin B2 contents of the white wines-1, 2 and 3 were decreased to 1.12, 1.16 and 1.35 mg/L, and 60.69, 66.88 and

69.02 mg/L respectively. Oak tannins interact with wine compounds, affecting the sensorial properties. In this study, both of (+)-catechin and procyanidin B2 were significantly ($p<0.05$) increased during resting in the presence of oak chips. Procyanidin B2 and (+)-catechin can give the astringency and bitterness to the wine by the assembling with proteins and glycoprotein in the saliva. These phenolic compounds have antioxidant and color effects in the wine. The most crucial factors affecting the types of flavanols in wine are the content of grapes, grape grown area, wine production technology, a contact time of juice with grape shell, alcohol amount, fermentation temperature and aging time (Uylaser and Ince, 2008).

Table 3. Changes of flavanols during white wine production (mg/L)*

	(+) – Catechin			**Procyanidin B2**		
	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3
Must	0.04 ± 0.01^{aA}	0.05 ± 0.01^{aA}	0.07 ± 0.02^{aA}	15.06 ± 0.10^{aA}	22.36 ± 0.28^{aB}	22.67 ± 0.32^{aB}
12	1.12 ± 0.03^{bA}	1.17 ± 0.02^{bAA}	1.32 ± 0.16^{bB}	63.05 ± 1.0^{bcA}	64.03 ± 0.40^{bA}	70.28 ± 0.54^{bB}
19	1.35 ± 0.06^{cA}	1.22 ± 0.03^{cB}	1.67 ± 0.02^{cC}	78.73 ± 0.20^{dA}	79.84 ± 0.34^{cB}	84.40 ± 0.48^{cC}
64	1.22 ± 0.03^{dA}	1.19 ± 0.03^{bcA}	1.44 ± 0.03^{bB}	65.58 ± 0.64^{cA}	69.72 ± 0.56^{bA}	76.84 ± 0.56^{dB}
154	1.12 ± 0.05^{bA}	1.16 ± 0.02^{bA}	1.35 ± 0.03^{bB}	60.69 ± 1.87^{bA}	66.88 ± 1.57^{bB}	69.02 ± 1.52^{bB}

*Standard deviations indicated in Table 1 subscript.

3.3. Changes in flavonols

The changes of flavonols contents during processing steps of white wines were given in Table 4. At the end of fermentation, myricetin contents of white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 0.14, 0.16 and 0.19 mg/L respectively. After the aging, the myricetin contents of the white wines-1, 2 and 3 were decreased to 0.11, 0.13 and 0.15 mg/L respectively. At the end of fermentation, quercetin content of the white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 0.62, 0.61 and 0.64 mg/L respectively. After aging, the quercetin contents of the white wines-1, 2 and 3 were 0.47, 0.50 and 0.59 mg/L respectively. At the end of fermentation, the rutin content of the white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 08, 0.10 and 0.11 mg/L respectively. During resting, maturation, and aging of the white wines, the rutin contents were slightly increased. Rutin capable of chelating metal ions (such as iron) causes the formation of oxygen radicals with their high antioxidant activity. Flavonols occur as the glycoside structure of grapes. They are hydrolyzed during juice extraction and fermentation. Quercetin gives a bitter taste to the whine. Flavonol contents of wines depending on the intensity of sunlight where the grape cultured, the thickness of the grape skin, the type of grape and the technological processes applied in wine production (Jackson, 2000).

3.4. Phenolic acids

The changes of phenolic acids contents during processing steps of white wines were given in Table 5. After maturation and aging, gallic acid contents were decreased. Gallic acid is released from grapes and formed during resting with oak chips. Gallic acid gives astringency aroma. At the end of fermentation, the chlorogenic acid content of the white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 7.52, 7.74 and 7.71 mg/L respectively. Final chlorogenic acid contents in white wines-1, 2 and 3 were decreased to 7.13 7.44 and 7.60 mg/L respectively.

Table 4. Changes of flavonols during white wine production (mg/L)*

	Myricetin			**Quercetin**			**Rutin**		
Days	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3
Must	0.02±0.00^{aA}	0.02±0.06^{aA}	0.04±0.02^{aA}	0.22±0.02^{aA}	0.32±0.02^{aB}	0.45±0.01^{aC}	0.03±0.01^{aA}	0.03±0.00^{aA}	0.03±0.01^{aA}
12	0.14±0.02^{bA}	0.16±01^{bA}	0.19±0.02^{bB}	0.62±0.01^{bA}	0.61±0.02^{bA}	0.64±0.01^{bA}	0.08±0.01^{bA}	0.10±0.00^{bA}	0.11±0.01^{bA}
19	0.14±0.03^{bA}	0.16±0.2^{bA}	0.18±0.03^{bcA}	0.72±0.010^{cA}	0.68±0.01^{cAB}	0.68±0.02^{cB}	0.11±0.01^{cA}	0.12±0.02^{bA}	0.18±0.01^{bcB}
64	0.14±0.01^{bA}	0.15±0.1^{bA}	0.16±0.01^{cA}	0.69±0.01^{dA}	0.74±0.01^{dA}	0.74±0.02^{dA}	0.22±0.01^{dA}	0.28±0.02^{cB}	0.22±0.01^{cA}
154	0.11±0.03^{bA}	0.13±0.01^{bA}	0.15±0.02^{cB}	0.47±0.02^{eA}	0.50±0.02^{eA}	0.59±0.01^{eB}	0.32±0.02^{eA}	0.30±0.02^{cA}	0.28±0.01^{dB}

*Standard deviations indicated in Table 1 subscript.

Table 5. Changes of phenolic acids during white wine production (mg/L)*

	Gallic acid			***p*-Coumaric acid**			**Chlorogenic acid**		
Days	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3
Must	3.49±0.02^{aA}	3.61±0.04^{aB}	3.48±0.03^{aA}	0.21±0.02^{aA}	0.22±0.01^{aA}	0.23±0.03^{aA}	7.17±0.04^{aA}	7.20±0.04^{aA}	7.19±0.04^{aA}
12	4.65±0.03^{bA}	3.69±0.02^{bB}	3.71±0.04^{bB}	0.66±0.02^{bA}	0.69±0.02^{bB}	0.78±0.01^{bC}	7.52±0.24^{bA}	7.74±0.04^{bB}	7.71±0.04^{bB}
19	5.04±0.05^{cA}	4.47±0.04^{cB}	4.43±0.02^{cB}	0.94±0.03^{cA}	0.76±0.02^{cB}	1.02±0.04^{cB}	7.73±0.07^{cA}	7.95±0.05^{cB}	7.88±0.04^{cC}
64	4.38±0.04^{dA}	4.35±0.04^{cA}	4.36±0.03^{cA}	1.19±0.03^{dA}	1.24±0.03^{dA}	1.31±0.04^{dA}	7.40±0.05^{dA}	7.78±0.04^{bB}	7.79±0.04^{dB}
154	4.29±0.04^{dA}	4.31±0.06^{dA}	4.33±0.02^{cB}	1.39±0.03^{eA}	1.41±0.02^{eA}	1.44±0.05^{eA}	7.13±0.07^{aA}	7.44±0.05^{dB}	7.60±0.07^{eC}

*Standard deviations recorded indicated in Table 1 subscript.

It is responsible for the sour taste in wine, easily oxidizes in the presence of polyphenol oxidase and converted to brown-colored compounds. At the end of fermentation, p-coumaric acid contents were significantly ($p<0.05$) increased to 0.66, 0.69 and 0.78 mg/L in the white wines-1, 2 and 3 respectively. The final content of the p-coumaric acid in aged white wines-1, 2 and 3 were significantly ($p<0.05$) decreased to 0.39, 0.41 and 0.44 mg/L respectively.

The hydroxybenzoic and hydroxycinnamic acids are derived from oak as well as from grapes. Together with anthocyanins, phenolic acids contribute important characteristic quality to white wines such as astringency and bitterness (Mendoza et al., 2011). The most crucial factors affecting the amount of the phenolic acid in wine are their contents in grapes, wine production technology, the contact time of shell during juice extraction, exposure time to oaks, ethyl alcohol amount, fermentation temperature and transformations during wine processes (Uylaser and Ince, 2008).

3.5. Resveratrol and tyrosol

The changes of stilbene and phenolic alcohol contents during processing steps of white wines were given in Table 6. At the end of fermentation, resveratrol contents of the white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 1.57, 1.93 and 1.82 mg/L respectively. Resveratrol contents of white wines were decreased after resting, maturation and aging. Resveratrol is in the skin of the grapes, dissolves during the juice extraction and fermentation process of white wine. At the end of fermentation, the tyrosol content of the white wines-1, 2 and 3 were significantly ($p<0.05$) increased to 36.55, 37.08 and 37.33 mg/L respectively. The tyrosol contents of the white wines were decreased during resting, maturation and aging periods. Tyrosol is phenolic alcohol and it is formed due to the sugar consumption by yeast.

Table 6. Changes of tyrosol and resveratrol amounts during white wine production (mg/L)*

		Tyrosol			**Resveratrol**	
Days	Wine-1	Wine-2	Wine-3	Wine-1	Wine-2	Wine-3
Must	0.11 ± 0.01^{aA}	0.12 ± 0.02^{aA}	0.14 ± 0.03^{aA}	0.13 ± 0.02^{aA}	0.16 ± 0.01^{aB}	0.17 ± 0.02^{aB}
12	36.55 ± 0.19^{cA}	37.08 ± 0.14^{bB}	37.33 ± 0.08^{bB}	1.57 ± 0.04^{bA}	1.93 ± 0.07^{bB}	1.82 ± 0.02^{bC}
19	36.38 ± 0.12^{bcA}	36.91 ± 0.08^{cB}	36.49 ± 0.11^{cA}	1.44 ± 0.05^{cA}	1.79 ± 0.04^{bB}	1.40 ± 0.03^{cA}
64	36.21 ± 0.09^{bA}	36.33 ± 0.14^{cA}	36.67 ± 0.09^{dA}	1.36 ± 0.06^{dA}	1.49 ± 0.05^{cA}	1.37 ± 0.04^{cA}
154	36.22 ± 0.09^{bA}	34.50 ± 0.15^{dB}	36.11 ± 0.10^{eA}	1.25 ± 0.03^{eA}	1.31 ± 0.06^{cA}	1.28 ± 0.03^{dA}

*Standard deviations indicated in Table 1 subscript.

Many remarkable features were observed, such as higher phenolic contents in the white wine-3 than the others and most of the literature results. Therefore, the sensory and color properties of white wine-3 are expected to be higher than other wines. This wine was produced from 70% Dökülgen+30% Paf grapes. However, the other two wines also contain a higher amount of phenolic compounds compared with most of the literature results. Paf grape was contributed a higher amount of phenolic compounds to the white wine than Dökülgen grape. Hence, the grape variety has a significant effect on the phenolic content of wines during fermentation. The bark of Dökülgen grape is "thin-skinned" and weaker. Paf grape is "thick-skinned". Dökülgen grape was contributed more sugar (21%). Procyanidin B2 was the most abundant phenolic in the white wines, while tyrosol was the second most abundant phenolic. Many published results for white wines indicated that the main individual phenolic compounds in white wines were (+)-catechine and gallic acid. This difference might be related to the 'terroir' of the zone, water deficits, fewer temperature differences between daytime and nighttime, and infertile soil. Phenolic compounds play a

primary role in defining the sensorial characteristics of wines, giving the "oak wood" taste typical of long-aged products, besides being largely responsible for the astringency and bitterness of young wines (Bianchini and Vainio, 2003). The results showed that Dökülgen and Paf grapes are suitable for high-quality white wine production. Since these grapes contribute higher amount of phenolic characteristics, better acidity, and Brix to white wine.

4. Conclusions

Procyanidin B2, tyrosol and chlorogenic acid were significantly ($p<0.05$) higher in all white wines than the other phenolics. Phenolic acid contents of Dökülgen and Paf grapes were suitable for white wine production. White wine with higher phenolic contents will associate with high antioxidant capacity. Many of the remarkable features of the phenolic profiles and Brix of grape varieties could help us to characterize Gaziantep White wines. The results from this study provide valuable information about the white wine produced from the ancient grape variety of the South-east region. This study presents original data for phenolic compounds of Gaziantep white wines. These results could be of great interest to nutritionists and dietitians for the assessment of dietary phenolic compounds intake. However, considering the different sugar, acidy and phenolic concentrations, grape varieties will be used separately and mixtures in the production of the white wines to indicate quality characteristics and acceptability by the panelist.

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