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Oxidative stability of soybean and corn oils enriched with *Pluchea quitoc* hydroalcoholic extract

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SUMMARY: Soybean and corn oils are among the most popular vegetable oils, and are ingredients which are widely used in cooking and in the food industry. These oils contain many unsaturated fatty acids such as oleic, linoleic and linolenic acids, which makes them easily oxidized by oxygen. Extensive efforts are being made to prevent or minimize vegetable oil oxidation through the development of antioxidants. Phenolic antioxidants which are present in some extracts can be used as food additives to prevent lipid oxidation. In this study chromatographic analyses (HPLC and GC) of the *Pluchea quitoc* hydroalcoholic extract were performed. The content of phenolic compounds by the Folin-Ciocalteau method and the antioxidant properties against radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) were also evaluated. The effect of samples prepared with soybean and corn oils enriched with *Pluchea quitoc* hydroalcoholic extract was determined and compared with samples of these oils which were free of antioxidants and with samples containing the synthetic antioxidant BHT. The results showed potential for application of the extract. A high content of phenolic compounds (314 milligrams of gallic acid equivalents (GAE)/g of extract) and good IC50 values were detected for the inhibition of the radicals DPPH and ABTS (13.2 $\mu g \cdot mL^{-1}$ and 5.6 $\mu g \cdot mL^{-1}$). In the evaluation of the oxidative stability of the oils enriched with this extract, it was found that at 1% concentration it was possible to obtain values of induction period (IP) close to the samples with added BHT.

KEYWORDS: *Hydroalcoholic extract; Induction period (IP); Lipid oxidation; Pluchea quitoc*

RESUMEN: ***Estabilidad oxidativa de aceites de soja y maíz enriquecidos con extracto hidroalcohólico de*** **Pluchea quitoc.** Los aceites de soja y maíz se encuentran entre los aceites vegetales más populares, ingredientes ampliamente utilizados en la cocina y también en la industria alimentaria. Estos aceites contienen muchos ácidos grasos insaturados como los ácidos oleico, linoleico y linolénico que se oxidan fácilmente con el oxígeno. Se están realizando grandes esfuerzos para prevenir o minimizar la oxidación de los aceites vegetales mediante el desarrollo de antioxidantes. Los antioxidantes fenólicos presentes en algunos extractos se pueden utilizar como aditivos alimentarios para prevenir la oxidación de lípidos. En este estudio se realizó la obtención y análisis cromatográficos (HPLC y GC) del extracto hidroalcohólico de *Pluchea quitoc*. El contenido de compuestos fenólicos se evaluó por el método de Folin-Ciocalteau y las propiedades antioxidantes frente a radicales DPPH y ABTS. Se determinó el efecto de muestras preparadas con aceites de soja y maíz enriquecidas con extracto hidroalcohólico de *Pluchea quitoc* y se comparó con muestras de estos aceites libres de antioxidantes y con el antioxidante sintético BHT. Los resultados mostraron potencial para la aplicación del extracto. Se detectó un alto contenido de compuestos fenólicos (314 mg GAE) y buenos valores de IC50 para la inhibición de los radicales DPPH y ABTS (13.2 $\mu g \cdot mL^{-1}$ y 5.6 $\mu g \cdot mL^{-1}$). En la evaluación de la estabilidad oxidativa de los aceites enriquecidos con este extracto, se encontró que para la concentración del 1% es posible obtener valores de período de inducción (IP) cercanos a las muestras adicionadas con BHT.

PALABRAS CLAVE: *Extracto hidroalcohólico; Oxidación de lípidos; Período de inducción (IP); Pluchea quitoc*

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1. INTRODUCTION

Lipid oxidation is a spontaneous and inevitable phenomenon, with direct implication in the commercial value of fatty compounds and all products that are formulated from them (e.g. foods, cosmetics, medicines) (Carocho *et al.*, 2018; Shahidi, 2005). The prevention or delay of lipid oxidation is considered one of the main factors in vegetable oil deterioration, which can be affected by the addition of antioxidants which preserve the quality and extend the shelf-life of the products (Klein *et al.,* 2020; Mohamed *et al.,* 2018; Ramalho and Jorge, 2006).

Nowadays, synthetic antioxidants are the most commonly used by industries, and include butylhydroxyanisol (BHA), butylhydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (GP). However, the continued use of these antioxidants has been the subject of debate because of the possibility of causing toxic effects in the body, which makes the use of these compounds questionable, as shown by recent data in the literature (Mikołajczak *et al*., 2020; Akoh and Min, 2008; Gunstone, 2011). Therefore, butylated hydroxyanisole (BHA) was removed from the list of compounds generally recognized as safe (GRAS) (Li *et al.,* 2021). Currently, due to the great demand for natural ingredients in various applications, consumers have demanded the gradual substitution of synthetic compounds by natural ones (Taghvaei *et al*., 2014; Rostagno and Prado, 2013). Consequently, the search for and evaluation of natural materials with antioxidant properties has become a trend, with plants and extracts receiving special focus.

Thus, in an attempt to substitute synthetic antioxidants, many scientific researchers have been looking at options of natural and sustainable raw materials. mainly those that possess antioxidant and antimicrobial activities in their extracts and can produce a protective action in food (Shahidi and Ambigaipalan, 2015). Extracts obtained from plants are constantly used as functional foods, ingredients, additives (dyes, antioxidants, etc.) or as final products (nutraceuticals and supplements), and many of them have high antioxidant power (Javadian *et al.,* 2017; Shahidi and Ambigaipalan, 2015). Some procedures have been used to enrich or incorporate antioxidants in vegetable oils and have provided the development of functional edible oils with potential health-promoting potential, as they are improved with extracts rich in phenolic compounds (Fregapane *et al*., 2020).

Due to questions and certain insecurity related to the consumption of synthetic antioxidants, research has focused on obtaining natural products with antioxidant activity which serve to replace synthetics (Sousa *et al.,* 2014; Sousa *et al.,* 2019). Interest in natural phenolic compounds as antioxidants has grown rapidly in recent years due to evidence of nutritional properties for human health. Phenolic compounds, in addition to having high antioxidant activity, reduce the risk of certain types of diseases through consumption in foods (Bravo, 1998; Wu *et al.,* 2019)

Many plants are recognized for their antioxidant properties, and it has been observed that the extracts of some species have potential equivalent to synthetic or isolated natural antioxidants. A strategy currently employed is to carry out tests with combinations of natural antioxidants (extracts or isolates) with synthetic antioxidants in order to minimize the use or decrease the concentration of synthetics in formulations (Li *et al*., 2021). Some results have shown to be very promising, such the combination of extracts with traditional antioxidants, highlighting the synergistic effect (Hraš *et al.,* 2000; Marinova *et al.,* 2008; Thoo *et al.,* 2013; Yanishlieva *et al.,* 2006). In most cases, the antioxidant capacity of the extracts is attributed to phenolic compounds, which are important in the development of plants which are efficient in preventing auto-oxidation (Angelo and Jorge, 2007).

As well as to the protective action against oil oxidation, the preparation of oils enriched with extracts is a good strategy to ensure the intake of bioactive polyphenols through the diet (Fregapane *et al*., 2020). The development of these functional oils can help prevent chronic diseases (such as cardiovascular disease, immune weakness, aging disorders and degenerative diseases) and improve the quality of life for many consumers by reducing health costs (Reboredo-Rodríguez *et al*., 2017). Many efforts are being made to contribute to the developmental processes of functional oils, including computational modeling of the enrichment process with phenolic compounds/extracts, kinetic and thermodynamic studies and the application of artificial neural networks, which enable evaluation and improve the quality of vegetable oils treated with phytochemicals (Gülmez and Sahin, 2019; Sahin *et al.,* 2017; Sahin *et al.,* 2019; Sahin *et al.,* 2020; Samli *et al.,* 2020).

The *P. quitoc* species is a shrub belonging to the Asteraceae family, popularly known as "quitoco". This species is common in the tropical region, located in different countries of Latin America, including the south of Brazil. It is a perennial, erect and aromatic plant with a characteristic mild odor. In the literature it is possible to find records of the popular use of this plant as an agent to combat respiratory and stomach disorders. It is carminative, with indications for the home treatment of digestive problems and colds and also acts as a stimulant. Other studies have shown that *P. quitoc* extracts have antimicrobial, anti-inflammatory, antioxidant and anticancer effects (Simionatto *et al.,* 2007b; Guilhon and Muller, 1996).

In order to contribute to studies on the possibilities of natural antioxidants, in the present study the effectiveness of the *P. quitoc* hydroalcoholic extract was compared to the synthetic antioxidant butylhydroxyltoluene (BHT) and flavonoid rutin, for delaying the oxidation of the vegetable oils soybean and corn.

2. MATERIALS AND METHODS

2.1. Chemicals

Ethanol, methanol, glacial acetic acid, sodium acetate, acetonitrile (99.9%), caffeic acid (98.0%), ferulic acid (98.0%), gallic acid (97.5%), rosmarinic acid (98.0%), *p*-coumaric acid (98.0%), rutin (94%), quercetin (95.0%), campesterol (65%), β-sitosterol (95%), lupeol (94%), lupeol acetate (95.0%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±) -6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ethyl benzothiazoline-6-sulfonic acid diammonium 2,2'-azino-bis (ABTS), sodium chloride, aluminum chloride, sodium acetate, sodium carbonate, potassium persulfate and butylhydroxyltoluene (BHT), were all purchased from Sigma-Aldrich, São Paulo - SP, Brazil. Additive-free vegetable oils were supplied by the company Cocamar, Maringá-PR, Brazil.

2.2. Plant material

The aerial parts of *P. quitoc* were collected in Ibarama-RS, Brazil, in January 2018. The collected material was dried in a place with low luminosity and humidity, at 35 ºC for 5 days, and maintained at room temperature for further processing. The voucher specimen was deposited in the Herbarium of the Faculty of Biological Sciences of the Federal University of Grande Dourados - UFGD (voucher no. 8507).

2.3. Preparation of hydroalcoholic extract

The *P. quitoc* aerial part (1.0 kg) was crushed and subjected to exhaustive extraction with ethanol/water (70/30, 2L) at room temperature and with occasional stirring. The extraction process was carried out over 10 days, and every 2 days in maceration, the hydroalcoholic extract was filtered and stored in amber bottles. The hydroalcoholic extract was obtained by removing the solvents in a rotary evaporator (50 °C) under vacuum. The drying was carried out at room temperature, 10 mL of the extract were deposited onto plates and kept for 48 hours. This process resulted in a solid, which was removed and conditioned.

2.4. Determination of the total phenolic content

The total phenolic content was determined according to the Folin–Ciocalteu's reagent method (Djeridane *et al.,* 2006). In a short period of time, 100 µL of the aqueous extract solution (1 $mg \cdot mL^{-1}$) were added to 1000 µL of ultrapure water and 500 µL of the Folin–Ciocalteu's reagent (1/10) in water. After 1 min 1500 µL of Na_2CO_3 (20% w/v) were added. The final mixture was shaken and incubated for 2 hours in the dark. The absorbance was read by a spectrophotometer (FENTO 700 PLUS) (λ=760 nm). Gallic acid (Sigma-Aldrich, USA) was used as standard at concentrations varying from 5 to 1000 $\mu g \cdot mL^{-1}$, $r^2 = 0.9992$. The results were expressed in mg of gallic acid per g of dry weight of aqueous extract. All tests were performed in triplicate.

2.5. Evaluation of free radical scavenging activity

2.5.1. DPPH assay

The free radical scavenging activity of extracts was evaluated by the modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and according to the Trolox equivalent antioxidant capacity (TEAC) (Simionatto *et al*., 2007a; Klein *et al*., 2020). A stock solution of DPPH in 0.004% methanol was prepared and 50 µL of the concentrations 1.25 $mg \cdot mL^{-1}$, 0.625 $mg \cdot mL^{-1}$ and 0.312 $mg \cdot mL^{-1}$ of *P. quitoc* extracts were added to 5 mL of the DPPH solution. After 30 minutes of incubation in a dark environment and at room tem-

perature, the absorbance was analyzed at a wavelength of 517 nm. Rutin was used as standard. The calculation to verify the inhibition of free radical DPPH (I%) is described in the equation:

$$I\% = [(Aa - Ab) / Aa] \cdot 100$$

With Aa being the control Absorbance and Ab the absorbance of the reaction. The extract concentration ($\mu g \cdot mL^{-1}$) giving 50% inhibition of the DPPH radicals (IC50) was obtained by linear regression analysis by interpolation.

The Trolox equivalent antioxidant capacity (TEAC) was performed using a standard curve constructed with concentrations of 2000 μM, 1500 μM, 1000 μM, 500 μM and 100 μM of Trolox. A DPPH 0.004% solution was used as the reagent and the concentrations of the hydroalcoholic extract of *P. quitoc* were 5 $mg \cdot mL^{-1}$, 2.5 $mg \cdot mL^{-1}$, 1.25 $mg \cdot mL^{-1}$, 0.625 $mg \cdot mL^{-1}$ and 0.312 $mg \cdot mL^{-1}$. The values obtained were expressed in μMtrolox/g of crude extract.

2.5.2. ABTS assay

The activity of elimination of free radical extracts was also determined by ABTS assay. The percentage of inhibition was evaluated according to the methodology described by Re *et al*. (1999) and the method described by Rufino *et al.* (2007). The ABTS radical was prepared from the reaction of 140 mM potassium persulfate with 7 mM ABTS, stored in the dark at room temperature for 16 hours, and then diluted in 95% ethyl alcohol until the absorbance value of 0.700 ± 0.020 at the wavelength 734 nm.

To measure the percentage of antioxidant inhibition, sample aliquots of 30 μL at concentrations of 1.25 $mg \cdot mL^{-1}$, 0.625 $mg \cdot mL^{-1}$ and 0.312 $mg \cdot mL^{-1}$ were transferred to test tubes and added with ABTS (3 mL). After 6 minutes of reaction in a dark place at room temperature, the absorbances were analyzed at 734 nm using ethanol as blank and rutin as standard. The calculation to verify the inhibition of free radical ABTS (I%) is described in the equation:

$$I\% = [(Aa - Ab) / Aa] \cdot 100$$

The extract concentration ($\mu g \cdot mL^{-1}$) giving 50% inhibition of the DPPH radicals (IC50) was obtained by linear regression analysis by interpolation.

The Trolox equivalent antioxidant capacity (TEAC), was performed using a standard curve constructed at 2000 μM, 1500 μM, 1000 μM, 500 μM and 100 μM Trolox concentrations, using the ABTS radical as reagent, and the extracts at concentrations of 5 $mg \cdot mL^{-1}$, 2.5 $mg \cdot mL^{-1}$, 1.25 $mg \cdot mL^{-1}$, 0.625 $mg \cdot mL^{-1}$ and 0.312 $mg \cdot mL^{-1}$. The values obtained are expressed in μMtrolox/g crude extract.

2.6. HPLC analysis of *P. quitoc* extract

The sample was solubilized in water: methanol (7:3 v:v) filtered through 0.45 μm ultrafilter. and evaluated in a LC analytical column (LC-6AD Shimadzu, Kyoto, Japan) with the assistance of a photodiode array detector (DAD) system which was monitored between wavelengths λ = 200-800 nm. In an LC analytical apparatus; the column was ODS HYPERSIL (C-18, 150 mm long x 4.6 mm diameter, Thermo Electron Corporation, United States). The flow rate and the injection volume were 1 $mL \cdot min^{-1}$ and 20 μL, respectively. All chromatographic analyses were performed at 25 ºC. Eluent A was composed of a binary mobile phase of water with 6% acetic acid and 2 mM sodium acetate, and eluent B was composed of acetonitrile and the following gradient was applied: 0 min 5% B; 20 min 15% B; 30 min 60% B; and 40 min 100% B. Standard samples of gallic acid, ferulic acid, caffeic acid, ferric acid, rosmarinic acid, *p*-coumaric acid, rutin, quercetin, luteolin and apigenin were used (Sigma), prepared in methanol-water (7:3 v:v) in 1000 $\mu g.mL^{-1}$ concentration. The elution method developed for LC aimed at the identification of phenolic compounds with the assistance of the DAD scanning detector in the spectral range of 200-800 nm did not reveal interferences in the retention times. The absorption spectra and the retention time of the standards were the parameters for the identification and quantification of the compounds. Co-injection experiments, in which extracts and standard aliquots were mixed and diluted to a known volume, were also carried out to unequivocally identify the compounds. The calibration curves were determined by linear regression using LC. The linearity for standards was assessed for 10 concentration ranges. The average standard errors for the peak areas of replicated injections (n = 5) were less than 2%, thus showing good repeatability of the calibration curve. The respective coefficients of determination (r^2) were 0.9994 for caffeic

acid, ferulic acid and gallic acid and $r^2 = 0.9996$ for rutin and quercetin.

2.7. Chromatographic analysis by GC-MS

Sample preparation for GC-MS analysis was as follows; 100 mg of the *P. quitoc* extract were added to 1 mL of water and 1 mL of hexane and followed by separation of the hexane fraction. 2 mL of hexane were added to the aqueous fraction and the process was repeated again. These two hexane fractions were dried and suspended in 1.0 mL of hexane. For GC-MS analysis the solution was first filtered through a 0.45 μm ultrafilter.

To identify the compounds present in the sample, it was also evaluated by mass spectrometry (GC-MS). The GC-MS analysis was carried out on a GC-2010 Plus (Shimadzu, Kyoto, Japan), equipped with a mass spectrometry detector (GC-MS Ultra 2010), using LM-5 (5% phenyldimethylpolysiloxane), fitted with a capillary column of fused silica (15 m length x 0.2 mm id, and 0.2 μm-thick film). The analysis was performed under the following conditions: helium make up gas (99.999% and flow rate 1 $mL \cdot min^{-1}$), 1 μL of injection volume, split ratio (1:20), initial oven temperature adjusted to 150 ºC and heating from 150 ºC to 280 ºC at 15 $ºC \cdot min^{-1}$ and held at 280 ºC for 15 min. The injector temperature was 280 °C and the quadrupole detector temperature was 300 °C. An electron impact ionization voltage of 70 eV, a mass range of 45-600 nm *m/z* and a scanning interval of 0.3 s were the MS scanning parameters.

Compounds identification was accomplished by comparing the mass spectra obtained in the NIST21 and WILEY229 libraries. In some cases, reference compounds were co-chromatographed and the identification of the components was made by comparison of their retention times with standards. Standards for stigmasterol, campesterol, β-sitosterol, lupeol and lupeol acetate (Sigma) were prepared in hexane in the concentration of 1000 $\mu g \cdot mL^{-1}$. The compound concentrations were determined by external calibration. The linearity of standards was assessed for 5 concentration ranges. The average standard errors for the peak areas of replicated injections (n = 5) was less than 2%, thus showing good repeatability of the calibration curve. The respective coefficients of determination (r^2) were 0.9996 for stigmasterol, campesterol, β-sitosterol and lupeol and $r^2 = 0.9994$ for lupeol acetate.

2.8. Preparation of *P. quitoc* extract - enriched soybean and corn oils

Samples of soybean and corn oils were enriched with the *P. quitoc* hydroalcoholic extract to reach concentrations of 0.62, 1.25 and 2.5% (extract in oil). The extracts were added directly to the oils, followed by slow stirring for homogenization. Samples of soy and corn oils were also enriched with the synthetic antioxidant BHT at 0.02% (w/v) concentration (Brazil-Anvisa, 2005). BHT was added directly to the oils, followed by slow stirring until dissolved.

2.9. Rancimat test

The Rancimat test was used to assess the oxidative stability of the additive oils (with BHT and *P. quitoc* extract) and of the additive-free oils. The analyses were performed using a Rancimat apparatus (Metrohm, model 893, Herisau, Switzerland). The analyses were performed in a fixed amount of oil sample (3 g) at 110 °C and 10-$L \cdot h^{-1}$ air flow (Tinello and Lante, 2020). The oxidative stability was expressed as the induction period (IP) corresponding to the time (h). The induction period was determined using the software provided by the equipment manufacturer (StabNet).

2.10. Schaal oven test and evaluation of acid value

The Schaal oven test, which aims to assess the effect of accelerated storage conditions on the oxidative stability of oils, was performed as described by Yang *et al.* (2016) in soybean and corn oils without any addition (C), with *P. quitoc* hydroalcoholic extract (1%, 2.5% and 5.0%), and with butylated hydroxytoluene (BHT) as synthetic antioxidant at 0.02% (w/w) concentration, which corresponds to the maximum level established by Codex Alimentarius (2019). In detail, the oil samples were accurately weighed (40 g ± 0.01 g) in beakers and stored in an oven at a constant temperature of 62 °C for 28 days. Every 7 days the samples were collected and submitted to analysis.

2.10.1. Acid value

For the measurement of the acid value, the official method of AOCS (1993) was followed. 15 g of each oil were weighed into a container, dissolved in 50 mL of ethanol/diethyl ether (1:1 v/v) and then titrated with potassium hydroxide in the presence of phenolphthalein until persistence of pink coloring.

2.11. Statistical analysis

Results are presented and expressed in terms of mean and standard deviations. Significant differences were calculated using ANOVA in combination with the Duncan's test with significance level of α=0.05, supplemented, when necessary, by the Tukey test with Statistic Software (version 7.0).

3. RESULTS AND DISCUSSION

3.1. Characterization of the *P. quitoc* hydroethanolic extract

The hydroalcoholic extract of the *P. quitoc* aerial part was obtained st a yield of 10.2%. This yield can be considered good and comparable to the extracts obtained from from *Schinus molle* (fruits and leaves) (12 and 16%), *Psidium firmum* (11.5%) and lower than *Rosmarinus officinalis* (16-19%) (Klein *et al.,* 2020; Peres *et al.,* 2013, Wang *et al.,* 2018). The extract was initially characterized following the Folin-Ciocalteau method for the presence of phenolic compounds attributed to the concentration of 314 mg GAE (gallic acid equivalent)/g of *P. quitoc* extract. This result serves to classify this plant as rich in phenolic compounds at levels close to the extracts widely known and already highlighted as having high contents of these compounds. Several works highlight the phenol content identified in peanut skin extracts, ginger (*Zingiber officinale*), turmeric (*Curcuma longa*) and rosemary, which have phenol contents close to those found in the *P. quitoc* hydroalcoholic extract (Franco *et al.*, 2018; Tinello and Lante, 2020; Wang *et al.*, 2018).

The extract was analyzed by HPLC, and initially detected the presence of seven peaks of greater intensity. With the use of standards and spectral analysis, it was possible to identify five compounds. Figure 1 shows the chromatographic profile of the extract and the identification of gallic acid (peak 1), caffeic acid (peak 2), ferulic acid (peak 3), rutin (peak 4) and quercetin (peak 5). It is worth noting that these phenolic compounds found in the *P. quitoc* extract have already been reported as good natural antioxidants (D'Andrea, 2015; Shahidi and Ambigaipalan, 2015). Considerable research has been done with the objective of obtaining extracts enriched with classes of phenolic compounds such as flavonoids, and organic phenolic acids, among others (Taghvaei *et al*., 2014).

The hydroalcoholic extract of *P. quitoc* was also subjected to extraction with hexane and after filtration, analyzed by gas chromatography (Figure 2). Through this analysis it was possible to identify and quantify five compounds: campesterol (22.0 $mg \cdot g^{-1}$), stigmasterol (26.5 $mg \cdot g^{-1}$), β-sitosterol (57.1 $mg \cdot g^{-1}$), lupeol (47.4 $mg \cdot g^{-1}$) and lupeol acetate (48.9 $mg \cdot g^{-1}$).

Table 1. Compounds identified by liquid chromatography (HPLC) the hydroalcoholic extract of the aerial parts of *P. quitoc.*

RT (min)	Peak	Compounds	Hydroalcoholic extract ($mg.g^{-1}$ ± SD)
2.31	1	Gallic acid	159.6 ± 1.1^{c}
6.31	2	Caffeic acid	82.6 ± 1.2^{b}
8.83	3	Ferrulic acid	217.5 ± 1.0^{e}
21.75	4	Rutin	69.8 ± 0.4^{a}
24.42	5	Quercetin	162.7 ± 0.2^{d}

Data are shown as mean ± standard deviation (SD). Different letters in the same lines represent significant differences ($p < 0.05$); equal letters do not differ significantly. Duncan test ($p < 0.05$) was used for the comparison of means. All experiments were carried out in triplicate. RT: retention time.

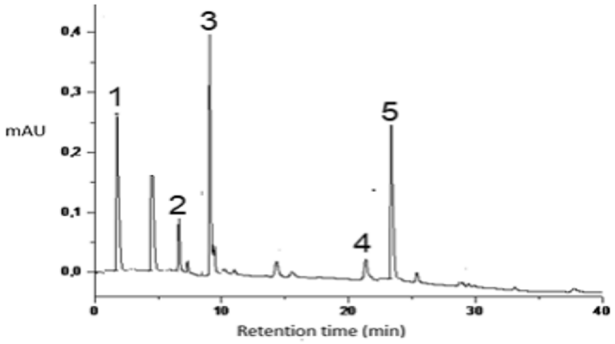

Figure 1. Chromatographic profile (280 nm) of the hydroalcoholic extract of *P. quitoc*. Identification of compounds: gallic acid (1), caffeic acid (2), ferrulic acid (3), rutin (4), quercetin (5).

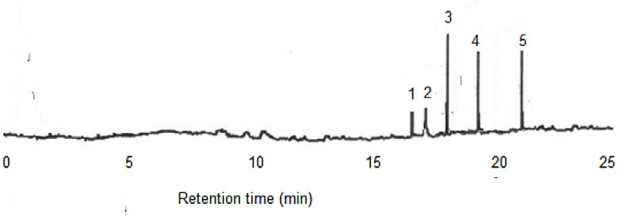

Figure 2. Chromatogram (GC-MS) of the hexane fraction obtained from the hydroethanolic extract of *P. quitoc*. Identification of compounds: campesterol (1), stigmasterol (2), β-sitosterol (3), lupeol (4) and lupeol acetate (5).

3.2. Evaluation of the antioxidant activity of hydroethanolic extract

Table 1 shows the results of the antioxidant activity against the DPPH and ABTS radicals, where it can be seen that the extract has high activity. The IC50 values for the extract were 13.2 $\mu g \cdot mL^{-1}$ and 5.6 $\mu g \cdot mL^{-1}$, in the tests with DPPH and ABTS radicals, respectively. According to the results, there is a variation in the IC50 values obtained by both methods. This variation has also been observed in other studies on the evaluation of the antioxidant properties of extracts using the radicals DPPH, ABTS and other methods (Franco *et al.,* 2018; Pedro *et al.,* 2018; Tinello and Lante, 2020). The high activity of the extract is verified against the two radicals in comparison to the standard of synthetic antioxidant BHT and to the natural flavonoid rutin, whose extracts presented IC 50 values close to the values for these standard compounds. Another parameter analyzed for the antioxidant potential of *P. quitoc* extract was the determination of the Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC results also showed that the extract had significant activity, obtaining the values of 1024.8 and 1943.43 mM Trolox/g of hydroalcoholic extract, for the tests with the radicals DPPH and ABTS, respectively. The extract showed higher TEAC compared to the flavonoid rutin using the ABTS method. This behavior can be related to a probable synergistic effect of the phenolic compounds present in the extract of *P. quitoc*, acting in the inhibition of the radical ABTS. The TEAC values obtained for the *P. quitoc* extract and standards can be compared to those obtained from green tea ethanolic extract (3028 mM Trolox·g^{-1}), green tea aqueous extract (1723 mM trolox·g^{-1}) and extracts obtained from *Mangifera indica* (2750 mM trolox·g^{-1}) (Leite *et al.,* 2012; Sánchez-Camargo *et al.,* 2020). The results obtained in the present study are relevant to the data on antioxidant activity, demonstrating that *P. quitoc* extract has great capacity to eliminate radicals, suggesting that the extract could act as a preventive or blocker of the formation of reactive oxygen species chains, which could delay damage to certain systems and materials.

Some plant extracts are already mentioned in the literature as promising natural antioxidants, verified by several methods. Plant extracts that can be prepared from herbs, leaves, vegetable residues and fruit residues stand out as important sources of phenolic compounds (Sharma *et al.*, 2019). It is also intensively mentioned in the literature that the content of simple phenols and phenolic acid compounds is linked to the antioxidant effects of plant extracts (Bodoira *et al.*, 2017).

This antioxidant action potential may be associated with the phenolic compounds present in the *P. quitoc* extract. The chromatographic analysis showed that the extract consisted of three groups of compounds, two of which were phenolic, flavonoids and phenolic acids. Many authors have already reported the ability of phenolic extracts, flavonoids and phenolic acids to eliminate ABTS and DPPH radicals (Zahran and Najafi, 2020; Wang *et al.,* 2018; Klein *et al.,* 2020). Among the phenolic compounds, flavonoid quercetin and ferulic acid were the most representative compounds of the *P. quitoc* extract with concentrations of 162.7 $mg \cdot g^{-1}$ and 217.5 $mg \cdot g^{-1}$, respectively. These compounds (quercetin and ferulic acid) are reported in the literature because both have important antioxidant effects, and present high activity (D'Andrea, 2015; Shahidi and Ambigaipalan, 2015). All other phenolic components (gallic acid, caffeic acid and rutin) of the *P. quitoc* hydroalcoholic extract are also highlighted for their important antioxidant effects (Shahidi and Ambigaipalan, 2015), which justify the high activity of this extract. The an-

Table 2. Concentration which inhibits 50% of radicals (IC50 values in $\mu g.mL^{-1}$) and Trolox Equivalent Antioxidant Capacity (TEAC) values (in $\mu mol.g^{-1}$) according to the ABTS and DPPH methods for the hydroalcoholic extract of *P. quitoc.*

Samples	Method	
	DPPH	ABTS
IC50 - extract *P. quitoc*	13.2±0.6[a]	5.6±0.2[b]
IC50-rutin	9.2±0.5[b]	16.0±0.3[a]
IC50-BHT	9.6±0.6[b]	4.2±0.3[c]
TEAC – extract *P. quitoc*	1024.8±24.8[c]	1943.43±27.4[b]
TEAC-rutin	2588.61±36.7[b]	966.4±16.2[c]
TEAC-BHT	2981.09±41.2[a]	2531.62±36.1[a]

Data are shown as mean ± standard deviation (SD). Different letters in the same column represent significant differences ($p < 0.05$); the same letters do not differ significantly. The Duncan test ($p < 0.05$) was used for the comparison of means. All experiments were carried out in triplicate. IC50: concentration which inhibits 50% of DPPH/ABTS radicals. TEAC: Trolox equivalent antioxidant capacity.

tioxidant potential of phenolic compounds depends on the number and arrangement of hydroxyl groups in the molecules. Phenolic substances, such as those found in the extract of *P. quitoc*, can act as antioxidants by donating hydrogen atoms to lipid radicals and produce lipid derivatives and phenoxyl radicals, which are more stable and have less potential for self-oxidation (Kiokias *et al.,* 2008). The other compounds characterized are steroids (campesterol, stigmasterol, β-sitosterol) and triterpenoids (lupeol and lupeol acetate).

3.3. Soybean and corn oils enriched with hydroalcoholic extract: Analysis of oxidative stability by Rancimat and acid value

After the characterization of the *P. quitoc* hydroalcoholic extract and verification of potential antioxidant activity (against DPPH and ABTS radicals), the extract was evaluated for its activity in the inhibiting of lipid oxidation, once added to vegetable oils. The Rancimat analysis was performed at 110 °C and the induction period was evaluated until the end point of the samples' stability. Different concentrations of the extract were added to soybean and corn oils and the effects of these oils enriched with the extracts were compared to the synthetic standard BHT. Table 4 presents the results of the induction period of soybean and corn oils added with different concentrations of *P. quitoc* hydroethanolic extract, with BHT, and without the addition of any antioxidants.

According to the results, considering antioxidant-free oils, it is noted that corn oil had greater oxidative stability in comparison to soybean oil, the difference being statistically evidenced, according to the Tukey test ($p < 0.05$). It can be attributed to the differences in the oils' compositions; corn oil has a higher content of saturated compounds and less content of unsaturated compounds in comparison to soybean oil, parameters that contribute to the greater stability of corn oil (Dweck and Sampaio, 2004). In oils enriched with extracts, it was observed that there was an increase in the induction period (IP), demonstrating the effectiveness of the extract in protecting the oil in relation to oxidative degradation. Analyzing corn oil specifically, the control sample showed a significant difference compared to the oil samples incorporated into the extract, with an increase in the induction period (IP) from 1.2 h, 1.4 h and 2.0 h to 1.0 2.5 and 5.0%, concentrations, respectively. As for soybean oil, the increases in induction periods in relation to the control sample were 0.7 h, 1.4 h and 1.7 h at concentrations of 1.0, 2.5 and 5.0%, respectively. Once the action of *P. quitoc* extract was compared to the synthetic antioxidant BHT, promising effects were also observed, since in both oils evaluated, the inhibition of oxidation was more effective with the use of the extract. The increase in the induction period (IP) of the oils added with BHT in relation to the control was 0.7 h and 0.4 h for corn and soybean oils, respectively; whereas with a concentration of 1% of extract, the oils presented increases in the induction period of 1.2 h and 0.7 h in relation to the control for corn and soybean oils, respectively. Therefore, oils already enriched with extract at a concentration of 1% performed better than the antioxidant BHT.

The acid value was selected as a parameter, with the objective of evaluating the stability of soybean and corn oil samples with or without antioxidants during storage at 62 °C for 28 days (Schall oven test). Acceptable acid values for vegetable oils must be below 0.6 mg KOH per g of oil, as established by the ANVISA (National Health Surveillance Agency) standard for vegetable oils (Brazil-Anvisa, 2005). Table 4 shows the results obtained from the determination of acid values. By comparing the acid values of oils without additives to the oils enriched with extracts, it was noted that the additive oils showed a beneficial effect in reducing acid values. According to the values, it appeared that all samples had an acid value below 0.6 mg $KOH \cdot g^{-1}$ at the time of preparation (day zero), demonstrating that the addition of

Table 3. Oxidative stability (Induction Period) of soybean and corn oils at different concentrations of *P. quitoc* extract.

Samples	Induction period (h)	
	Corn oil	Soybean oil
pure oil (control)	$9.5 \pm 0,54^{Ab}$	$7.3 \pm 0,11^{aA}$
Oil + BHT	$10.2 \pm 0,02^{baB}$	$7.7 \pm 0,42^{Ba}$
Oil + 1% of extract	$10.7 \pm 0,51^{cbB}$	$8.0 \pm 0,23^{cA}$
Oil + 2.5% of extract	$10.9 \pm 0,11^{dcB}$	$8.7 \pm 0,15^{Da}$
Oil + 5% of extract	$11.5 \pm 0,13^{edB}$	$8.9 \pm 0,11^{Da}$

Data are shown as mean ± standard deviation (SD). Values with same lowercase letters in same column and values with the same uppercase letters within the same row were not statistically different ($p < 0.05$). Duncan test ($p < 0.05$) was used for the comparison of means. All experiments were carried out in triplicate. BHT: butylhydroxyltoluene. Analysis conditions: 3 g of sample, at 110 °C and 10-$L \cdot h^{-1}$ air flow.

TABLE 4. Effect on the acid value of antioxidant-free corn and soybean oils, added with BHT and enriched with the hydroalcoholic extract of *P. quitoc* during 28 days storage at 62 °C.

Samples	Acid value (mg KOH·g^{-1})				
	Day 0	Day 7	Day 14	Day 21	Day 28
Corn oil					
Pure oil (control)	0.47 ± 0.4^{cA}	2.04 ± 0.01^{cB}	2.72 ± 0,02^{cC}	2.72 ± 0,01^{cC}	9.50 ± 0,01^{dD}
Oil + BHT	0.24 ± 0.1^{aA}	0.68 ± 0.02^{aB}	0.68 ± 0,02^{aB}	1.36 ± 0,01^{bC}	4.75 ±0,03^{bD}
Oil+1% extract	0.40 ± 0.01^{bA}	0.68 ± 0.01^{bC}	1,36 ±0,02^{bC}	1.36± 0,04^{aB}	8.15 ± 0,01^{cD}
Oil + 2.5% extract	0.40 ± 0.03^{bA}	1.36 ± 0.01^{bB}	1.36 ± 0,01^{bB}	1.36 ± 0,02^{bB}	2.72 ± 0,02^{aC}
Oil + 5% extract	0.40 ± 0.01^{bA}	1.36 ± 0.01^{bB}	1.36 ± 0,01^{bB}	1.36 ± 0,01^{bB}	2.72 ± 0,01^{aC}
Soybean oil					
Pure oil (control)	0.54 ± 0.01^{dA}	4.07 ± 0.02^{dB}	5.43 ± 0,06^{cC}	8.82 ± 0,02^{dD}	14.26 ± 0,02^{eE}
Oil + BHT	0.34 ±0.01^{aA}	0.68 ± 0.02^{aB}	0.68 ± 0,05^{aB}	3.39 ± 0,03^{cC}	5.43 ± 0,01^{aD}
Oil + 1% extract	0.44 ± 0.01^{cA}	1.36 ± 0.01^{cC}	2.04 ± 0,01^{bB}	2.04 ± 0,01^{aC}	9.50 ± 0,03^{dD}
Oil + 2.5% extract	0.40 ± 0.02^{bA}	1.36 ± 0.04^{bB}	1.36 ±0,01^{bB}	3.39 ± 0,01^{cC}	8.15 ± 0,02^{cD}
Oil + 5% extract	0.40 ± 0.01^{bA}	1.36 ± 0.01^{bB}	1.36 ± 0,03^{bB}	2.72 ± 0,02^{bC}	6.11 ± 0,02^{bD}

Data are shown as mean ± standard deviation (SD). Values with the same lowercase letter in the same column and values with the same uppercase letters within the same row were not statistically different ($p < 0.05$). All experiments were carried out in triplicate.

the extract to the oil samples did not affect this parameter. The tendency to increase the acid value of soybean and corn oils samples was slow in the first 14 days, while a sharp increase was observed until the end of thermal storage.

The antioxidant-free oils showed the highest acid values, and presented increases in all analyses of the period for soybean and corn oils, reaching high values on day 28, at 9.50 and 14.26 mg KOH·g^{-1}, respectively. With the extract addition, the samples showed a less accelerated behavior in the variation in acid values, which also presented lower indexes in comparison to oils without additives, suggesting that the extracts contributed to the control of this parameter.

The highest extract concentrations added to the oils (5%) provided samples with less acidity at the end of the analysis period (day 28), showing better behavior than the BHT standard for corn oil, with an acid value of 2.72 mg KOH·g^{-1}; whereas for the oil with BHT standard the acid value was 4.75 mg KOH·g^{-1}. For soybean oil, samples with BHT and 5% extracts also showed similar acid values, with 5.43 mg KOH·g^{-1} and 6.11 mg KOH·g^{-1}, respectively. According to the results obtained, soybean oil showed the highest acidity values at the end of the analysis period (day 28). The lipid composition of each oil can influence these acidity values. There are differences in the proportions of saturated/unsaturated compounds in soybean and corn oils, which are approximately 15%/84% and 18%/81%, respectively (Dweck and Sampaio, 2004). This higher proportion of unsaturated compounds in soybean oil may contribute to less oil stability, which may result in higher acidity values. In addition, the oils differ in terms of their contents of polyunsaturated compounds. Soy oil has approximately 58%, while corn oil has 45% (Dweck and Sampaio, 2004). Oils that contain a greater abundance of polyunsaturated fatty acids are vulnerable to oxidative degradation (Sharma *et al.,* 2019).

Generally, there was an increase in the acid value with the time of exposure of the sample to heat. This behavior is in agreement with other studies found in the literature (Yldirim, 2009; Souza *et al.,* 2014; Souza *el al.,* 2019). For the oils analyzed, the greatest variation in acid values occurred in the period of 21-28 days, both for oils with extracts or with BHT. In this period of analysis, it was observed that the oils added with the *P. quitoc* extract had significantly lower acid values than the oil which was free of antioxidants, demonstrating the positive effect of the extract in the control of acidity.

4. CONCLUSIONS

The hydroalcoholic extract of the aerial part of *P. quitoc* has a composition with high content of

phenolic compounds. Tests related to the evaluation of antioxidant activity demonstrated high activity for this extract. It was possible, for the first time, to determine the behavior of this extract in the lipid oxidation inhibition in vegetable oils (soybean and corn). It can be concluded that the *P. quitoc* hydroalcoholic extract can be used as stabilizer against the oxidation reactions of these oils. Under conditions of accelerated oxidation (Rancimat), all tested concentrations (1%, 2.5% and 5%) showed better effects as inhibitors of oil oxidation, providing an increase in induction periods in comparison to the samples exempt of antioxidants and samples containing the antioxidant BHT. In the highest concentration tested (5%), the *P. quitoc* hydroalcoholic extract also promoted control of the acid value of the oil samples, and these samples presented lower indexes than the control and standard samples. The protective effect of *P. quitoc* hydroalcoholic extract against the damage of primary oxidative reactions in soybean and corn oils may be highly correlated to its content of phenolic compounds and may be an important source of phytonutrients.

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A comparative evaluation of chemical composition and antimicrobial activities of essential oils extracted from different chemotypes of *Cinnamomum camphora* (L.) Presl

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ABSTRACT: The purpose of this study is to determine the chemical composition of the essential oils of *Cinnamomum camphora* (L.) Presl leaves (CCPL) from 5 different habitats in China by GC-MS, and to evaluate their antimicrobial activities against 3 foodborne pathogens, using a paper disc diffusion method. A total of 30 compounds were identified with a predominance of oxygenated monoterpenes, including linalool (42.65%-96.47%), eucalyptol (39.07%-55.35%) and camphor (26.08%) as well as monoterpene hydrocarbons such as sabinene (6.18%-12.93%) and α-terpineol (8.19%-13.81%). Through cluster analysis, CCPL from 5 different habitats can be well divided into 2 categories. Combining with principal component analysis, the habitats can be better correlated with the chemical constituents of the essential oils. The antimicrobial activities of 5 extracted essential oils against 2 gram-negative bacteria and one gram-positive bacteria were assessed. It showed that the essential oil extracted from the CCPL harvested in Jinxi had the strongest antibacterial property. The results of this study provided basis for resource identification of CCPL and quality difference identification of essential oils. Research on the antibacterial properties of several pathogenic strains has proved its application value as a natural food preservative.

KEYWORDS: *Antimicrobial activities;* Cinnamomum camphora *(L.) Presl; Essential oils; Foodborne bacteria; Linalool*

RESUMEN: ***Evaluación comparativa de la composición química y las actividades antimicrobianas de los aceites esenciales extraídos de diferentes quimiotipos de* Cinnamomum camphora *(L.) Presl.*** El objetivo de este estudio es determinar la composición química de los aceites esenciales de hojas de *Cinnamomum camphora* (L.) Presl (CCPL) de 5 hábitats diferentes de China mediante GC-MS, y evaluar sus actividades antimicrobianas contra 3 patógenos transmitidos por los alimentos, utilizando un método de difusión de disco de papel. Se identificaron un total de 30 compuestos, con predominio de monoterpenos oxigenados, entre ellos linalol (42,65%-96,47%), eucaliptol (39,07%-55,35%) y alcanfor (26,08%) así como hidrocarburos monoterpenos como el sabineno (6,18%-12,93%) y α-terpineol (8,19%-13,81%). A través del análisis de conglomerados, los CCPL de 5 hábitats diferentes se pueden dividir bien en 2 categorías. En combinación con el análisis de componentes principales, los hábitats se pueden correlacionar mejor con los componentes químicos de los aceites esenciales. Se evaluaron las actividades antimicrobianas de 5 aceites esenciales extraídos contra 2 bacterias gramnegativas y una bacteria grampositiva. Se demuestra que el aceite esencial extraído del CCPL cosechado en Jinxi tenía la propiedad antibacteriana más fuerte. Los resultados de este estudio proporcionaron la base para la identificación de recursos de CCPL y la identificación de diferencias de calidad de los aceites esenciales. La investigación sobre las propiedades antibacterianas de varias cepas patógenas ha demostrado su valor de aplicación como conservante natural de alimentos.

PALABRAS CLAVE: *Aceites esenciales; Actividades antimicrobianas; Bacterias transmitidas por los alimentos;* Cinnamomum camphora *(L.) Presl; Linalol*

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1. INTRODUCTION

Food safety is a global concern, with at least 1 in 10 people falling ill yearly from the consumption of contaminated food and 2 million deaths occurring as a result, mostly children, according to the World Health Organization (WHO) (Alegbeleye *et al.*, 2018). Foods containing harmful bacteria, viruses, parasites or chemicals can cause more than 200 diseases, from diarrhea to cancer. *Salmonella sp*., *Staphylococcus sp*. and *Escherichia coli* account for 28, 7 and 5% of foodborne bacterial infections, respectively (Dussault *et al.*, 2014; Mutlu-Ingok *et al.*, 2020; Scallan *et al.*, 2011). Foodborne pathogens are widely distributed in the environment and are transmitted through various food vehicles, including vegetables, meats, poultry products, ready-to-eat foods and dairy products (He *et al.*, 2016; Lee *et al.*, 2018). Antimicrobials, such as antibiotics, are essential for treating infections caused by bacteria. However, in the last decade we have witnessed a dramatic increase in the proportion and an absolute number of bacterial pathogens resistant to multiple antibacterial agents (Roca *et al.*, 2015). The overuse and misuse of antibiotics in veterinary and human medicine have had their efficacy and acceptance compromised (Dannenberg *et al.*, 2019; Van Boeckel *et al.*, 2015). In this context, there is a need to develop alternative strategies for the effective control of microbial pathogens in food, especially natural ones rather than traditionally synthetic preservatives.

As an alternative to synthetic preservatives, essential oils have attracted particular attention due to their antibacterial properties, biodegradable nature and their potential for commercial application (Limam *et al.*, 2020; Liu *et al.*, 2006). *Cinnamomum camphora* (L.) Presl (CCP), a member of the *Lauraceae* family, is a valuable timber and economic forest species as a special product in southeastern China (Imai *et al.*, 2009). The essential oil from the roots, barks, branches, leaves and fruits of *Cinnamomum camphora* has long been prescribed as economic importance as a source of food preservative and additive and as raw materials for the cosmetic and pharmaceutical industries (Chen *et al.*, 2018; Jiang *et al.*, 2016). According to the different chemical components and the main constituents of essential oils from leaves, *Cinnamomum camphora* was classified into 5 different chemotypes, including linalool-type (58–92%), borneol-type (67–82%), camphor-type (54–97%), cineole-type (32–52%), and nerolidol-type (16–57%) (Guo *et al.*, 2017).

Biological activity depends on the type, chemical composition, and concentration of the spice or essential oils. In previous studies, CCPL essential oil was used in the preparation of a strong fungistatic agent against *C. cucurbitarum* infection (Pragadheesh *et al.*, 2013). It was also considered an environmentally friendly plant-based preservative to resist the decay of bamboo (Xu *et al.*, 2013). Marasini *et al.* (2015) summarized the values of the use of the plant and the use of the extracts from the leaves, seeds, and bark of *Cinnamomum camphora* for the remedies of bronchitis, bronchopneumonia, epilepsy and wound infections. The inhibitory effects of the inflammatory phenomena were investigated using *Cinnamomum camphora* (Lee *et al.*, 2006; Xiao *et al.*, 2020). The essential oil from CCPL has been found to possess contact and fumigant activities towards *Lasioderma. Serricorne* (a cigarette beetle) (Chen *et al.*, 2014), while linalool was found to be a significant contributor to the insecticidal and repellent activities of cotton aphids (Jiang *et al.*, 2016).

All the above-mentioned biological activities of the essential oils from CCPL are justified by the chemical composition of all parts of the plant. The essential oil which contains large amounts of active constituents such as linalool, camphor, borneol, eucalyptol and α-terpineol, is crucial to biological activities. To the best of our knowledge, despite all mentioned studies concerning the composition of the single type CCPL essential oil in antibacterial activity, the comparison of different chemotypes, especially different habitats has not yet been investigated. In view of the need to make better use of *Cinnamomum camphora* resources to develop future technology application and discover new antimicrobials, the objective of this study is to report the chemical composition of CCPL essential oils from 5 different habitats in China and to investigate their antimicrobial activities against 3 foodborne bacteria. Through principal component analysis and systematic cluster analysis, the relationship between the habitats of CCPL and the components of essential oils was clarified, which further provided research basis for resource identification of CCPL and quality difference identification of its essential oils.

2. MATERIALS AND METHODS

2.1. Plant materials and chemicals

5-year-old plants of *Cinnamomum camphora* (L.) Presl were selected, and the fresh CCPL was obtained from upper branches collected during October to November in 2018 from Jinxi (Jiangxi Province, China), Nanchang (Jiangxi Province, China), Chengdu (Sichuan Province, China), Mianyang (Sichuan Province, China), and Kunming (Yunnan Province, China). The plants were identified by Prof. Ming Yang from the Jiangxi University of Traditional Chinese Medicine.

The nutrient broth and nutrient agar were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All solvents and other chemicals of analytical grade were purchased from Sigma Chemicals Co., Ltd.

2.2. Extraction of essential oils

Each essential oil was obtained from the fresh CCPL (150 g) by using a Clevenger-type apparatus as described by Ait Babahmad *et al.* (2018). In the extractions, the relationship between leaf mass and water volume was 3/50, at a temperature of 100 °C, for a period of 5 h. The obtained oils were dried over anhydrous sodium sulfate, weighed, and stored at 4 °C until use.

2.3. Gas chromatography-mass spectrometry

The determination of the chemical composition of the essential oils was made by means of an Agilent 7890A/5975C Gas Chromatography-Mass Spectrometer, equipped with a FID detector and a HP-5 ™ fused silica capillary column (30 m × 0.25 µm× 0.25 µm film thickness). The injector and detector temperature were set at 250 and 230 °C, respectively; while the detector operated in the electron-impact ionization (EI) mode with a mass scan range from m/z 40 to 400 at 70 eV. The ion source temperature was 230 °C. Helium was used as the carrier gas at a flow of 1 mL/min, and split ratio of 1:50. The oven temperature was programmed to start at 50 °C for 1 min, followed by an increase of 5 °C/min to 200 °C, then linearly increased by 10 °C/min to a final temperature of 250 °C, where it remained for 5 min.

The identification of components was mainly based on the comparison of their GC Kovat retention indices (RI), determined by reference to a homologous series of C7–C32 n-alkanes. GC retention times were also analyzed. Computer matching with NIST 11 library and comparison of the fragmentation patterns with those reported in the literature were also performed to ensure accuracy.

2.4. Antibacterial Activity

2.4.1. Microbial strains

A total of 3 bacterial species, selected as representative of foodborne bacteria, were tested: (1) 2 gram-negative bacteria, namely: *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC9027), and (2) one gram-positive bacteria, namely: *Staphylococcus aureus* (ATCC 25923). The bacteria were obtained from the Nanchang Institute of Microbiology.

2.4.2. Antimicrobial activity assay

The antimicrobial activity of essential oils was determined according to the NCCLS 2015 guideline. The strains were prepared with 0.9% sterile sodium chloride solution yielding the concentration of 1.5×10^8 CFU/mL (0.5 McFarland). The 0.2 mL aliquot inoculum was spread onto the surface of MacConkey-Sorbitol agar plates for *S. aureus*, *P. saeruginosa* and *E. coli* with sterile swabs. Sterile circular filter papers with a diameter of 6 mm were arranged on the plate and 6 µl of each EO was added to each disc. After 24 h of incubation at 37 °C, the inhibition zones were measured by a vernier caliper and expressed in mm. All experiments were performed in triplicate.

2.5. Data analysis

All analyses were conducted in triplicate. Data were analyzed using one-way analysis of variance ANOVA and Duncan's mean comparison test. The software (IBM SPSS Statistics 21) was used to calculate the eigenvalues and cumulative contribution rate of samples from different habitats by principal component analysis. Using Euclidean square distance as the measurement, a cluster analysis of samples from different habitats was carried out. Tukey test was used to detect significant differences ($p \leq 0.05$) among the mean values obtained from the antimicrobial activity assay.

3. RESULTS AND DISCUSSION

3.1. Characterization of essential oils

The essential oils of CCPL from 5 different habitats in China gave nearly colorless oil with a yield of 2.07% (Nanchang), light yellow oil with a yield of 1.52% (Jinxi), nearly colorless oil with a yield of 1.39% (Chengdu), light yellow oil with a yield of 1.11% (Kunming) and golden oil with a yield of 0.80% (Mianyang). The essential oil from Nanchang had the highest oil yield, while Mianyang had the lowest oil yield. Similar yields (1.83% and 1.3%) of essential oils extracted from CCPL were reported by Chen HP *et al.* (2014) and Satyal *et al.* (2013), respectively. The different yield obtained may be explained by different developmental stages and also by the environmental conditions as well as ontogenetic developments which influence the biosynthetic pathway of oil compounds (Jamali *et al.*, 2013; Kizil *et al.*, 2008).

The composition of the essential oils of CCPL were identified using GC–MS. The chemical compositions of the essential oils are reported in Table 1 with their retention index (RIs), the molecular formula and the relative areas of compounds. A total of 30 compounds were identified in the essential oils of CCPL from 5 different habitats. Among these compounds, only 5 compounds were identified in the essential oil of CCPL from Jinxi; while 17 compounds were identified in the essential oil of CCPL from Mianyang, Kunming, Chengdu and Nanchang.

According to the GC–MS quantitative analysis, the collected results highlighted the domination of the essential oil of CCPL from Jinxi by oxygenated monoterpenes, where linalool (96.47%) was distinguished as the chief component. Its proportion was more than twice that of Mianyang's. Among the 17 compounds identified in the leaves' essential oil from Mianyang, the main constituents in descending order of content were linalool (42.65%), camphor (26.08%), borneol (5.62%), nerolidol (3.59%), caryophyllene oxide (3.34%), bornyl acetate (3.12%) and eucalyptol (2.33%). The above main components accounted for 86.73% of the total amount of essential oil. A total of 17 components were detected in the essential oil of CCPL from Kunming, which accounted for 95.82%. They were mainly composed of the oxygenated monoterpenes eucalyptol (39.07%) followed by nerolidol (16.9%), 3,3,6-trimethyl-1,5-heptadine-4-one (13.42%) and α-terpineol (8.19%). Moreover, no significant difference was found between the quantitative and qualitative composition of the oils obtained from the CCPL of Chengdu or Nanchang. Comparing the composition of the essential oils of Chengdu and Nanchang, oxygenated monoterpenes (71.55 and 72.67%, respectively) and monoterpene hydrocarbons (26.41 and 24.89%, respectively) were found to be the important constituents in the study. For instance, linalool (42.65-96.47%) was identified as the major compound of the leaves' essential oil from Jinxi and Mianyang; while 3 others were dominated by eucalyptol (39.07-55.35%). Therefore, combined with the previous studies reported by Chen *et al.* (2018), the CCPL from Jinxi and Mianyang belonged to the linalool type and 3 others belonged to the cineole type because of rich linalool and eucalyptol, respectively. As natural linalool is an important source for domestic products, cosmetics and fragrance applications, this significant type of *Cinnamomum camphora* from Jinxi is economically and practically ideal (Amiri *et al.*, 2016; Herman *et al.*, 2016)

In fact, these differences in chemical compounds of essential oils in quantitative and qualitative terms could be caused by several factors such as environment (Mutlu-Ingok *et al.*, 2020), harvest time, local climate, extraction technique and variety (Harkat-Madouri *et al*., 2015). As essential oil of the *Cinnamomum* genus (*Lauraceae*) is an important source for chemical and pharmaceutical use (Dai *et al.*, 2020), various profiles of famous medicinal herbs such as *cinnamon* (*C. cassia* Presl), *Ceylon cinnamon* (*C. zeylanicum* Bl.), *Chai Gui* (*C. tamala)*, *sassafras* (*C. porrectum.*) and *Chuan Gui* (*C. wilsonii* Gamble) were previously described based on their major compounds of essential oils. Also, recent research works were focused on the *Cinnamomum* genus essential oils containing different major compounds. As shown in Table 2, aromatic compounds and oxygenated monoterpenes were reported as the main constituents in the essential oils of *Cinnamomum* genus. The essential oil also contained elements which characterize certain genera belonging to the *Cinnamomum* genus, such as linalool and camphor, which were the basis of the *Sect. Camphora* (Trew) Meissn. Compounds; whereas eugenol and borneol were the main compounds in *Sect. Cinnamomum*.

TABLE 1. Chemical compositions and relative area of CCPL essential oils from five different locations in China

No.	Compounds	Molecular formula	RI[a]	Relative area (%)				
				A	B	C	D	E
1	α-Thujene	$C_{10}H_{16}$	934	-[b]	-	0.43	0.74	0.72
2	α-Pinene	$C_{10}H_{16}$	942	-	1.78	2.33	4.38	4.11
3	Camphene	$C_{10}H_{16}$	959	-	1.08	-	-	-
4	Sabinene	$C_{10}H_{16}$	989	-	-	6.18	12.93	12.69
5	β-Pinene	$C_{10}H_{16}$	993	-	0.59	1.84	3.27	3.01
6	Myrcene	$C_{10}H_{16}$	1007	-	-	0.65	1.36	1.25
7	α-Terpinene	$C_{10}H_{16}$	1029	-	-	0.58	0.96	0.87
8	p-Cymene	$C_{10}H_{14}$	1037	-	0.57	-	-	-
9	D-Limonene	$C_{10}H_{16}$	1040	-	1.39	-	-	-
10	Eucalyptol	$C_{10}H_{18}O$	1042	-	2.33	39.07	52.2	55.35
11	γ-Terpinene	$C_{10}H_{16}$	1067	-	-	1.02	1.72	1.48
12	trans-4-Thujanol	$C_{10}H_{18}O$	1075	-	-	-	0.59	0.63
13	Terpinolene	$C_{10}H_{16}$	1094	-	0.87	-	0.39	0.34
14	Tricyclene	$C_{10}H_{16}$	1104	-	-	-	0.66	0.42
15	Linalool	$C_{10}H_{18}O$	1105	96.47	42.65	0.89	-	-
16	Camphor	$C_{10}H_{16}O$	1153	-	26.08	0.48	-	-
17	3-Methyldecane	$C_{11}H_{24}$	1159	0.55	-	-	-	-
18	Borneol	$C_{10}H_{18}O$	1180	-	5.62	-	-	-
19	Myrcenol	$C_{10}H_{18}O$	1181	-	-	0.72	1.04	0.82
20	Terpinen-4-ol	$C_{10}H_{18}O$	1195	-	-	2.58	3.91	3.25
21	α-Terpineol	$C_{10}H_{18}O$	1209	-	1.10	8.19	13.81	12.62
22	Bornyl acetate	$C_{12}H_{20}O_2$	1313	-	3.12	-	-	-
23	β-Caryophyllene	$C_{15}H_{24}$	1444	0.58	-	1.00	0.65	0.78
24	α-Humulene	$C_{15}H_{24}$	1482	-	-	0.54	0.84	0.87
25	β-Selinene	$C_{15}H_{24}$	1516	-	0.89	-	-	-
26	3,7,11,11- Tetramethylbicyclo[8.1.0]2,6-undecadiene	$C_{15}H_{24}$	1526	1.69	-	-	0.55	0.78
27	Nerolidol	$C_{15}H_{26}O$	1590	-	3.59	16.90	-	-
28	Spathulenol	$C_{15}H_{24}O$	1604	0.70	1.51	-	-	-
29	Caryophyllene oxide	$C_{15}H_{24}O$	1610	-	3.34	-	-	-
30	1,5-Heptadien-4-one,3,3,6-trimethyl-	$C_{10}H_{16}O$	1763	-	1.25	13.42	-	-
	Total identified			99.99	97.76	96.82	100	99.99
	Oxygenated monoterpenes			96.47	82.15	65.35	71.55	72.67
	Monoterpene hydrocarbons			-	6.28	13.03	26.41	24.89
	Oxygenated sesquiterpenes			0.70	8.44	16.90	-	-
	Sesquiterpenes hydrocarbons			2.27	0.89	1.54	2.04	2.43
	Aliphatic compounds			0.55	-	-	-	-

Notes: Means of relative area (%) are the average of three determinations (n = 3).
CCPL, *Cinnamomum camphora* (L.) Presl leaves.
A, essential oil of camphor leaves from Jinxi, (Jiangxi Province, China).
B, essential oil of camphor leaves from Mianyang, (Sichuan Province, China).
C, essential oil of camphor leaves from Kunming, (Yunnan Province, China).
D, essential oil of camphor leaves from Chengdu, (Sichuan Province, China).
E, essential oil of camphor leaves from Nanchang, (Jiangxi Province, China).
RI[a], Retention index
-[b], not detected

Table 2. The major compounds of essential oils, classification, geographical distribution and extraction techniques.

Plant	Major compounds	Classification	Geographical distribution	Extraction techniques	Reference
Cinnamomum porrectum	Safrole (93.92%)	aromatic compounds	China, Pakistan, India, Malaysia and Indonesia	HD	(Sukcharoen *et al.*, 2017)
Cinnamomum kanehirae	Linalool (64.4%)	oxygenated monoterpenes	Taiwan (China)	HD	(Cheng *et al.*, 2015)
Cinnamomum septentrionale	Camphor (30.53%)	oxygenated monoterpenes	China	HD	(Yang *et al.*, 2017)
Cinnamomum agasthyamalayanum	Camphor (70.8%)	oxygenated monoterpenes	India	HD	(Sriramavaratharajan *et al.*, 2016)
Cinnamomum cassia Presl	(E)-cinnamaldehyde (79.39%)	aromatic compounds	China, India, Laos, Vietnam and Indonesia	HD	(Sun *et al.*, 2016)
Cinnamomum zeylanicum	Benzyl benzoate (64.36%)	aromatic compounds	Sri Lanka, China and tropical Asian countries.	HD	(Lobo *et al.*, 2018)
Cinnamomum tamala	Eugenol (52.54%)	aromatic compounds	China, Nepal, Bhutan, and India	HD	(Heer *et al.*, 2016)
Cinnamomum japonicum Sieb.	Borneol (41.91 and 36.15% for SFMD–HE and HD, respectively)	oxygenated monoterpenes	China, Korea and Japan	SFMD–HE and HD	(Zhao *et al.*, 2018)

Notes: HD, hydrodistillation.
SFMD–HE, solvent-free microwave-assisted distillation followed by homogenate extraction.

3.2. Principal component analysis of essential oils

3.2.1. Correlation analysis of chemical constituents of CCPL essential oils from five different habitats in China

According to the analysis of the correlation matrix of CCPL essential oils from 5 different habitats in China (as shown in Table 3), the following conclusions can be drawn: (1) the similarity of CCPL essential oils between Jinxi and Mianyang was very large, which showed that there was little difference between the 2 samples; (2) the similarity of CCPL essential oils between Kunming, Chengdu and Nanchang was large, which indicated that the 3 sample components were less different. Generally, the correlation coefficient was more than 0.6, which can be considered a significant correlation.

3.2.2. Principal component analysis of CCPL essential oils from five different habitats in China

The percentage of the peak area values corresponding to 30 retention indexes was taken as the

Table 3. The correlation matrix of essential oils from five different habitats in China

Column heading		Correlation Matrix				
		Jinxi	**Mianyang**	**Kunming**	**Chengdu**	**Nanchang**
Correlation	**Jinxi**	1.000	0.840	-0.059	-0.066	-0.063
	Mianyang	0.840	1.000	-0.041	-0.071	-0.066
	Kunming	-0.059	-0.041	1.000	0.863	0.867
	Chengdu	-0.066	-0.071	0.863	1.000	0.999
	Nanchang	-0.063	-0.066	0.867	0.999	1.000

Notes: The analysis of the correlation matrix of *Cinnamomum camphora* (L.) Presl leaves' essential oils from 5 different habitats were based on the average of three determinations.

Table 4. Initial eigenvalues and contribution rate of principal components in CCPL essential oils

The principal components	Initial eigenvalues		
	eigenvalues	variance contribution rate (%)	cumulative contribution rate (%)
1	19.039	63.464	63.464
2	7.391	24.637	88.101
3	3.444	11.479	99.580

Notes: CCPL, *Cinnamomum camphora* (L.) pressed leaves.
The analysis of initial eigenvalues and contribution rate of principal components in CCPL essential oils were based on the average of three determinations.

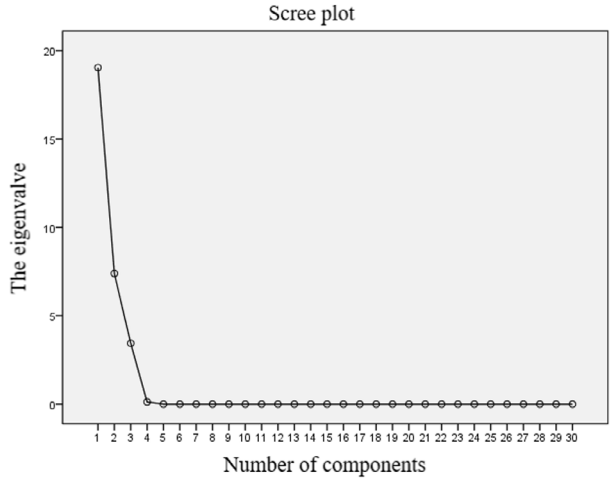

Figure 1. The scree plot of eigenvalues of 30 components.

Notes: The results of scree plot of eigenvalues of 30 components were based on the average of three determinations.

analysis object, marking the percentage of 30 peak area values in turn as A1-A30. After the data were standardized, PCA analysis was conducted. As can be seen from Table 4, the characteristic values of the first 3 principal components were all greater than 1 and the cumulative contribution rate to the total variance was 99.58%, indicating that the first 3 factors played a leading role in influencing the evaluation index of the volatile quality and can objectively reflect the quality represented by the volatile components of CCPL. Generally, the eigenvalue of each scree in the scree plot in Fig.1 was the basis of the characteristic quantity value, which was used to determine the number of principal components to be retained. It can be seen from Fig.1 that the difference among the eigenvalues of factors 1, 2 and 3 was large; while the difference among factors 4-30 was small. A preliminary conclusion can be drawn that most of the information can be generalized by retaining 3 factors. Therefore, the first 3 principal components should be selected for comprehensive comparison.

The information provided by the 3 principal components accounted for 99.580% (> 85%) of the total information, which were 63.464, 24.637 and 11.479%, respectively. The eigenvectors corresponding to the first principal component (PC1) were larger in compounds 2, 5, 21, 6, 4, 1, 7, 11, 24, 10, 20, 19 and 15, indicating that the first principal component was greatly affected by these components. Interestingly, linalool (15) showed the highest relative content in Jinxi and Mianyang. The second principal components corresponding to the larger load were 22, 9, 3, 29, 8, 18 and 25. The above 7 components were not detected in CCPL essential oils from Kunming, Chengdu or Nanchang. The third principal components corresponding to a larger load were compounds 27 and 30, which had no significant influence on the differentiation of the essential oils of *Cinnamomum camphora* from Kunming.

3.2.3. Cluster analysis of CCPL essential oil from five different habitats in China

According to Figure 2, the cluster can be divided into 2 categories: first was No.1 and No.2, i.e. CCPL essential oils from Jinxi and Mianyang; the second was No.3, No.4 and No.5, i.e. CCPL essential oils from Kunming, Chengdu and Nanchang. Among the 5 samples, linalool was 96.47 and 42.65% from Jinxi and Mianyang, respectively, and it had components that were not detected in some other 3 areas. Therefore, it was classified into one group by systematic clustering. Further combined with the principal component analysis results, it can be seen that there were up to 13 components of CCPL essential oils from Kunming, Chengdu and

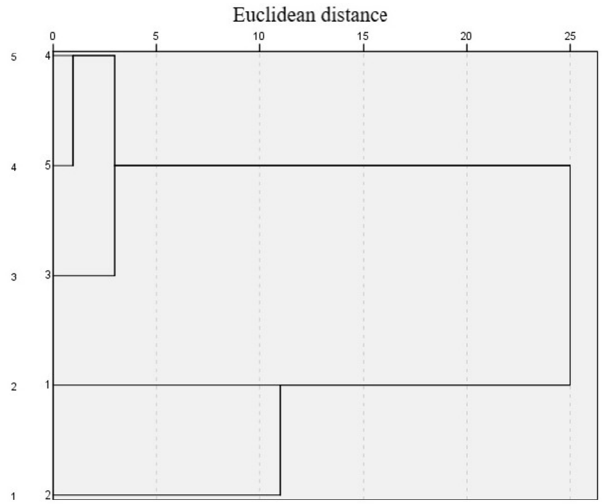

Figure 2. Cluster analysis of CCPL essential oil from five different habitats in China.

Notes: The results of cluster analysis of CCPL essential oil from five different habitats were based on the average of three determinations. CCPL, Cinnamomum camphora (L.) Presl leaves.
1, essential oil of camphor leaves from Jinxi, (Jiangxi Province, China).
2, essential oil of camphor leaves from Mianyang, (Sichuan Province, China).
3, essential oil of camphor leaves from Kunming, (Yunnan Province, China).
4, essential oil of camphor leaves from Chengdu, (Sichuan Province, China).
5, essential oil of camphor leaves from Nanchang, (Jiangxi Province, China).

Nanchang, and their relative contents were similar, and therefore they were classified into one category by systematic clustering.

3.3. Antibacterial activity of essential oils

The antimicrobial properties of CCPL essential oils from 5 different habitats in China were assessed against one gram-positive bacteria (*S. aureus*) and 2 Gram-negative bacteria (*E. coli* and *P. aeruginosa*) by the paper disc diffusion method which was used to measure the diameter of the inhibition zone. As shown in Table 5, the obtained data suggested that all essential oils possessed different degrees of inhibition against all tested strains, apart from CCPL EO from Kunming, which had no inhibitory effects on *P. aeruginosa*. Among the 5 essential oils, Jinxi's showed the strongest antibacterial properties (with 14.29±0.02mm as IZ) especially when tested against *S. aureus;* while the other 4 essential oils showed low bacteriostasis (6.30±0.02-8.79±0.09). Furthermore, the EO of Jinxi's on *E. coli* and *P. aeruginosa* had larger inhibition zones (11.33±0.18mm and 7.89±0.09mm, respectively), compared to the other 4 essential oils (from 6.16±0.03 mm to 9.14±0.02 mm and from 6.47±0.15 mm to 7.37±0.02 mm, against *E. coli* and *P. aeruginosa,* respectively). Moreover, considering the antibacterial properties, the essential oils from Mianyang, Chengdu and Nanchang showed no statistical differences ($p > 0.05$) against *S. aureus* and *E. coli,* respectively.

Table 5. The antibacterial activity of CCPL essential oils from 5 different habitats in China.

Essential oils	Average value of inhibition zone (mm)		
	S. aureus	E. coli	P. aeruginosa
A	14.29±0.02a	11.33±0.18a	7.89±0.09a
B	8.73±0.05b	8.93±0.05b	6.47±0.15d
C	6.30±0.02c	6.16±0.03c	NT
D	8.79±0.09b	9.14±0.02b	7.37±0.02b
E	8.68±0.09b	9.05±0.04b	6.82±0.09c

Notes: CCPL, *Cinnamomum camphora* (L.) Presl leaves.
A, essential oil of camphor leaves from Jinxi, (Jiangxi Province China).
B, essential oil of camphor leaves from Mianyang, (Sichuan Province China).
C, essential oil of camphor leaves from Kunming, (Yunnan Province China).
D, essential oil of camphor leaves from Chengdu, (Sichuan Province China).
E, essential oil of camphor leaves from Nanchang, (Jiangxi Province, China).
NT, Not tested
* Tukey test was used to detect significant differences ($p \leq 0.05$) among the mean values obtained from three replicates performed. Different letters in each column indicate significance (* $p < 0.05$).

Previous studies have shown that the most diverse group of isoprenoids, sesquiterpenes and monoterpenes accounted for the largest proportion of essential oils in plants (Sarikurkcu *et al.*, 2018). In addition, Chen *et al.* (2018) reported that different major monoterpenoids primarily led to differences in different chemical types of volatile oils. Therefore, the similar antibacterial effects of CCPL essential oils from Chengdu and Nanchang can be attributed to the similar proportion of the major compound eucalyptol (52.2 and 55.35%, respectively). As for the EO from Mianyang, the high concentration of other constituents, such as linalool (42.65%) and camphor (26.08%) also contributed to its synergistic effects

on antimicrobial activity. This antibacterial potency of the EO from Jinxi can probably be explained by the known antimicrobial effects of linalool as it was identified as the major compound (96.47%) in the essential oil of CCPL from Jinxi. Indeed, Herman *et al.* (2016) confirmed that the combination of tested essential oil with linalool showed a greater efficacy than the essential oil and linalool separately against Gram-negative bacteria including *P. aeruginosa* and *E. coli*, also against *C. albicans* (fungus). This conclusion is strongly supported by a study by Schmidt *et al.* (2012), who proved the antibacterial activity of linalool chemotype essential oil, which can be related to the interaction of the main constituent linalool. The study by Chen *et al.* (2020) also showed that the essential oil from *C. camphora,* which is rich in linalool had good activity against MRSA, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Salmonella gallinarum* and *Escherichia coli*.

Generally, comparing the gram-negative bacteria, gram-positive bacteria proved to be more sensitive to the essential oils. As a matter of fact, the essential oils from different habitats had the strongest inhibitory effects on *S. aureus* and the weakest inhibition on *P. aeruginosa*. Moreover, Kunming's oil had the minimal inhibition zones (6.30±0.02mm and 6.16±0.03mm against *S. aureus* and *E. coli,* respectively) on both tested strains, and even no effect on *P. aeruginosa*. This variation in antibacterial behavior could be explained by the fact that the cell wall structure of bacteria was different. Gram-negative has a special component of the outer membrane layer embedded with lipopolysaccharide (Nisar *et al.*, 2018; Otoni *et al.*, 2014). The addition of an outer lipophilic membrane increased the penetration of hydrophobic compounds (linalool, eucalyptol and camphor) through the membrane (Fisher *et al.*, 2006).

4. CONCLUSIONS

In this study, CCPL essential oil of from Jinxi is a promising source of linalool, which was proved to have strong antimicrobial activities, which make it a natural food preservative due to its high concentration of linalool (96.47%). Based on principal component analysis and systematic cluster analysis, a comprehensive comparison of 30 compounds contained in CCPL essential oils from 5 different locations showed that the chemical components of Jinxi and Mianyang were significantly different from those from Kunming, Chengdu and Nanchang, providing a basis for resource substitution. The antibacterial activity obtained from CCPL essential oils from 5 different habitats in China were mostly attributable to rich bioactive oxygenated monoterpene hydrocarbons, such as linalool, eucalyptol and camphor. The essential oils were natural products, and can inhibit the growth and reproduction of foodborne pathogens (*S. aureus*, *E. coli* and *P. aeruginosa*) in this test, hereby serving as a basis to justify and guide further work aiming toward suitable concentrations of these essential oils or their bioactive components for the application of CCPL extract as a natural food preservative.

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6. ETHICAL STATEMENTS

This study does not involve any human or animal testing.

7. CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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Antioxidant activity, polyphenolic composition and *in vitro* antibacterial and antifungal activities of tea seed oil

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SUMMARY: The polyphenolic composition and antioxidant activity of tea seed oil from *C. sinensis* TRFK 301/5 (green colored) and TRFK 306 (purple colored) and *C. oleifera* were evaluated. The total polyphenolic content, total catechins and catechin fractions were significantly different in the oils. *C. oleifera* contained significantly ($p \leq 0.05$) higher amounts of catechins and polyphenols than *C. sinensis*. *C. oleifera* also exhibited a higher DPPH radical scavenging activity (18.81 ± 0.46%) compared to *C. sinensis* (TRFK 306; 15.98 ± 0.13 and TRFK 301/5; 14.73 ± 0.47%). The antimicrobial activities of tea seed oil and two selected oils (olive and eucalyptus oil), were also evaluated against *Escherichia coli, Staphylococcus aureus*, *Candinda albicans*, *Cryptococcus neoformans* and *Trichophyton mentagrophytes*. *S. aureus* was significantly inhibited by the oils compared to *E. coli*. The oils inhibited the growth of *T. mentagrophytes* and *C. albicans,* although they had no effect on *C. neoformans*. Tea seed oil is a potential source of beneficial phytochemicals and potent antimicrobial agents.

KEYWORDS: *Anti-microbial activity; Antioxidant activity; Catechins; Polyphenol; Tea seed oil*

RESUMEN: ***Actividad antioxidante, composición polifenólica y actividades antibacterianas y antifúngicas in vitro del aceite de semilla de té.*** Se evaluó la composición polifenólica y la actividad antioxidante del aceite de semilla de té de *C. sinensis* TRFK 301/5 (color verde) y TRFK 306 (color púrpura) y *C. oleifera*. El contenido polifenólico total, catequinas totales y fracciones de catequinas fueron significativamente diferentes en los aceites. *C. oleifera* contenía cantidades significativamente mayores ($p \leq 0.05$) de catequinas y polifenoles que *C. sinensis*. *C. oleifera* también exhibió una mayor actividad de eliminación de radicales DPPH (18,81 ± 0,46%) en comparación con *C. sinensis* (TRFK 306; 15,98 ± 0,13 y TRFK 301/5; 14,73 ± 0,47%). También se evaluó la actividad antimicrobiana del aceite de semilla de té y dos aceites seleccionados (aceite de oliva y de eucalipto) frente a *Escherichia coli, Staphylococcus aureus*, *Candinda albicans, Cryptococcus neoformans* y *Trichophyton mentagrophytes*. *S. aureus* fue inhibido significativamente por los aceites en comparación con *E. coli*. Los aceites inhibieron el crecimiento de *T. mentagrophytes* y *C. albicans*, sin embargo, no tuvieron ningún efecto sobre *C. neoformans*. El aceite de semilla de té es una fuente potencial de fitoquímicos beneficiosos y potentes agentes antimicrobianos.

PALABRAS CLAVE: *Aceite de semilla de té; Actividad antimicrobiana; Actividad antioxidante; Catequinas; Polifenol*

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1. INTRODUCTION

Tea is used to manufacture tea products that are used in beverages all over the world. The products come mainly from the leaf. The tea plant has been given a lot of attention by researchers due to its immense health benefits (Schneider and Segre, 2009). In China for example *C. oleifera*, is the most important and widely distributed of the *camellia* species and is used in seed oil production which is important in traditional therapy for burns and stomach aches, ringworms and dandruff in China. The oil is used as a crop food oil, and additives for cosmetic products among other uses, while tea seed cake, a by-product, is utilized as feed additives in aquaculture and livestock production (George *et al.,* 2013)iodine value, saponification value, peroxide value, free fatty acids, total polyphenols and antioxidant activity were determined. The oil yields ranged between 16 to 25% w/w. Iodine value was in the range of 86 to 91 g I 2 /100 g, peroxide value < 3.5 meq O_2/kg, saponification value between 182 to 187 mg KOH/g, free fatty acid < 1.5% oleic acid, total polyphenols 0.036 to 0.043 mg/L gallic acid and antioxidant activity of between 14 to 21% 2,2-diphenyl-1-picrylhydrazyl (DPPH. Whole tea seed powder is mainly used in aquaculture as feed additives to help prevent bacterial infections in fish (Rico *et al.,* 2012).

C. sinensis is majorly grown for its leaves used in the manufacture of black tea, the most consumed beverage second only to water. However, *C. sinensis* has been seen to produce a lot of seeds especially when under stressful environmental conditions. The tea seed oil from *C. sinensis* is rich in phytochemicals such as polyphenols, catechins, saponin and squalene. In other studies, these phytochemicals have been demonstrated as antimicrobial agents and also have been shown to exhibit antioxidant properties.

Infectious diseases caused by various microorganisms are a burden globally. To combat these diseases, it is essential that novel antimicrobial agents are developed. Doctors utilize synthetic antibiotics all over the world to manage and treat microbial related disease. However, most antibiotics are synthetic and may have adverse side effects on many occasions (Cunha, 2001).

The antimicrobial properties of various plant extracts are due to the various phytochemicals available in them which are used to carry out life functions as well as defense mechanisms against predators (Tayel *et al.,* 2011). Most of these phytochemicals have been found useful by humans especially for treating various diseases. Phytochemicals are usually found in the barks, leaves, roots and other parts of plants. The presence of phytochemicals in plants is a major contributor of the use of plants as natural medicine worldwide (Ahmad and Beg, 2001).

The in vitro antimicrobial activity of extracted tea seed oil has not been researched extensively. The eradication of multidrug resistant microbes is currently a challenging task in the fight against both existing and emerging infectious diseases and as such there is need for continued search for new antimicrobial products for use in the fight against microorganisms. Therefore, the main aim of this study was e to determine the antioxidant activity, polyphenolic composition and establish in vitro antimicrobial activities of tea seed oil against *E. coli, S. aureus, C. albicans, C. neoformans* and *T. mentagrophytes.*

1.1. Uses of tea seeds

Tea seeds have been perceived to be a rich source of saponin. Five saponins have been distinguished in tea seeds, particularly Theasapogenal A, B, C, D and E. These saponins have been observed to be pharmalogically dynamic with articulated antiexudative and disease prevention properties. Saponin from tea has a wide array of biological activities that include but are not limited to foam-stabilization and emulsification. Tea saponin has been used in aquaculture to kill unwanted fish as well as insects in prawn ponds and in the control of pests and mites (Yamauchi *et al.,* 2001). Other physiological functions displayed by tea saponin include anti-inflammatory and expectorant properties (Morikawa *et al.,* 2006).

The phytochemicals in tea have been extensively studied and are well known for their myriad biological activities which include but are not limited to antibacterial activity, antifungal activity anticancer properties, anti-inflammation properties, antiviral activity, prevention of cardiovascular disorders and also anti-obesity and hypo-lipidemic effects (Lin and Lin-Shiau, 2006). The polyphenols in tea are mainly catechins which include epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), (+) catechin (C), gallic acid (GA) and epigallocatechin gallate (EGCG). The main mechanisms of these polyphenols are that they have anti-oxidative activity and can scavenge reactive nitrogen and oxygen

species by chelating redox-active transition metal ions. The tea seed oil from *C. sinensis* also has been shown to contain these phytochemicals (Njuguna *et al.,* 2014).

Tea seed component determination has shown that tea seed oil is composed of linolenic, palmitic, linoleic and oleic acids. According to (Guynot *et al.,* 2003), the fatty acid chains, just like the polyphenols, have exhibited antibacterial and antifungal activities.

2. MATERIALS AND METHODS

2.1. Sample collection and preparation

The seeds of *Camellia sinensis* (L. O Kuntze), were collected in September 2019 at the Tea Research Institute seed Barrie located at the KALRO-TRI, (0.3722 °S, 35.3483 °E: Elevation 2180 m above sea level). Samples were collected in triplicate. Only healthy and mature seeds were picked. The seeds were de-husked, cleaned and rinsed using distilled water and then dried.

2.2. Chemical and reagents

The standards used for chromatography were purchased from Sigma Aldrich and included Gallic acid (GA), Epigallocatechin (EGC), caffeine, (+) catechin (C), epigallocatechin gallate (EGCG) and Epicatechin gallate (ECG). The reagents used for the study included Folin-ciocalteu reagent, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH), Methanol, *n*-hexane, Acetonitrile, Acetic acid, Ethylenediaminetetraacetic acid (EDTA), Dimethyl sulfoxide (DMSO), Ascorbic acid, and culture media (Milton Hueller (MH) and the Sabour and dextrose agar (SDA) used were analytical grade and were obtained from Sigma-Aldrich Chemical Company, Germany through Kobian Suppliers.

2.3. Methodology

2.3.1. Extraction of tea seed oil

Dried tea seed samples were de-husked and ground using a pestle and mortar and dried at low temperatures for the subsequent extraction of seed oil. Extraction was done according to the soxhlet method with hexane as solvent. Extraction was done for 8 hours with a sample-to-solvent ratio of 1:20. The oil extract was then put into a rotary evaporator to concentrate and expel the hexane solvent at 60 ºC. The concentrated sample was then placed in an oven at 80 ºC to remove residual solvent. The samples were weighed and then transferred to sterilized bottles for further analysis.

2.3.2. Extraction for catechin, polyphenols and antioxidant assay

Sample extraction for catechins, total polyphenols and antioxidant activity determination were done simultaneously using a similar method. The extraction method was liquid-liquid extraction (LLE), which involved the use of n-hexane, methanol and water to extract the catechins and total polyphenols from the seed oil. Two grams of seed oil were dissolved in 2.0 mL of n-hexane in a graduated 10-mL tube. The mixture was mixed in a vortex machine for 1 minute. This was followed by liquid-to-liquid extraction. 4 mL of 80:20 methanol to water were added to the graduated tube and vortexed for 10 seconds. The mixture was then centrifuged at 3500 RPM for 10 minutes. The supernatant was then decanted and poured into a graduated extraction tube. The supernatant was further subjected to a second and third extraction with 4 mL with an 80:20 methanol-to-water ratio. After the third extraction the sample was centrifuged and the supernatant put in sterilized sample tubes and stored at -15 ºC for further analysis.

2.4. Empirical studies

2.4.1. Determination of the polyphenolic composition and profiles of camellia oil of Camellia sinensis *and* Camellia oleifera

Total catechin and individual catechin levels of *C. sinensis* and *C. oleifera* seed oil profile were determined using the HPLC method and the polyphenol levels were measured using Folin Ciocalteu reagent method developed in 1999 with slight modifications (Singleton *et al*., 1999). In this study all the standards and reagents used were pure analytical grade with 99.9 % purity and were obtained from Sigma-Aldrich chemical company.

2.4.2. Determination of polyphenol from tea seed oil

The total phenolic content from tea seed oil was determined according to a method described by (Singleton *et al*., 1999). The method utilizes Folin-Cio-

calteau reagent. 1 mL of sample was pipetted into a 100 mL volumetric flask, with 5 mL of Folin-Ciocalteau reagent added and a further 10 mL of 20% w/v sodium carbonate solution. The mixture was then topped up with distilled water to the 100-mL mark. The mixture was mixed thoroughly and left to stand for 1 hour. The absorbance was then determined at a wavelength of 725 nm using UV-Vis spectrophotometer (UV 1800, Shimadzu, Japan). The result was expressed as a gallic acid equivalent.

2.4.3. Determination of total catechin and individual catechin fractions from tea seed oil

THE Catechin fraction detection and quantification employed a Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method. Catechin standards were obtained from Sigma and Aldrich i.e. (+) - Catechin (C), (-) - epicatechin (EC), epigallocatechin (EGC), (-) - epicatechingallate (ECG) and (-) - epigallocatechingallate (EGCG). Double distilled water was used for dilutions. All other reagents used were HPLC grade i.e. n-hexane, methanol, ethylenediaminetetraacetic acid (EDTA) and ascorbic acid. The catechin contents in the methanolic extracts of the crude oils were quantitatively estimated by RP-HPLC (Shimadzu LC 20, Japan). 1.0 mL of sample was pipetted into a graduated tube, 10% v/v stabilizing solution was added to make up 5 mL. The mixture was then passed through a 0.45 µm nylon membrane filter and transferred to sample vials. A Shimadzu LC 20 AT HPLC system fitted with a SIL 20A auto-sampler, an SPD-20 UV-Visible detector, a class LC10 chromatography work station and a Gemini 5 µm C6 Phenyl 110Å, 250 mm × 4.6 mm i.d (Phenomenex, Torrance, CA, USA) separation column was used. Catechin quantification was done using the caffeine calibration curve with individual catechin relative response factors. Total catechin (TC) was calculated by adding up individual catechin fraction i.e. ECG + EGC + EC + EGCG + C = % TC.

2.4.4. Determination of the antioxidant activity of **Camellia sinensis** *and* **Camellia oleifera** *seed oil*

The antioxidant activity was measured using the DPPH radical scavenging method developed in 1958 (Blois, 1958) and later modified in 2001 (Morales and Jiménez-Pérez, 2001). The procedure was slightly modified to fit this particular study.

2.4.5. Antioxidant activity assay

The extracted sample was brought to room temperature and a 400 µl aliquot of the oil sample was mixed with 3 mL of 2, 2-Diphenyl-1- picrylhydrazyl (DPPH). The DPPH was 74 mg/L in 80% Methanol. The resulting mixture was vortexed for 1 minute and put in a dark room for 1 hour. The absorbance of the blank solution i.e. DPPH and sample was then read at 520 nm in a UV-Vis spectrophotometer. The percentage antioxidant activity was calculated using the formula:

100(CC-CD/CC);

*where CC is the absorbance of the blank sample, CD the absorbance of the test sample.

2.4.6. **In-vitro** *antibacterial and antifungal activities of* **C. sinensis, C. oleifera** *seed oil, eucalyptus oil and olive oil*

The microbes used for the study were American Type Culture Collection (ATCC), *Escherichia coli* (ATCC 25922), *Staphylococcous aureus* (ATCC 25923*)* and fungi *Candida albicans* (ATCC 90028), and the other two were *Cryptococcus neoformans* and *Trichophyton mentagrophytes*.

The antimicrobial activities of the oils were determined according to protocols from the National Committee of Clinical and Laboratory Standards (NCCLS). Bacteria cultures were kept in Milton-Hueller (MH) while those of the fungi were kept in a Sabouraud Dextrose Agar (SDA) medium supplemented with Chloramphenicol (50 mg/mL) and Streptomycin sulfate (500 µg/mL) and stored at +4 °C. The Barium sulphate standard equivalent to McFarland No. 0.25 was used to give a cell density of 1.5 (x) 108/mL. The inoculum of approximately 1.5 (x) 108 cells was spread uniformly onto MH agar containing 0.2% glucose and 0.5 g/mL of methylene blue dye. Excess inoculum was drained and allowed to rest for approximately 1 hour. The medium was then left to stand for 30 minutes to allow the solution to diffuse into the agar medium. The discs impregnated with the respective concentrations of oil were then put on the plates and incubated for 24 hrs at 35 ˚C and later the zones of inhibition were measured in millimeters. Every assay was done in triplicate.

2.5. Statistical Analysis

ANOVA was used to determine the mean differences in the biochemical properties of the camellia

Table 1. Total catechin levels and catechin fractions of tea seed oil

	Tea varieties		
	***C. sinensis* - TRFK 306**	***C. sinensis* -TRFK 301/5**	***C. oleifera***
Gallic Acid	0.54±0.01[b]	0.54±0.03[b]	0.62±0.04[a]
Epigallocatechin	1.06±0.25[a]	0.87±0.18[a]	0.99±0.00[a]
+Catechin	0.14±0.02[a]	0.14±0.01[a]	0.16±0.01[a]
Epicatechin	0.71±0.08[a]	0.62±0.05[b]	0.70±0.05[a]
Epigallocatechin-3-Gallate	0.96±0.12[ab]	0.90±0.06[b]	1.07±0.02[a]
Epicatechingallate	1.22±0.11[b]	1.26±0.03[b]	1.67±0.02[b]
Total Catechin	4.62±0.27[b]	4.33±0.21[b]	5.22±0.09[a]

Means with the same letter in the same row are not significantly different at P ≤ 0.05 according to the Tukey test p < 0.05. Number of replicates = 3. C. sinensis = camellia sinensis, C. oleifera = Camellia oleifera, TRFK 306– Tea research foundation of Kenya tea variety 306/1, TRFK 301/5– Tea research foundation of Kenya tea variety 301/5

oil and to compare the differences in the antibacterial and antifungal activity of the oils studied among the test microbes. Graphs were plotted and LSDS used to separate the means.

Probability was set at 95% for the significant confidence interval to determine the differences in the mean diameters of the inhibition zones.

A correlation analysis was carried out using Excel 2013 to determine the relationships among the catechin fractions, total catechins, antioxidant activity and total phenolic content of camellia oil its antimicrobial activity. All Statistical analysis was done using SAS 9.1 and Excel 2013.

3. RESULTS

3.1. Polyphenolic composition and profiles of *Camellia sinensis* and *Camellia oleifera* seed oil. Catechin profile of tea seed oil

The catechins identified in tea oils were epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). The two main gallated catechins present were EGCG and ECG, while the others were non-gallated. Generally, the TC levels were higher in *C. oleifera* than those of C. sinensis.

The various individual catechin profiled showed a significant difference ($p \leq 0.05$) among the tea oils. Epicatechingallate was the most abundant in all the oils assayed while +C was the least abundant. The total catechin levels ranged from 4.08 ± 0.27% to 4.60 ± 0.09%. *C. oleifera* had the highest total catechin content at (4.60 ± 0.09%) while the *C. sinensis* clone TRFK 301/5 had the lowest at (3.79 ± 0.2 %). It is also notable that the *Camellia sinesis* varieties (TRFK 301/5 and TRFK 306) did not differ significantly at $P \leq 0.05$ (Table 1).

Table 2. Catechin retention times

Catechin fractions	RT (Mins)
Gallic acid	4.61
Epigallocatechin	8.92
Epicatechin	15.58
Epigallocatechin gallate	19.82
Epicatechin gallate	24.91

Catechin retention times in minutes; RT – retention time; Mins - minutes

Under the present conditions, the retention times (min) for the standard compounds were 4.61, 8.920, 15.58, 19.82, 24.91 for Gallic acid, Epigallocatechin, Epicatechin, Epigallocatechin gallate, Epicatechin gallate, respectively (Table 2). The HPLC chromatogram graphs obtained had similar chemical compositions but with different peak areas. There were statistical differences ($p < 0.05$) in all catechin fractions assayed in this study. *C. sinensis* exhibited lower levels of catechins for all the analyzed catechin fractions than its counterpart *C. oleifera.* ECG (1.67%) was the most predominant catechin fraction in the oils. However, EGCG was not significantly different ($p \leq 0.05$) for the three oils. Gallic acid did not show any significant difference ($p < 0.05$) with levels ranging from 0.54 ± 0.01%, 0.54 ± 0.03% and 0.62 ± 0.04% for *C. sinensis* TRFK 306, *C. sinensis* 301/5 and *C. oleifera* oils, respectively. +C had the

lowest levels (0.14 to 0.16%) compared to the other catechin fractions (Table 5).

3.2. Total polyphenol levels in tea seed oil

The results revealed that oils from the different plants (*C. sinensis* clone TRFK 306 and TRFK 301/5 and *C. oleifera*) differed significantly ($p = 0.001$) in the levels of total polyphenols. The total polyphenolic content ranged from 5.53 to 7.24%. *C. oleifera* had the highest polyphenol content at 7.24 ± 0.29% and *C. sinensis* clone TRFK 301/5 had the lowest at 4.783 ± 0.16%.

3.3. Antioxidant activity of *Camellia sinensis* and *Camellia oleifera* seed oil

The antioxidant activities ranged from 14.73 to18.81%. The results indicated that there were significant differences ($p \leq 0.05$) in the antioxidant activity among the different types of tea oils ($p=0.0005$). The antioxidant activity of *C. oleifera* was 18.81 ± 0.46%, *C. sinensis* TRFK 306, 15.98 ± 0.135 and *C. sinensis* 301/5, 14.73 ± 0.47%.

3.4. Correlation between antioxidant activity, polyphenolic composition and profiles of *Camellia sinensis* and *Camellia oleifera* seed oil

Total catechin positively correlated with all the individual catechin fractions including total polyphenol ($r = 0.998$). Total catechin also positively correlated with the antioxidant activity of the oils ($r = 0.954$). The individual catechin C, EGCG and ECG positively and significantly correlated with antioxidant activity (Table 3).

Table 3. Correlation matrix of the biochemical parameters of tea seed oil

	GA	EGC	+C	EC	EGCG	ECG	TC	TP	AA
GA	1								
EGC	0.150210	1							
+C	1.000000	0.150210	1						
EC	0.409644	0.963428	0.409644	1					
EGCG	0.937509	0.484836	0.937509	0.701470	1				
ECG	0.996771	0.070336	0.996771	0.335068	0.906541	1			
TC	0.935507	0.489823	0.935507	0.705529	0.999984	0.904115	1		
TP	0.955144	0.436256	0.955144	0.661424	0.998502	0.928279	0.998173	1	
AA	0.954256	0.438937	0.954256	0.663658	0.998661	0.927165	0.998349	0.999996	1

TP- total polyphenols; AA- antioxidant activity; GA- gallic acid; EGC- epigallocatechin; +C- catechin; EC- epicatechin; EGCG- epigallocatechin gallate; ECG- epicatechin gallate, AA – antioxidant activity

Table 4. Zones of inhibition (mm) against two bacterial strains

Plants	Variety	S. aureus IZDs(mm)	E. coli IZDs (mm)
Tea	C. sinensis (TRFK 301/5)	11.00±1.00^{c}	8.00±0.00^{b}
	C. sinensis (TRFK 306)	12.00±1.00^{c}	8.00±1.00^{b}
	C. oleifera	11.67±0.58^{c}	8.33±0.58^{b}
Eucalyptus	Eucalyptus Citriodora	20.67±0.58^{a}	13.67±0.58a
Olives	Olea europaea	18.33±0.58^{b}	8.67±0.58^{b}
DMSO (0.5 %)		6.00	6.00
Control – Chloramphenicol (0.6µg)		27.00	20.00

Means with the same letter in the same row are not significantly different at $P \leq 0.05$ according to the Tukey test $p < 0.05$. Number of replicates = 3; IZDs – inhibition Zone diameters (mm); CONC – concentration; DMSO - Dimethyl sulfoxide; *S. aureus* - Staphyloccocus aureus; *E. Coli* - *Escherichia coli*; *C. sinensis* - *Camellia sinensis*; *C. oleifera* - *Camellia oleifera*; TRFK 306/1– Tea research foundation of Kenya tea variety 306; TRFK 301/5– Tea research foundation of Kenya tea variety 301/5

3.5. Antibacterial activities of *Camellia sinensis* (TRFK 306, TRFK 301/5), *C. oleifera*, eucalyptus and olive oil

It was found that *E. coli* (ATCC 25922) was inhibited weakly by tea seed oil (Table 4). TRFK 301/5, TRFK 306 and *C. oleifera* at a concentration of 100% showed mean zones of inhibition of 8.00 mm, 8.00 mm and 8.33 mm respectively. *Eucalyptus citriodora* gave the highest inhibitory effects of 13.67 ± 0.58 mm while olive oil gave a zone of inhibition of 8.67 mm. The trend was similar when the concentration was reduced to 50% whereupon *C. oleifera, C. sinensis* teas (TRFK 301/5 and TRFK 306) showed no inhibition. However, *Eucalyptus citriodora* and olive oil showed significant inhibition even at this low concentration.

S. aureus a gram-positive bacterium was inhibited by all the test samples at a concentration of 100%. Eucalyptus exhibited the highest bio-activity with IZD of 20.67 mm followed by Olive oil (18.33mm) (Table 4). The tea samples had low activity especially at 50% concentration.

Eucalyptus oil showed the highest antibacterial activity with zones of inhibition of 20.67 mm and 13.67 mm for *S. aureus* and *E. coli,* respectively (Table 4). Tea seed oil exhibited the lowest antibacterial activity. *C. oleifera* exhibited antimicrobial activity compared to *C. sinensis*, although IZDs values did not differ significantly ($p \leq 0.05$) for *S. aureus* or *E. coli*. Olive oil inhibited the growth of *S. aureus* with an IZD of 18.33 mm, which was significantly ($p \leq 0.05$) higher than those of the camellia oils.

3.6. Antifungal activity of *Camellia sinensis* (TRFK 306, TRFK 301/5), *C. oleifera*, eucalyptus and olive oil

There was minimal antifungal activity of tea seed oila gainst *C. albicans, C. neoformans* and *T. mentagrophytes*. *Eucalyptus Citriodora* oil (Essential oil), and Olive oil (seed oil) exhibited antimicrobial activity. *Eucalyptus citriodora* showed activity against *C. albicans* and *T. mentagrophytes*. TRFK 301/5 seed oil showed activity against *C. albicans* at a concentration of 100%. The antifungal activity of tea seed oil was low. *Cryptoccus neoformans* was not inhibited by any of the oils. TRFK 301/5 oil exhibited bioactive activity against *C. albicans*, and other tea oils had no activity, although Eucalyptus and olive oil exhibited antimicrobial activity at a 100% concentration. At 50% concentration no activity was noted. *T. mentagrophytes* was inhibited by all tea seed oil except seed oil from TRFK 301/5 seeds (Table 5).

3.7. Correlation between antioxidant activity, polyphenolic composition, catechin fractions and inhibition zone diameters

The inhibition zone diameters positively correlated with total polyphenols (r = 0.975), antioxidant activity (r = 0.976), gallic acid (r = 0.866); epigallocatechin (r = 0.624); catechin (r = 0.866); epicatechin (r = 0.811); epigallocatechin gallate (r = 0.986); epicatechin gallate (r = 0.823) (Table 13). Correlation analyses between the inhibition zone diameters of *S. aureus* with biochemical parameters of tea seed oil

Table 5. Zones of inhibition of three fungal strains

Plants	Variety	*C. albicans* IZDs (mm)	*T. mentagrophytes* IZDs (mm)	*C. neoformans* IZDs (mm)
	C. sinensis (TRFK 301/5)	6.00±0.00^{d}	6.00±0.00^{d}	6.00±0.00^{a}
Tea	*C. sinensis* (TRFK 306)	6.67±0.58^{bc}	7.00±0.58^{c}	6.00±0.00^{a}
	C. oleifera	6.00±0.00^{cd}	6.67±0.58^{d}	6.00±0.00^{a}
Eucalyptus	*Eucalyptus Citriodora*	10.33±0.58^{a}	10.33±0.58^{a}	6.00±0.00^{a}
Olives	*Olea europaea*	8.00±0.00^{b}	8.00±0.00^{b}	6.00±0.00^{a}
DMSO (0.5 %)		6.00	6.00	6.00
Control – Nystatin (30µg)		26.00	27.00	25.00

Means with the same letter in the same row are not significantly different at $P \leq 0.05$ according to the Tukey test $p < 0.05$. Number of replicates = 3. IZDs – inhibition Zone diameters (mm); CONC – concentration; DMSO - Dimethyl sulfoxide; *C. alibicans = Candida albicans*; *T. Mentagrophytes – Trichophyton mentagrophytes; C. Neoformans – Cryptoccus neoformans*; *C. sinensis = camellia sinensis; C. oleifera = Camellia oleifera*, TRFK 306/1– Tea research foundation of Kenya tea variety 306, TRFK 301/5– Tea research foundation of Kenya tea variety 301/5

Table 6. Correlation matrix of inhibition zone diameters to total phenolic content, total catechin and catechin fractions and antioxidant activity.

	GA	EGC	+C	EC	EGCG	ECG	TC	TP	AA
S. aureus	0.866	0.624	0.866	0.811	0.986	0.823	0.987	0.975	0.976
E. coli	1.000	0.150	1.000	0.410	0.938	0.997	0.936	0.955	0.954
C. albicans	0.500	0.931	0.500	0.995	0.770	0.429	0.774	0.734	0.736
T. mentgrophytes	0.632	0.901	0.515	0.981	0.986	0.429	0.936	0.736	0.780

TP- total polyphenols; AA- antioxidant activity; GA- gallic acid; EGC- epigallocatechin; +C- catechin; EC- epicatechin; EGCG- epigallocatechin gallate; ECG- epicatechin gallate; *C. alibicans = Candida albicans; T. Mentagrophytes – Trichophyton mentagrophytes; S. aureus -Staphyloccocus aureus; E. Coli -Escherichia coli.*

(Table 6) revealed that the inhibition zone diameters significantly correlated with following biochemical properties of tea seed oil: epigallocatechin gallate (r = 0.938), epicatechin gallate (r = 0.997), total catechin (r = 0.936), total polyphenol (r = 0.955) and antioxidant activity (r = 0.954).

Correlation analyses between inhibition zone diameters of *E. coli* ATCC 25922 with biochemical parameters of tea revealed that the inhibition zone diameters significantly correlated with following biochemical properties of tea: EGCG (r = 0.938), ECG (r = 0.997), TP (r = 0.955). Antioxidant activity also showed significant positive correlation (r = 0.954*) influence on the inhibition zone diameter.

The inhibition zone diameter of a *C. albicans* with biochemical parameters of the assayed tea samples positively correlated with total polyphenols GA (r = 0.500), EGC (r = 0.931), +C (r = 0.500), EC (r = 0.995), EGCG (r = 0.770), TP (r = 0.734) and AA (r = 0.736) (Table 3).

The correlation between the inhibition zone diameters of *T. mentagrophytes* with total polyphenols and individual catechins and antioxidant activity is presented in Table 6. The TP (r = 0.734), GA (r = 0.500), EGCG (r = 0.770), EGC (r = 0.931) and antioxidant activity (r = 0.736) significantly correlated with the inhibition zone diameters.

4. DISCUSSION

In this study the total polyphenol contents of oils were determined. The results confirmed the presence of polyphenols in tea seed oil. However, the levels varied among the oils and *C. oleifera* exhibited the highest levels. Earlier, it had been demonstrated that the extent of variation in total polyphenol contents between different tea oils is as a result of the genotype of the tea plant under study and other factors such as geographical origin. In the current study it was evident that genotype played an important role. *C. sinensis* tea varieties TRFK 306 and TRFK 301/5 with similar genotype had lower levels of polyphenols than *C. oleifera* oil.

The results of this study showed that the tea cultivars (*Camellia sinensis*) had significantly lower levels of polyphenols in their seed oil compared to those of its leaves. The polyphenol levels of the leaves of the tea cultivars studied ranged from 21.90% (TRFK 306) to 22.07% (TRFK 301/5) as seen in a study done by (Karori *et al.,* 2014) on the polyphenol composition of tea leaves. The polyphenol content of the tea seed oil was in agreement with work reported by George (2013). The low levels of polyphenols in the seed oil could have been due to the fact that most of the polyphenols in the seed of plants were not easily extractable using aqueous or aqueous-organic solvents as opposed to the polyphenols found in the leaves and skin of most plants.

This study showed that the catechins in the oils were similar to those found in the leaves of *Camellia* plants i.e. (-)-epigallocatechin (EGC), (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (EGC). From the chromatograms it was seen that the gallated catechins were the last to be eluted. The difference in retention times between the gallated and non gallated catechins was because of their polarity to the C6 column used in the HPLC system. Gallated catechins bind tightly to the C6 column unlike non gallated catechins that bind loosely and are not eluted first. In a review by Stalikas, (2007) on extraction, separation, and detection methods for phenolic acids and flavonoids, he indicated that phenolic acids are eluted from RP columns according to decreasing po-

larities. This was the case in this study whereby the gallated catechins have less hydroxy groups which exhibit less polarity, while the non gallated catechins have more of the hydroxyl groups exhibiting increased polarity, and thus the decreased polarity increased their retention time.

This study also investigated the radical scavenging activity of DPPH by tea seed oil to determine its antioxidant activity. The results obtained indicated that oils have the ability to scavenge for free radicals. This phenomenon is attributed to the polyphenolic composition in the oils. In a study done at the Tea Research Institute Kenya, by George (213), the antioxidant capacities of tea seed oil and the whole tea seed meal expressed as gallic acid equivalent showed that the seed cake had significantly higher antioxidant activity than the oil. In addition, the metal chelating capacity of tea seed cake was as high as 90% at 25 mg sample equivalent/mL, demonstrating that tea seed products can provide lasting health benefits. In an article written by (Bernatoniene and Kopustinskiene, 2018) on 'the Role of Catechin in Cellular Responses to Oxidative Stress", it was noted that catechin had the ability to donate hydrogen ions to stabilize free radicals. This was considered the first mechanism in the antioxidant efficacy of catechin. Other mechanisms included indirect ways such as the induction of antioxidant enzymes that inhibit the pro-oxidant enzymes and also the production of detoxification enzymes and antioxidant enzymes in a stage referred to as PHASE II (Bernatoniene and Kopustinskiene, 2018). However, the antioxidant activity of the tea seed oil in this study was lower than that observed for the leaf as seen in other studies (Karori *et al.,* 2007)twelve different types of commercial tea samples were assayed to determine their phenolic composition and antioxidant activity. Reverse phase high performance liquid chromatography using a binary gradient system was used for the identification and quantification of individual catechins. Subsequently, total phenolic content was determined spectrophotometrically according to the Folin-ciocalteus method. Total theaflavins and thearubigins were also determined. The radical scavenging behavior of the polyphenols on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH. Despite the low antioxidant activity of the tea seed oil, it can be seen as a potential source of naturally high value edible oil. Various studies have outlined the beneficial effects of tea seed oil such as hepatoprotection (Lee *et al.,* 2007), and reduction in weight gain (Kim *et al.,* 2008). In a study done by Zhang *et al.* (2014), an ability to clear lipid peroxidation in rat livers was detected, hence alleviating liver disease.

The tea plant in other countries such as China is not only grown for the leaf but also for the seed. The common tea plant planted for seeds in China is *C. oleifera.* The seeds of *C. oleifera* are used to produce tea seed oil which is an edible oil and can also be used in the cosmetic and pharmaceutical industries (Wang *et al.,* 2017). *C. sinensis* planted mainly in Kenya has seeds that can be exploited to produce oil for consumption and commercial use (George *et al.,* 2016). In this study the oils from two tea varieties, *Camellia oleiefera* and two *C. sinensis* cultivars, were evaluated for their antimicrobial activity against five microbes viz. *E. coli*, *S. aureus*, *C. albicans, C. neoformans* and *T. mentagrophytes*. The results obtained confirmed that tea seed oil indeed has antimicrobial properties. The tea plants were able to inhibit the growth of *E. coli* and *S. aureus*. At an oil concentration of 100%, *S. aureus,* a gram-positive bacteria, was the most susceptible microbe to the oils. This was due to the much greater binding of negatively charged EGCG to the positively charged lipid bilayer constituent of the cell membrane of gram-positive bacteria. The bactericidal effect of EGCG was attributed to membrane perturbation (Koech *et al.,* 2013). Eucalyptus oil and olive oil had better activity than the tea oils. *C. oleifera* exhibited the most activity among the teas. The low activity of tea seed oil against the microbes under study might have been due to the low levels of catechin and polyphenols. Catechin (flavonoids) contains two benzene rings A and B, which have been identified as the main bactericidal components. EGCG is the main catechin component among the catechin which highly inhibits bacterial growth (Yamada, 2013). EGCG is known to even inhibit the activity of HIV and *S. aureus* in various study models (Yamaguchi *et al.,* 2002). Another catechin, EGC, has been demonstrated to disrupt the activity of the gyrase enzyme on the DNA gyrase b subunit of bacteria after binding to the ATP site (Hoshino *et al.,* 1999). Apart from catechins, the other tea flavanols like Quercitin, Kaempferol and Myricetin were also found to have antibacterial activity against gram-positive bacteria at varying concentrations (Yoda *et al.,* 2004). Quercitin exhibited

remarkable activity against *S. aureus,* even higher than EGCG (Yoda *et al.,* 2004). Over four thousand compounds have been detected in tea. Amongst the 4000 compounds about a hundred of them have been shown to exhibit remarkable antimicrobial activity against various microorganisms (Yoda *et al.,* 2004).

5. CONCLUSIONS

From this study it is evident that tea seed oil is a potential source of beneficial phytochemicals that can be utilized for their health promoting effects. It was also seen that the oils have antioxidant capacity and thus can be used as a good source of food that can help prevent pathophysiological diseases associated with oxidative stress. The correlation of the antimicrobial activity of oils to its biochemical/phytochemical constituents was positively significant, suggesting that phytochemicals in oils are the main bioactive constituents that give them potent antimicrobial activity. From this study TP, EGC, EGCG, ECG, and GA were identified as the most potent antimicrobial biochemical in the assayed teas. Therefore, antimicrobial activity was higher in tea extracts containing high levels of TPP, EGCG, ECG and EGC. The *C. sinensis* and *C. oleifera* tea seed oil possessed both antifungal and antibacterial activities.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Textural and rheological properties of soybean oil organogels structured with polyglycerol and propylene glycol esters during storage

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SUMMARY: Organogels have emerged as an alternative to the intake of saturated fats. Organogels of soybean oil (SBO) structured with polyglycerol esters (PGE) or propylene glycol esters (PPGE) at different concentrations (0.5, 1.0, 2.0, 3.0, or 4.0%) were formulated. Both emulsifiers at 4% (w/w) concentrations were able to form solid-like organogels and showed thixotropy and low mechanical resistance when compression forces were applied. However, the SBO/PGE (4%) organogels presented lower values for flow curves and micrographs showed a more organized network compared to the SBO/PPGE at 4%. However, higher flow curve values, larger crystals, and mechanical resistance on compression were observed after a two-month storage period of SBO/PPGE compared to SBO/PGE organogels. Both organogels have the potential to be used for diverse food applications although the SBO/PGE was more stable throughout storage.

KEYWORDS: *Fatty acids; Hardness; Organogels; Polyglycerol ester; Propylene glycol ester; Saturated thermal stability*

RESUMEN: ***Propiedades texturales y reológicas de organogeles de aceite de soja estructurados con ésteres de poliglicerol y propilenglicol durante el almacenamiento.*** Los organogeles surgieron como una alternativa a la ingesta de grasas saturadas. Se formularon organogeles de aceite de soja (SBO) estructurados con ésteres de poliglicerol (PGE) o ésteres de propilenglicol (PPGE) a diferentes concentraciones (0,5, 1,0, 2,0, 3,0 y 4,0%). Ambos emulsificantes fueron capaces de formar organogeles sólidos con un 4% (p/p) y mostraron tixotropía y baja resistencia mecánica cuando se aplicaron fuerzas de compresión. Sin embargo, los organogeles SBO/PGE (4%) presentaron valores más bajos de curvas de flujo y las microfotografías mostraron una red más organizada en comparación con el SBO/PPGE al 4%. Sin embargo, se observaron valores de curva de flujo más altos, cristales más grandes y resistencia mecánica a la compresión después de dos meses de almacenamiento en el SBO/PPGE en comparación con los organogeles de SBO/PGE. Ambos organogeles tienen potencial para ser usados en la industria alimentaria aunque el organogel SBO/PGE fue más estable durante almacenamiento.

PALABRAS CLAVE: *Ácidos grasos saturados; Dureza; Estabilidad Térmica; Ester de poliglicerol; Ester de propilenglicol; Organogeles*

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1. INTRODUCTION

Fats are a vital part of the human diet, not only due to their sensory attributes but also for providing essential fatty acids and energy (Dorni *et al.,* 2018). The food industry currently uses processed, fractionated, or interesterified fats with high contents of saturated fatty acids (SFA). A high intake of SFA is associated with increased levels of blood cholesterol and a high mortality rate from cardiovascular diseases (Hunter *et al.,* 2009). The World Health Organization (WHO) recommends that the total daily intake of SFA should not exceed 10% of the total caloric intake and diets should contain higher levels of polyunsaturated fatty acids (PUFAs) n-3 and linoleic acid (n-6) (WHO, 2008). Therefore, efforts have been made to replace SFA with sources rich in PUFAs (Wang, 2018) with little success due to the technological properties that SFAs provide to foods. PUFAs are mostly liquid at room temperature and may not have the desired functionality compared to solid shortenings such as creaming, high viscosity, and enhanced flavor, odor, and texture in bakery products (Buitimea-Cantúa *et al.,* 2017; Abramovič *et al.,* 2018). Because of this, the organogel technology for structuring PUFA-rich oils was developed as a viable alternative to considerably reduce saturated and *trans* fats, and recently have been utilized for the manufacturing of an array of lipid-based products (Chaves *et al.,* 2018).

Organogels impart the desired functionality to foods without generating *trans* fatty acid (TFA) and with reduced SFA contents (da Silva *et al.,* 2018a). Oleogelation has several advantages compared to hydrogenation and interesterification, the most relevant being that no biochemical processes are involved (Sellami *et al.,* 2012; Dinç *et al.,* 2014).

During the preparation of organogels, one or more structuring agents can be used to produce a change in the physical properties of the vegetable oil that facilitate the formation of semi-solid or solid plastic materials. The vegetable oils from safflower, rice bran, sunflower, canola, high-oleic sunflower and soybean, and cod liver oil have been successfully structured into organogels (Rocha *et al.,* 2013; Lopez-Martinez *et al.,* 2015; Sintang *et al.,* 2017a, b; Palla *et al.,* 2017; Yang *et al.,* 2018; da Silva *et al.,* 2018a; Wijarnprecha *et al.,* 2018). The quite inexpensive and highly available soybean oil has the advantage of containing a relatively high proportion of polyunsaturated fatty acids, which are known to decrease serum cholesterol and cardiovascular diseases (Chou *et al.,* 2018). Several investigations have demonstrated the feasibility of producing different functional organogels with diverse structurants such as carnauba (*Copernica cerifera*), sugarcane (*Saccharum officinarum*), candelilla (*Euphorbia cerifera*), sunflower (*Helianthus annuus*), rice (*Oryza sativa*) bran, and monoglycerides (Marangoni, 2012; Rocha *et al.,* 2013; Patel *et al.,* 2015; Lim *et al.,* 2017; Öğütcü and Yılmaz 2014; Chaves *et al.,* 2018; da Silva *et al.,* 2018b; Buitimea-Cantúa *et al.,* 2020). The organogels formulated with different vegetable oils (oil phases) produced different crystallization behaviors and consequently formed organogels with different thermal stability, rheological and textural properties (Marangoni, 2012; Rocha *et al.,* 2013; Öğütcü and Yılmaz 2014; Lim *et al.,* 2017; Cotabarren *et al.,* 2019; Buitimea-Cantúa *et al.,* 2020).

Other researchers have focused on the influence of diverse structurant agents such as waxes and emulsifiers added in small concentrations (<10%, w/w) on the mechanical, rheological, and textural properties of organogels (Pernetti *et al.,* 2007; Rocha *et al.,* 2013; Sintang *et al.,* 2017b). Among the emulsifiers used for producing organogels are sorbitan tristearate and mono- and diacylglycerols (Ojijo *et al.,* 2004; Hughes *et al.,* 2009; Rocha-Amador *et al.,* 2014; López-Martínez *et al.,* 2015; Sintang *et al.,* 2017a; Palla *et al.,* 2017; Fayaz *et al.,* 2017; Cotabarren *et al.,* 2019). In particular, organogels formulated with monoglycerides increase their stability, change their microstructural crystal network and produce plastic structures typical of hardstock fats that impart the desired functionality to foods without TFA and with low SFA contents (Dassanayake *et al.,* 2011; López-Martínez *et al.,* 2015; Palla *et al.,* 2017; Fayaz *et al.,* 2017; Cotabarren *et al.,* 2019). Polyglycerol esters are emulsifiers which are commonly used in the food industry (Curschellas *et al.,* 2013) and they could be a new alternative for the production of organogels. López-Martínez *et al.* (2015) reported that the utilization of a mixture of monoglycerides in safflower oil developed mixed self-assembled structures that resulted in organogels with improved rheological properties compared to the use of pure monoglycerides. Recently, Cotabarren *et al.* (2019) concluded that organogel mixtures of

monoglyceride and phytosterols produced by extrusion-based 3D printing showed crystals in organogels of irregular, elongated, fibrillar, or needle-like shapes. Meng *et al. (*2019) utilized sodium stearoyl lactylate as a gelling agent at concentrations of 7. 9, 11, and 13% (w/w) with sunflower oils to structure organogels and concluded that higher concentrations of the oleogelator resulted in a denser crystalline network, which provided stronger mechanical strength and enhanced the ability to retain the oil phase.

An organogel is a self-assembled or crystalline particle structure formed by the entanglement of one or more structurant units such as crystals, fibrillar networks, or suspended polymer strands (Sawalha *et al.,* 2011; Sintang *et al.,* 2017a; Sintang *et al.,* 2017b). The structuring mechanisms involved in these systems can be divided into a dispersion of the fat phase as crystallized or uncrystallized solid particles or self-assembled complex structures, which are held together by specific supramolecular interactions (Pernetti *et al.,* 2007). It is essential to optimize and design the textural and rheological properties when developing a new organogel. However, during storage organogels may change their rheological properties and appearance (phase separation) due to oil exudation, which are major causes of rejection (Sintang *et al.,* 2017a; Sintang *et al.,* 2017b). This study was planned to evaluate the textural and rheological properties of soybean oil organogels structured with polyglycerol or propylene glycol esters at different concentrations during two months of storage at room temperature. Both structurants are considered GRAS by the FDA and are derived from mono and diglycerides, acetylated mono and dyglycerides, phosphated mono and diglycerides, and esters of propilenglycol, sorbitan, phosphate, sucrose, polyglycerol lactate, and lecithin (Hasenhuettl, 1997). The polyglycerol esters and propylene glycol esters are used as emulsifiers in foods, in amounts not greater than that required to produce the intended physical or technical effect.

2. MATERIALS AND METHODS

2.1. Materials

Refined soybean oil (SBO) was bought at a local supermarket (Monterrey, N.L., Mexico). The structurants used were: 1) A mixture of mono-diglycerides and polyglycerol esters (Polyglycerol Esters of Fatty Acids) or PGE (Fusion point = 55-61 °C) (Admulse MSPG-40) and 2) Propylene Glycol Esters of Fatty Acids or PPGE (Fusion point = 55-60 °C) (Admulse MEPG-AL). The structurants were kindly provided by ADIPLEX, S.A. de C.V (Monterrey, Nuevo León, Mexico).

2.2. Preparation of organogels

The preparation of organogels consisted of a standard methodology described by Rocha *et al.* (2013), in which samples were prepared by heating soybean oil to 80 °C under continuous stirring. When the temperature of 80 °C was reached, the structurant was added at different concentrations (0.5, 1.0, 2.0, 3.0, or 4.0% w/w) and mixed until complete dissolution. The mixture was kept under agitation for about 3 min to assure complete melting. The resulting blends of soybean oil with PEG or PPGE were stored at 20 °C for 24 h to enhance the formation of gels and kept at this temperature for two months. The organogels were identified as SBO/PEG (soybean oil with polyglycerol esters) and SBO/PPGE (soybean oil with propylene glycol esters).

2.3. Visual appearance

After 24 hours of storage of the organogels, a visual assessment was performed. Organogels were subjectively classified into five types: 1, 2, 3, 4, and 5, corresponding to liquid, viscous liquid, high-flowing semisolid gel, low flowing semisolid gel, and a totally solid gel which maintained its structure and hardness, respectively (García *et al.,* 2013).

2.4. Thermal stability

The thermal stability of organogels was determined by a cyclization process described by Garcia *et al.* (2013). Briefly, samples of 30 mL of each organogel were placed in 50-mL beakers and then subjected to sequential temperature variations according to the following conditions: 25 °C for 24 h for complete crystallization, followed subsequently by 5 °C for 24 h, 25 °C for 24 h, 5 °C for 48 h, 35 °C for 24 h, 25 °C for 24 h, 35 °C for 48 h and 5 °C for 48 h. Finally, the sample was stored at 25 °C for 24 h according to conditions specified in Table 1. After each storage condition, samples were immediately tested and classified according to the visual subjective appearance (type 1, 2, 3, 4, or 5). According to results

Table 1. Thermal stability of soybean oil organogels structured with polyglycerol esters (PGE) or propylene glycol esters (PPGE) tested sequentially at different temperatures and times*

Organogels	Esters (%)	Temperature /Time								
		25 °C /24 h	5 °C /24 h	25 °C /24 h	5 °C /48 h	35 °C /24 h	25 °C /24 h	35 °C /48 h	5 °C /48 h	25 °C /24 h
SBO/PGE	0.5	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a
	1	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a
	2	3±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b
	3	4±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c
	4	5±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d
SBO/PPGE	0.5	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a
	1	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a	1±0 a
	2	3±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b	2±0 b
	3	4±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c	3±0 c
	4	5±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d	4±0 d

*Subjectively rated according to Garcia *et al.* (2013) in type 1 (totally liquid), 2 (viscous liquid), 3 (high-flowing semisolid gel), 4 (low-flowing semisolid gel), and 5 (solid gel). Mean value ± SD (n=3). Tukey tests were employed for comparison of means ($p < 0.05$). Different lowercase letters within each column indicate significant differences ($p < 0.05$). The number of panelists who evaluated the physical status of the samples was three. SBO; soybean oil, PGE; polyglycerol esters, PPGE; propylene glycol esters.

of thermal stability, type-5 organogels stable under the cyclization conditions tested herein were stored at 25 ºC for 2 months. Both the visual appearance and thermal stability of the organogels were used as the main criteria to select the optimal concentration of SBO/PEG or SBO/PPGE.

2.5. Instrumental color measurements

The color of the SBO, PEG, PPGE, and their respective organogels were analyzed after 24 h at 25 °C. The color measurements based on the system CIEL*C*h (L = luminosity from zero (black) to 100 (white); $+a$ = red, $-a$ = green, $+b$ = yellow, and $-b$ = blue) were obtained with a Hunter Lab colorimeter (MiniScan PLUSXE, Hunter Lab, Reston, VA, U.S.A.) (da Silva *et al.,* 2018b) with slight modifications. The equipment was calibrated with standards provided by the supplier. E values were calculated by using the following equation: $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$.

2.6. Hardness (compression/extrusion)

The hardness of solid-like SBO/PGE and SBO/PPGE organogels structured with 4% concentration (w/w) were evaluated by compression/extrusion measurements using the texture analyzer Stable Micro Systems model TA-XT2i (Godalming-UK). 40 mL of organogels were conditioned in 50-mL glass containers of 35 mm internal diameter and 22 mm height. The glass containers were kept for 24 h at 20 °C for stabilization and after this period compressed with a 15-mm acrylic cylinder (25 mm diameter and 35 mm height) with a head cross speed of 1.0 mm/s (Rocha *et al.,* 2013). All determinations were performed in triplicate.

2.7. Microstructure

The organogels were placed on a glass slide and covered with a coverslip. Then, organogels were conditioned at 20 °C for 24 h and examined under constant temperature. The morphologies of the crystals of SBO/PGE and SBO/PPGE organogels (4% w/w) were viewed under a polarized light microscope (Olympus System Microscope model BX 50, Olympus America Inc., Center Valley, PA, USA) equipped with the digital camera Olympus EX300 (Olympus America Inc., Center Valley, PA, USA). Photographs were taken in different fields and visuals, and the resulting images were evaluated using the software Image Pro-Plus 7.0.1 for Windows by Media Cybernetics (Bethesda, MD, USA) with a magnification of 4x (Rocha *et al.,* 2013).

2.8. Rheological properties: Flow curve

The rheological properties were assessed using a Rheometer (Anton Paar, Graz, Austria). The flow

curves were acquired with sand-blasted rough plate geometry of 5 cm wide, a roughness of 5-7 μm, and a gap of 300 μm. The temperature throughout the analyses was maintained constant at 25 °C with shear rates ranging from 0 to 300 s^{-1} (Rocha *et al.,* 2013). All determinations were made in triplicate and the models adjusted according to the Power Law, which classifies fluids according to their behavioral index (n) into: Newtonian (n = 1 and τ0 = 0), pseudoplastic (0 <n <1) or dilating (1 <n <∞).

2.9. Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and Tukey tests were employed for comparison of means ($p < 0.05$). Data were reported as means and standard deviations. All statistical analyses were performed using the software JMP 5.0.1 (SAS Institute, Cary, NC, USA).

3. RESULTS AND DISCUSSION

3.1. Visual appearance and thermal stability

The SBO/PGE and SBO/PPGE organogels formulated with different concentrations and kept at different storage temperatures showed changes in stability. Their visual appearance, gel consistency, and stability were affected by the concentration of structurants (Table 1). As expected, the stability and consistency increased with a higher concentration of structurants for both the SBO/PGE and SBO/PPGE organogels. Gels formulated with 0.5% showed a total liquid consistency. However, both structurants supplemented at 2% (w/w) yielded high flowing organogels with semisolid features (type 3).

Among the experimental organogels, the SBO/PGE and SBO/PPGE gels formulated with 4% (w/w) were entirely solid and stable (Type 5) at 25 °C compared to their counterparts prepared with lower concentrations of structurants. Interestingly, both structurants kept forming solid-like organogels when added at 4% and stored at a higher temperature (35 °C). These organogels had a slightly lower consistency (type 3) and did not show any indication of liquid phase separation. The consistency of all organogels decreased 1-point unit after 24 h of storage. As the concentration of structurant decreased, the consistency/stability of organogels also decreased, particularly those formulated with concentrations of 0.5 and 1.0%. These organogels yielded liquid gels rated as Type 1. The higher stability in SBO/PGE and SBO/PPGE organogels formulated with a concentration of 4% (w/w) were attributed to the chemical composition of these pure structurants (Polyglycerol Esters or PEG and propylene glycol esters or PPGE). These structurants can potentially mimic triacylglyceride crystallization through molecular self-assembly, leading to the formation of the more stable three-dimensional gel network. Similarly, Öğütcü and Yilmaz (2014) reported that a 3% addition of monoglycerides in olive oil-based organogels yielded stable gels, whereas the candelilla wax could not create stable gels at the same concentration. Therefore, the results herein clearly indicate the potential for the use of SBO/PGE and SBO/PPGE for the production of gels with semi-liquid consistency at concentrations lower than 3% (w/w). PEG and PPGE structurants at concentrations of 0.5, 1.0, 2.0, or 3.0% w/w, showed total liquid and semi-liquid consistencies and negatively affected the thermal stability of organogels (Tables 1 and 2). Furthermore, a preliminary study was carried out to select the optimal storage of organogels. Organogels stored for two months showed particle aggregation and thus negatively affected the consistency. The best organogels were produced for the assessment of the microstructure, hardness, and rheological properties after 24 hours and two months of storage.

3.2. Color parameters

The color parameters of the organogels (SBO/PGE and SBO/PPGE) formulated with 4% PGE or PPGE are summarized in Table 2. The results indicate that the luminosity and yellowish coloration were not significantly affected by the two different polyglycerols. However, a significant difference in ΔE values was obtained in the organogels formulated with SBO/PPGE. The ΔE values of SBO/PPGE organogels indicated a greater color difference caused by the structurant type.

The luminosity, greenness and yellowish values in organogels formulated with 4% SBO/PGE were L= 24.49; a = 0.70, b = 3.94; whereas in SBO/PPGE counterparts L= 24.48; a = 0.72., b = 3.79 (Table 2). The observed differences can be attributed to the addition of higher proportions of soybean oil. The values of b parameter (b=) (yellowish) in soybean oil can be attributed to the presence of chlorophyll derivatives (pheophytin A) which are responsible

TABLE 2. Color parameters *L*, *a*, *b,* and *ΔE* and maximum force of soybean oil organogels structured with polyglycerol esters (PGE) or propylene glycol esters (PPGE) at 4.0% (w/w) after storage at 25 °C for 24 hours and two months. *

	Organogels	
Parameter	**SBO/PGE**	**SBO/PPGE**
Color		
L	24.49 ± 0.28 a	24.48 ± 0.34 a
a	0.70 ± 0.10 a	0.72 ± 0.99 a
b	3.94 ± 0.29 a	3.79± 0.31 a
ΔE	73.55 ± 0.45 b	78.62 ± 0.73 a
Hardness (N)		
24 h	0.06 ± 0.01 aA	0.06 ± 0.01 aA
2 months	0.06 ± 0.01 bA	0.08 ± 0.01 aB

* Mean value ± standard deviations (n=3). Tukey tests were employed for comparison of means ($p < 0.05$). Different letters within each row and uppercase letter in column per treatment and determination denote significant differences ($p < 0.05$). L= luminosity (100 = lightness and 0 = darkness), +a = increasing red and -a = increasing green, +b = increasing yellow and -b = increasing blue, and *ΔE* value = $[(DL^2) + (Da^2) + (Db^2)]^{1/2}$. SBO; soybean oil, PGE; polyglycerol esters, PPGE; propylene glycol esters. N; Newton.

for imparting green-yellow colorations (Fraser and Frankl, 1985). On the other hand, the SBO-free PGE presented higher luminosity values (*L*=27.64). The structurants showed higher luminosity because the PGE was devoid of chlorophyll. Among all organogels, the SBO/PGE containing 2, 3 or 4% presented the lowest color values (data not shown). Thus, the addition of PGE for the production of SBO/PGE organogels enhanced luminosity values.

3.3. Hardness

The concentration of 4% SBO/PGE or SBO/PPGE was selected as the best to form structured organogels that could withstand prolonged storage at room temperature. These organogels presented characteristics of ideal thermal stability. The organogel texture values measured as hardness (N) at 24 hours and after 2 months of storage at room temperature are summarized in Table 2. These results showed that higher concentrations of SBO/PPGE and SBO/PEG produced organogels with similar maximum force at 24 hours. However, both SBO/PGE and SBO/PPGE organogels stored for 24 hours showed lower mechanical resistance compared to counterparts stored for 2 months. Interestingly, the organogels SBO/PPGE (4%, w/w) after 2 months of storage showed a higher mechanical resistance (0.080 N) compared to counterparts formulated with SBO/PGE (0.065 N). The major textural changes occurred after 24 h of storage. After organogels were prepared, the crystals gradually rearranged, allowing growth formation, which consequently altered the texture. The rearrangement increased oil exudation (apolar liquid phase) from the fat crystals and also enhanced phase separation (post-hardening phenomena) (Hughes *et al.,* 2009). Moreover, the SBO/PPGE organogel formulated with a concentration of 4% showed two different crystal morphologies, consisting of larger fat crystal networks and spherulite crystals which were more evenly distributed compared to crystals formed in the SBO/PGE organogels. Likewise, the presence of two different morphologies of crystal explains the highest hardness and shear stress values observed for the SBO/PPGE organogel (Figure 1 A-B). This behavior is attributed to the different chemical compositions of the structurants. PEG is a mixture of mono-diglyceride and polyglycerol esters whereas PPGE consisted of propylene glycol esters of fatty acids. These results showed that a higher concentration of SBO/PPGE (4%, w/w) than SBO/

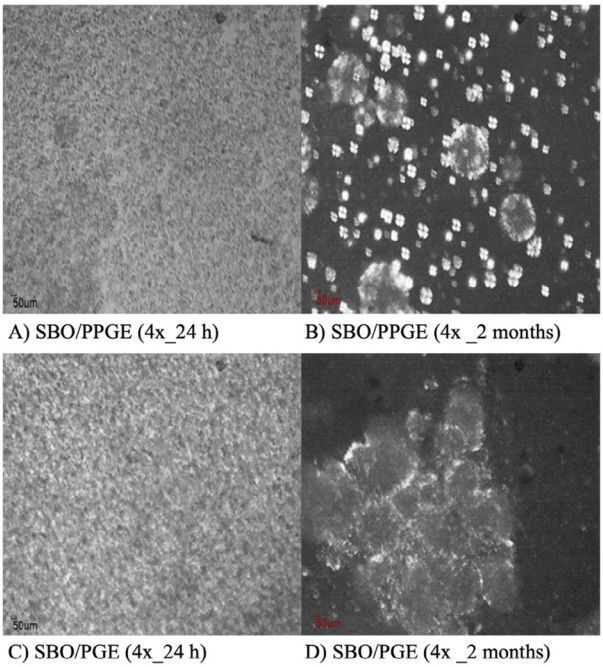

FIGURE 1. Morphological image obtained by polarized light microscopy in SBO/PPGE (A and B) and SBO/PGE (C and D) organogel with 4% of structurants, with the magnification of 4x at 20 °C after 24 h and 2 months. SBO; soybean oil, PGE; polyglycerol esters, PPGE; propylene glycol esters.

PEG produced stronger organogels after 2 months of storage. Pernetti *et al.* (2007) demonstrated that both diacylglycerols and monoacylglycerols were needed to produce organogels with softer textures. Regardless of the type of emulsifier, the longer the chain length the greater the firmness of the gel. However, these SBO/PGE and SBO/PPGE at 4% organogels showed lower hardness compared to organogels prepared with sugarcane wax (4%) and soybean oil (1.65 N) (Rocha *et al.,* 2013). Limited information exists about the presence of SBO/PGE and SBO/PPGE organogels, and this research contributes to new valuable information on the effects of PEG and PPGE addition and storage time on the hardness and related properties of the structured organogels.

3.4. Rheological properties: Flow curve

The organogels flow curves determined after 24 hours and 2 months of storage at 25 °C are depicted in Figures 2 A and B. The curves can be used for qualitative comparisons among organogels. Both SBO/PGE and SBO/PPGE organogels showed a characteristic thixotropic behavior (Steffe, 1996; Rocha *et al.,* 2013). However, organogels prepared with SBO/PGE stored for 24 h showed considerably lower shear stress values (24.16 Pa at 10.40 s^{-1}) (Figure 2A). However, a significant portion of the observed changes in shear stress occurred during storage because flow curve values increased with storage time for all treatments. Higher shear stress values were observed in organogels prepared with SBO/PPGE (Figure 2B). Shear stress values increased with the shear rate for the SBO/PPGE (4%) organogels after 2 months of storage (64.63 Pa at 300 s^{-1} at 25 °C). Also, the SBO/PGE organogels presented a fast increase and decrease in shear stress at low shear rates which are a consequence of the easier disruption of the structural network (Riscardo *et al.,* 2005; Perrechil *et al.,* 2010). The shear stress values for SBO/PGE organogels at 2 months storage were 78.50 Pa at 10.40 s^{-1}. Storage resulted in stronger structural deformational changes in gels kept for 2 months, likely due to the formation of stronger crystal network aggregates.

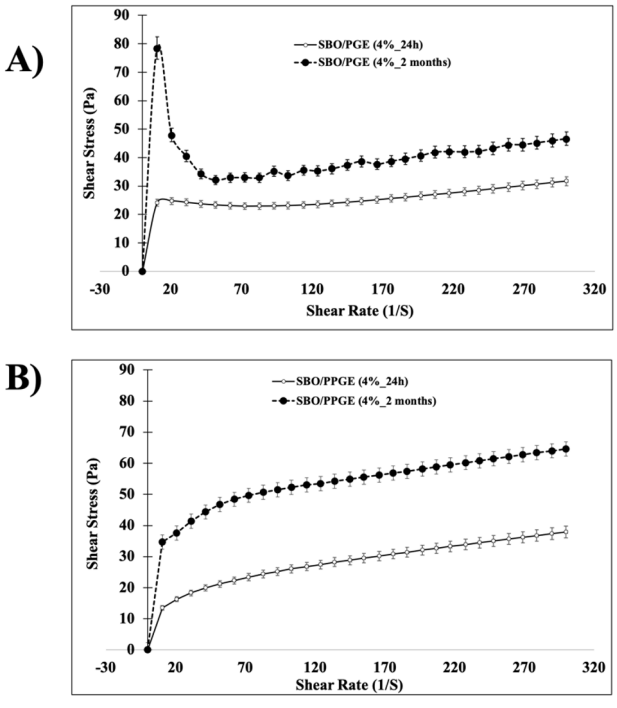

FIGURE 2. Flow curves of organogels A) SBO/PGE and B) SBO/PPGE with 4% of structurants after 24 h and 2 months. Mean value ± standard deviations (n=3) of SBO/PGE from 24 h (3.64) and 2 months (9.84); SBO/PPGE from 24 h (5.69) and 2 months (10.41). SBO; soybean oil, PGE; polyglycerol esters, PPGE; propylene glycol esters.

The observed negative effects of storage for 8-10 weeks on the structure of organogels formulated in combination with monoglycerides and phytosterols have been previously described by Sintang *et al.* (2017a). Similar results related to shear stress (62.2 at 3 s^{-1} at 25 °C) were obtained by Rocha *et al.* (2013), who used sugarcane wax (4%) to structure organogels. Recently, Buitimea-Cantúa *et al.* (2020) reported that organogels elaborated with refined carnauba wax (5.5%) increased when the shear rate increased (110 at 3 s^{-1}) from 0 to 50 1/s and this effect was higher in organogels stored for 2 months (300 at 3 s^{-1}). Results herein demonstrated that the combination of SBO/PGE or SBO/PPGE added at different ratios could provide an array of new organogels with desired rheological properties which can last up to two months in storage at 25 °C. All organogels showed pseudoplastic flow characteristics.

3.5. Microstructure

Polarized light microphotographs of the SBO/PGE and SBO/PPGE organogels at 4% of concentration and stored for 24 h or 2 months are depicted in Figure 1. The micrographs show that the crystal networks of the SBO/PPGE organogels at 24 hours of storage tended to be smaller, more uniform, smoother, and more evenly distributed (Figure 1A) compared to the crystal arrangements of the SBO/PGE counterparts (Figure 1C).

A significant portion of the organogel crystal structure changed after 2 months of storage at 25 °C because these gels had larger crystal aggregations (Figures 1B and D). The structure formed by the SBO/PGE organogels (spherulite-crystals) (Figure 1D) is an indication of weaker intermolecular interactions such as Van der Waals interactions and London dispersion forces (Sintang *et al.,* 2017a). Different complex phenomena took place after two-months' storage of solid-like organogels produced with 4% of PGE or PPGE. Post-crystallization events included polymorphic transitions from less stable to more stable polymorphs, the appearance of new crystalline particles, sintering, and Ostwald ripening (Johansson and Bergenståhl, 1995; Ojijo *et al.,* 2004). These phenomena yielded the formation of larger crystal clusters instead of smaller counterparts, thereby leading to a weaker gel (Ribeiro *et al.,* 2015; Tanaka *et al.,* 2007). These observations were previously documented by Doan *et al.* (2017) and recently by Buitimea-Cantúa *et al.* (2020) in organogels structured with refined carnauba wax.

This might explain the observed higher mechanical resistance at the beginning of the compression/extrusion tests. It is important to visualize the morphology and crystal networks because they reflect the spatial distribution of crystals that influences their rheological properties (Marangoni and Rousseau, 1996; Blake *et al.,* 2014).

4. CONCLUSIONS

The structurants PGE and PPGE were able to form semi-solid/solid-like organogels with soybean oil at 25 °C when used at 4%. The SBO/PGE organogel showed a more organized crystal network (smaller and more uniform crystals which were evenly distributed) compared to the crystals of the SBO/PPGE counterpart. The SBO/PGE organogel was softer and presented lower mechanical resistance compared to the SBO/PPGE counterpart. However, the prolonged storage of two months affected the structure of organogels formulated with either SBO/PGE or SBO/PPGE. These organogels presented larger crystal networks and higher shear stress values, which significantly affected hardness. These negative effects were more pronounced in organogels structured with SBO/PGPE. Therefore, both structurants were effective for the formation of organogels with concentrations higher than 4%, and with technological properties that can be applied in lipid-based food products.

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Solvent-free synthesis of oleic acid-based wax esters using recyclable acidic deep eutectic solvent

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SUMMARY: Wax esters have been widely used in cosmetics and pharmaceutical products. Oleic acid wax esters can be used to replace spermaceti oil or jojoba oil. In this work, the acidic deep eutectic solvent (DES) composed of choline chloride and *p*-toluenesulfonic acid (1:4, mol/mol) was used as an efficient recyclable catalyst for the synthesis of oleic acid-based liquid wax esters through an esterification reaction. The esterification conversion of cetyl alcohol reached 99.1% under the following optimal reaction conditions: 5% DES as catalyst, molar ratio of fatty acid to alcohol of 1.3:1 and reaction temperature of 70 °C for 3h. The catalyst recovery experiments showed that this low-price acidic DES catalyst could be reused five times with uniform activity. Moreover, DES-catalyzed solvent-free esterification could be applied in the preparation of other oleic acid-based wax esters and excellent conversions (> 96%) could be obtained under such mild conditions.

KEYWORDS: *Liquid wax esters; Esterification; Deep eutectic solvents; Solvent-free; p-toluenesulfonic acid*

RESUMEN: ***Síntesis libre de solventes de ceras a base de ácido oleico utilizando ácido eutéctico profundo reciclable.*** Las ceras se han utilizado ampliamente en productos cosméticos y farmacéuticos. Las ceras de ácido oleico se pueden utilizar para reemplazar al espermaceti o al aceite de jojoba. En este trabajo se utilizó el ácido eutéctico profundo (DES) compuesto por cloruro de colina y ácido *p*-toluensulfónico (1:4, mol/mol) como un catalizador reciclable eficiente para la síntesis de ceras líquida a base de ácido oleico mediante reacción de esterificación. La conversión de esterificación del alcohol cetílico podría alcanzar el 99,1% en las condiciones óptimas de reacción, mostrada como sigue: 5% de DES como catalizador, relación molar de ácido graso a alcohol de 1,3:1 y temperatura de reacción de 70 ℃ durante 3 h. Es importante destacar que los experimentos de recuperación del catalizador mostraron que este catalizador DES ácido de bajo precio podría reutilizarse cinco veces con una actividad uniforme. Además, la esterificación sin disolvente catalizada por DES podría aplicarse en la preparación de otras ceras a base de ácido oleico y podrían obtenerse excelentes conversiones (> 96%) en tales condiciones suaves.

PALABRAS CLAVE: *Ácido p-toluensulfónico; Cera líquida; Disolventes eutécticos profundos; Esterificación; Libre de disolventes*

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1. INTRODUCTION

Wax esters are the main components of natural waxes, which are composed of long-chain (>12 carbon atoms) fatty acids and alcohols (Doan *et al.*, 2017; Gunawan *et al.*, 2005). Wax esters are widely used in many fields. For example, they are the key ingredients in lipsticks and moisturizers in cosmetic formulations (Li, 1999; Keng *et al.*, 2009). In the pharmaceutical industry, wax esters are used as anti-foaming agents in penicillin production (Ungcharoenwiwat and H-Kittikun, 2013). In addition, wax esters are widely used in the food industry as lubricants, polishes and plasticizers (Salis *et al.*, 2003; Canizares *et al.*, 2020; Mandu *et al.*, 2020).

Wax esters are classified as saturated wax esters or unsaturated wax esters depending on their degree of unsaturation. The main raw materials of natural unsaturated wax esters are spermaceti oil and jojoba oil (Aissa *et al.*, 2012). However, the sperm whale is an endangered species, and the whaling ban has prompted researchers to look for the alternatives to natural spermaceti oil (Papadaki *et al.*, 2017). Jojoba oil has some similar properties to spermaceti oil, and can be used as a good substitute. However, the main limitations to the use of jojoba oil are its cost and availability (Keng *et al.*, 2009). Therefore, it is highly desirable to produce unsaturated wax esters using a chemically synthesize method.

The synthesis of wax esters has been studied for the past decades (Lima *et al.*, 2018). Wax esters can be synthesized via chemical (Aracil *et al.*, 1992) and enzymatic methods (Poisson *et al.*, 1999). Although the enzymatic reaction is mild, it also has many disadvantages, such as longer reaction time, use of organic solvents and high cost (Salis e*t al.*, 2003; Deng *et al.*, 2011). Traditionally, conventional chemical catalysts such as mineral acids (e.g., H_2SO_4), organic acids (e.g., *p*-toluenesulfonic acid) and heterogeneous catalysts (e.g., ion exchange resins, zeolites) are used as esterification catalysts (Al-Arafi and Salimon, 2012; Khalkar *et al.*, 2012; Kolah *et al.*, 2007). However, these traditional acid-catalysts have many problems, such as difficulty in catalyst recovery or separation and side reactions (Ieda *et al.*, 2018).

In recent years, acidic ionic liquids have been used for the synthesis of wax ester (Kohno *et al.*, 2019; Yıldırım *et al.*, 2018). For example, Brønsted acidic ionic liquid ($[C_{16}ImSO_3H]Cl$) has been used to synthesize wax esters from stearic acid with myristyl alcohol (Yıldırım *et al.*, 2018). However, ionic liquids (ILs) also have some limitations, such as high cost and complex preparation process (Han and Armstrong, 2007). As a new generation of ionic liquid or its substitute, deep eutectic solvent (DES) has attracted attention because of its unique physicochemical properties, such as biodegradability, non-toxic and non-volatile (Zhang *et al.*, 2012). DESs are usually composed of hydrogen bond donors (HBD) (such as alcohols, carboxylic acids, or metal halides) and salts (such as choline chloride), which exhibit high purity and environmentally friendly properties (Ünlü *et al.*, 2019). DESs have been widely used in many fields, such as extraction (Hadi *et al.*, 2015), separation (Shishov *et al.*, 2017), catalysis (Sert, 2015) and CO_2 absorption (Isaifan and Amhamed, 2018). For instance, DESs have been successfully used as catalyst for the esterification of carboxylic acid with simple alcohols (Santi *et al.*, 2012; Sunitha *et al.*, 2007; Yasmin *et al.*, 2018; Williamson *et al.*, 2017; Pan *et al.*, 2016; Cao *et al.*, 2016; Tang *et al.*, 2014).

With the aim of developing low-cost and sustainable catalysts for the preparation of liquid wax esters, an efficient synthesis of oleic acid-based wax esters using choline-based DESs as catalyst under mild conditions was investigated in this work. The DES composed of choline chloride and *p*-toluenesulfonic acid could promote the synthesis of wax esters through esterification of oleic acid with various long-chain fatty alcohols (e.g., cetyl alcohol) in solvent-free conditions. Recovery of the acidic DES catalyst was examined as well.

2. MATERIALS AND METHODS

2.1. Materials

Oleic acid (80%) and cetyl alcohol (99%) were purchased from Aladdin Chemical Reagent Co., Ltd (Shanghai, China). Choline chloride (99%), zinc chloride ($ZnCl_2$, 99%), zinc bromide ($ZnBr_2$, 98%), and *p*-toluenesulfonic acid (PTSA, 98%) were purchased from Macklin Biochemical Co. Ltd (Shanghai, China). Oleic acid (99%) was purchased from Sigma-Aldrich Co. Ltd (Shanghai, China). Methanol, anhydrous sodium sulfate (Na_2SO_4) and methanesulfonic acid (MSA, 98%) with analytical purity were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China). Lauryl alcohol

(99%), myristyl alcohol (98%) and stearyl alcohol (98%) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The *n*-hexane used was of HPLC grade, while other chemicals were of analytical reagent grade.

2.2. Preparation of the deep eutectic solvents (DESs)

The DESs based on choline chloride and hydrogen bond donors (HBD) with different molar ratios were prepared by continuously stirring in an oil bath at 80 °C until a uniform and transparent liquid was formed. The compositions of the acidic DESs used in this work are shown in Table 1.

2.3. Synthesis of wax esters

1.5 mmol of oleic acid (0.42g), 1.0 mmol of cetyl alcohol (0.24g) and catalyst 10% (w/w) of cetyl alcohol (0.024g) were added into the reaction tube. Then the mixture was heated with stirring in the aluminum heating block at 60 °C for 2 h. After the reaction, the mixture was cooled to room temperature and the product was extracted with *n*-hexane. The *n*-hexane layer (wax ester layer) was washed with warm distilled water more than three times to remove the catalyst until the aqueous solution was neutral. Before the product was analyzed, *n*-hexane was removed with a rotary evaporator under reduced pressure. Finally, the product was dried and analyzed by gas chromatography (GC).

2.4. Analytical methods

2.4.1. Determination of wax esters

The contents of wax esters, fatty acids and alcohols in the reaction mixture were quantified using a GC-7890B gas chromatography (Agilent) equipped with a DB-1ht capillary column (28 m×250 μm×0.1 μm) and a flame ionizing detector (FID). The column temperature was programmed at 100 ºC, held for 0 min, increased to 180 ºC at a rate of 10 ºC·min^{-1}, maintained for 2 min; then increased to 230 ºC at 10 ºC·min^{-1}; finally elevated to 330 ºC at 20 ºC·min^{-1}, and held for 2 min. The injector and detector temperatures were set at 350 and 360 ºC, respectively.

The content of the product was quantified with hexadecane as the internal standard. The residual of cetyl alcohol was calculated by the equation:

$$M_i = f \times m_{si} \times \frac{Ai}{Asi},$$

where M_i is the mass of residue cetyl alcohol, m_{si} is the mass of the internal standard hexadecane, A_i and A_{si} are the peak area of cetyl alcohol and hexadecane, respectively, f is the response factor:

$$(f = \frac{\frac{A_{si}}{M_{si}}}{\frac{A_i}{M_i}})$$

The conversion of cetyl alcohol was calculated by equation shown below:

$$\text{Conversion of cetyl alcohol (\%)} = (1\text{-}\frac{M_i}{M_o}) * 100\%$$

Where M_o is the total mass of cetyl alcohol.

2.4.2. Characterization of purified wax esters

^{1}H NMR and ^{13}C NMR spectroscopy were used to characterize the wax esters. ^{1}H NMR spectra were recorded on Bruker NMR spectrometer (500 MHz). Chemical shifts were recorded in parts per million

Table 1. Composition of the DESs used in this work

Abbreviation	HBA	HBD	Molar ratio
DES-1	Choline chloride	Zinc chloride	1:2
DES-2	Choline chloride	Zinc bromide	1:2
DES-3	Choline chloride	*p*-toluenesulfonic acid	1:1
DES-4	Choline chloride	Methanesulfonic acid	1:2
DES-5	Choline chloride	*p*-toluenesulfonic acid	1:2
DES-6	Choline chloride	*p*-toluenesulfonic acid	1:3
DES-7	Choline chloride	*p*-toluenesulfonic acid	1:4

DES-deep eutectic solvent; HBA-hydrogen bond acceptor; HBD-hydrogen bond donors.

Figure 1. Esterification of oleic acid and cetyl alcohol. DES-deep eutectic solvent

(ppm) referenced to 0.0 ppm for tetramethylsilane (TMS). The ^{13}C NMR spectra were recorded on a Bruker NMR spectrometer (125 MHz). Chemical shifts were reported in ppm referenced to the center line of a triplet at 77.16 ppm of Chloroform-d.

2.5. Recovery of DES catalyst

For investigating the reusability of acidic DES catalyst, the reaction mixture was extracted with *n*-hexane into a separation funnel after the reaction was terminated. As obvious stratification occurred, the lower layer (DES phase) was used for the next batch of esterification experiments after drying in vacuo at 60 °C for 3h.

2.6. Statistical analysis

All the experiments were replicated three times, and the results are expressed as mean ± standard deviation (SD). The ANOVA analysis was performed at 95% confidence level ($p < 0.05$), along with Duncan for comparisons between groups using Statistical Product and Service Solutions (SPSS).

3. RESULTS AND DISCUSSION

3.1. The composition of oleic acid

The commercial oleic acid (technical grade, ~80%) used in this experiment was analyzed by the GC method after methylation (Table 2). The fatty acid composition showed that the purity of oleic acid purchased (technical grade) was 79.16%, which met the demand of the large-scale preparation of wax esters. In fact, such purity of oleic acid (technical grade) was suitable for the preparation of liquid wax esters owing to its low price.

3.2. Screening of DES catalysts

The esterification of oleic acid and cetyl alcohol to synthesize wax esters was selected as the model reaction to optimize the reaction conditions (Figure 1).

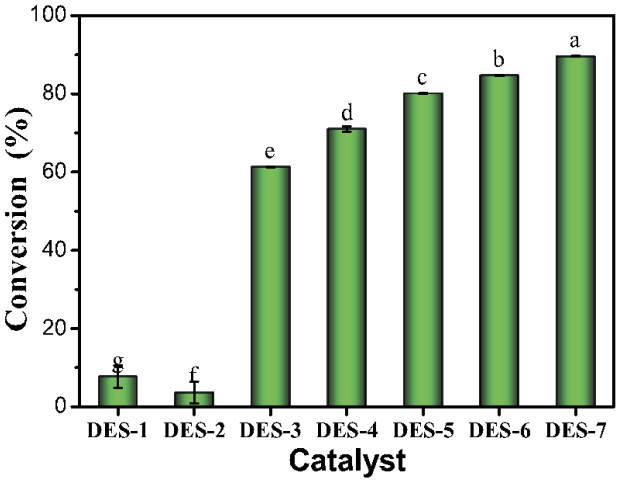

Figure 2. Effects of DES on the esterification of oleic acid and cetyl alcohol. Reaction conditions: temperature 60 °C, molar ratio of oleic acid to cetyl alcohol 1.5:1, catalyst10% (w/w) cetyl alcohol, reaction time 2h. Values are means ± SD of conversions of cetyl alcohol individually in three times. Duncan for comparisons between groups using Statistical Product and Service Solutions (SPSS). Different lower-case letters were significantly different ($p<0.05$)

Firstly, the effects of different DESs on the conversion of cetyl alcohol were studied. DESs were synthesized by combining ChCl with different HBDs (Table 1). As shown in Figure 2, seven acidic DESs (DES-1~DES-7) were used to catalyze the esterification of oleic acid and cetyl alcohol to synthesize wax ester (cetyl oleate). The catalytic efficiency of Lewis acidic DESs (DES-1: $ChCl/ZnCl_2$, DES-2: $ChCl/ZnBr_2$) was lower than that of Brønsted acidic DESs (DES-4: ChCl/MSA; DES-3, DES-5~7: ChCl/PTSA). For Brønsted acidic DES, the catalytic efficiency of PTSA-based DESs (DES-3, DES-5~7) were better than that of MSA-based DESs. Indeed, the original *p*-toluenesulfonic acid was more acidic than methanesulfonic acid. With the increase in the molar ratio of ChCl to PTSA (1:1 to 1:4), the catalytic efficiency of PTSA-based DESs was enhanced. The results showed that PTSA-based DES(DES-7) had the highest con-

version (89.6%) (Figure 2). Therefore, DES-7 (ChCl/PTSA=1:4, mol/mol) was chosen as the best catalyst for the synthesis of wax ester.

3.3. Effect of reaction temperature

It is well known that reaction temperature plays a crucial role in exothermic reactions such as esterification. Therefore, the effect of reaction temperature was studied (Figure 3a). The results showed that the occurrence of esterification reaction was found to be slow when the temperature was lower than 50 ºC, which might be because the melting point of cetyl alcohol (50 ºC) prevented the reaction process. When the reaction temperature was increased from 50 ºC to 70 ºC, the conversion of cetyl alcohol was increased significantly (97.8%). Continuing increase of the reaction temperature to 80 ºC caused the conversion of cetyl alcohol to remain unchanged. Therefore, 70 ºC was chosen as the suitable reaction temperature for the esterification reaction of oleic acid and cetyl alcohol. Comparison with the previous esterifications using Lewis acidic ionic liquid ChCl/$ZnCl_2$ or [$C_{16}ImSO_3H$]Cl as catalysts conducted at 110 ºC (Sunitha *et al.*, 2007; Yıldırım *et al.*, 2018) resulted in a more mild (70 ºC) reaction temperature.

3.4. Effect of the amount of DES catalyst

The amount of catalyst was evaluated in order to achieve the highest conversion of cetyl alcohol (Figure 3b). As the amount of DES-7 was increased (1-5%, w/w), the conversion of cetyl alcohol was increased (6.9-96.7%). However, further increase in the amount of DES-7 (10-20%, w/w) had no significant improve-

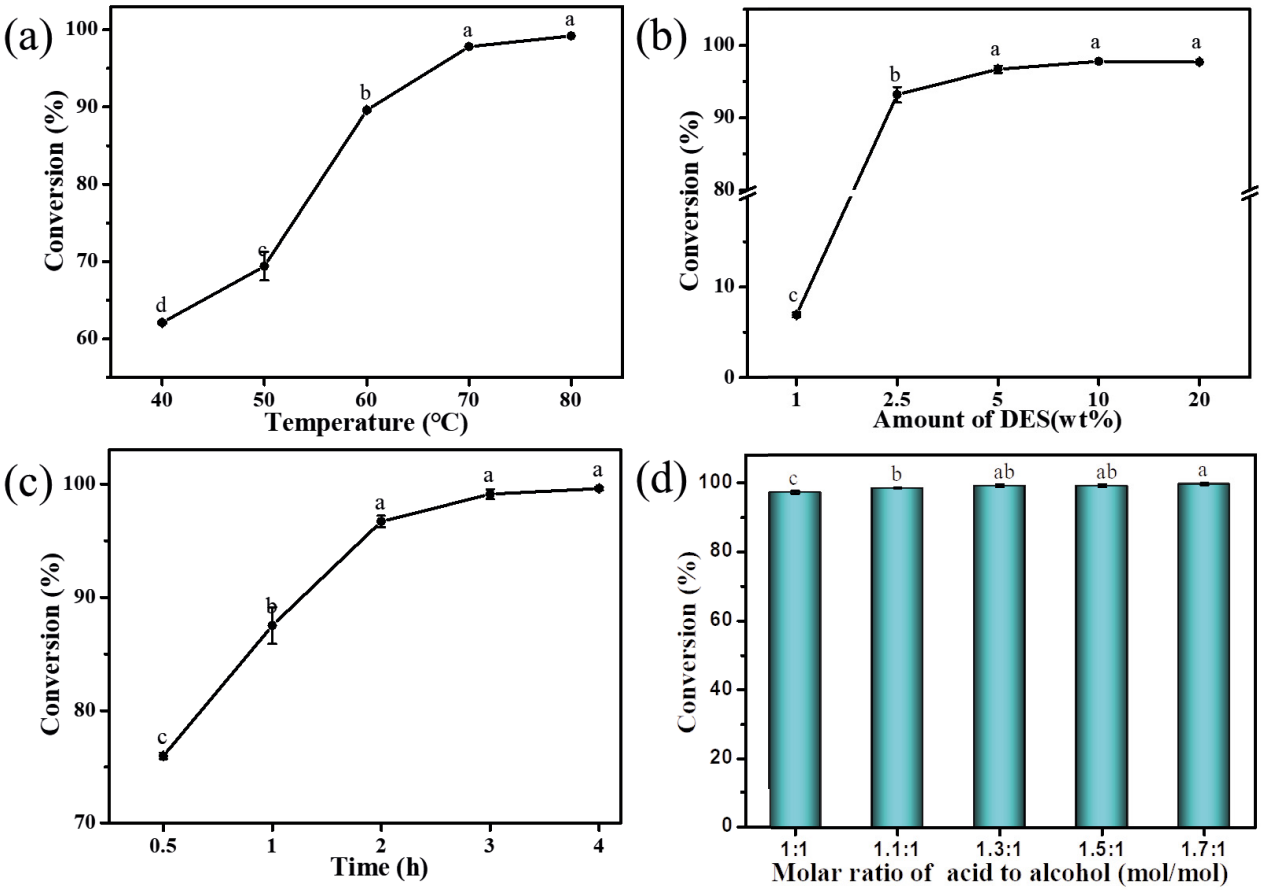

FIGURE 3. Effects of (a) reaction temperature, (b) amount of DES, (c) reaction time, (d) molar ratio of acid-to-alcohol on the conversion of cetyl alcohol. Reaction conditions: (a) 1.5:1, DES-7 concentration10% (w/w), 2h; (b) 70 ºC, 1.5:1, 2h; (c) 70 ºC, DES-7 concentration 5% (w/w), 1.5:1; (d) 70 ºC, DES-7 concentration 5% (w/w), 3h. Values are means ± SD of conversions of cetyl alcohol individually in three replicates. Duncan's test was used for comparisons between groups using Statistical Product and Service Solutions (SPSS). Different lower-case letters were significantly different ($p<0.05$)

ment on the conversion of cetyl alcohol (97.7-97.8%). Therefore, 5% DES-7 was selected as the optimal amount of catalyst for such esterification reactions.

3.5. Effect of reaction time

Reaction time is also one of the important factors for the reaction conditions. The reaction time varied from 0.5 to 4h to evaluate the suitable reaction conditions (Figure 3c). The conversion of cetyl alcohol was increased rapidly (76.0-99.1%) when the reaction time was increased from 0.5 to 3h. But further prolonging the reaction time (4h) led to no further increment in the conversion of cetyl alcohol. It was observed that the conversion of cetyl alcohol reached its maximum (99.1%) after a reaction time of 3h. Therefore, 3h was chosen as the optimal reaction time for the esterification reaction.

3.6. Effect of molar ratio of oleic acid to cetyl alcohol

As the molar ratio of substrates is also one of the important factors affecting the conversion of cetyl alcohol, the molar ratio of substrates (oleic acid/cetyl alcohol) was studied (Figure 3d). When the molar ratio of oleic acid to cetyl alcohol was 1:1, a higher conversion of 97.2% was detected. When the molar ratio of oleic acid to cetyl alcohol was increased from 1:1 to 1.3:1, the conversions of cetyl alcohol were increased from 97.2 to 99.1%. Then the conversion of cetyl alcohol exhibited no change as the molar ratio of oleic acid to cetyl alcohol increased to 1.5:1. Because the esterification of oleic acid with cetyl alcohol was an equilibrium-limited chemical reaction, the use of an excess of oleic acid would be better to promote the conversion of cetyl alcohol. Notably, an excess amount of oleic acid could not only cause a waste of starting materials, but it also affects the separation and purification process of final wax ester products. Therefore, 1.3:1 was selected as the optimal molar ratio of oleic acid to cetyl alcohol.

3.7. Catalyst recovery

The reusability of the acidic DES catalyst used in this work was evaluated and the results are listed in Figure 4. DES containing ChCl and PTSA could be reused for the esterification of oleic acid with cetyl alcohol. It was found that the conversion of cetyl alcohol decreased slightly (93.9%) after re-using the DES catalyst five times. These results are in agreement with the observations described by Taysun *et al.* (Taysun

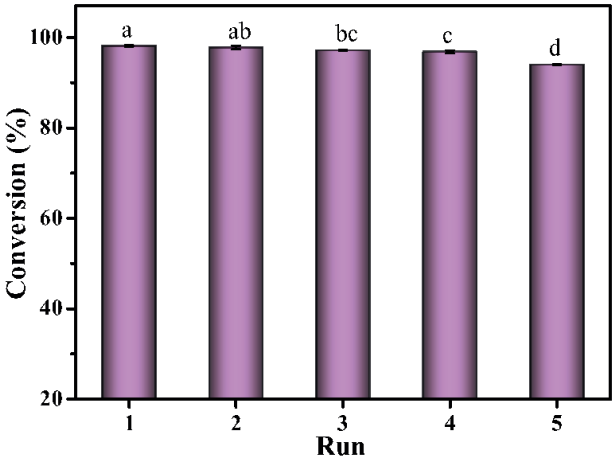

FIGURE 4. Reusability of DES-7 for the esterification of oleic acid and cetyl alcohol. Reaction conditions: temperature was 70 ºC, catalyst 5% (w/w) of cetyl alcohol, molar ratio of oleic acid to cetyl alcohol was 1.3:1, reaction time was 3h. Values are means ± SD of conversions of cetyl alcohol individually in three replicates. Duncan's test was used for comparisons between groups using Statistical Product and Service Solutions (SPSS). Different lower-case letters were significantly different ($p<0.05$)

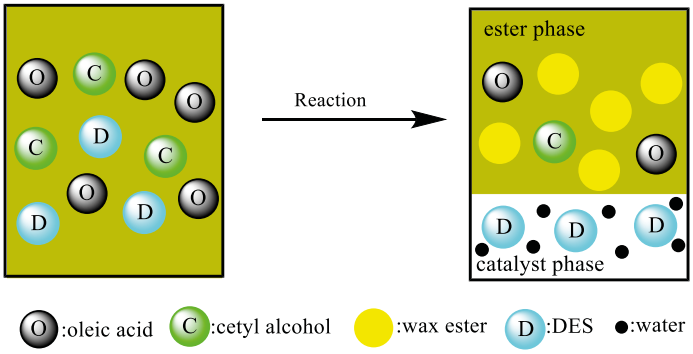

FIGURE 5. Phase separation of DES-7 after the esterification reaction

TABLE 2. Composition analysis of oleic acid (industrial purity) *

Fatty acids	Structure	Content (wt%)
Lauric acid	C12:0	1.92±0.09^{d}
Myristic acid	C14:0	0.41±0.01^{e}
Palmitic acid	C16:0	4.44±0.07^{c}
Stearic acid	C18:0	1.89±0.05^{d}
Oleic acid	C18:1	79.09±0.09^{a}
Linoleic acid	C18:2	12.24±0.06^{b}

Different lower-case letters were significantly different ($p<0.05$)
*Values are means ± SD of composition analysis of oleic acid individually in three replicates. Duncan's test was used for comparisons between groups using Statistical Product and Service Solutions (SPSS).

et al., 2017), who proved the good reusability of DES catalysts (4-8 cycles without any treatment) (Sunitha *et al.*, 2007). Indeed, the above results demonstrated that the acidic DES catalyst had good catalytic activity and stability in the reaction using for several batches of esterification reactions. Importantly, the DES phase could be separated easily from the wax ester phase through static layering after the esterification reaction (Figure 5). Therefore, the acidic DES catalyst was also an efficient recyclable catalyst for esterification reactions.

3.8. Comparison of acidic catalysts

The catalytic activity of three acidic catalysts with the same amount of PTSA was compared (Table 3). The results showed that there was no significant difference in the catalytic activity of the three catalysts (DES-7, ChCl&PTSA and PTSA). It could be observed that when PTSA was solely added as esterification catalyst, the final product's mixture presented a homogeneous system with a brown color (Figure 6c). However, with the addition of ChCl with PTSA as co-catalyst (ChCl&PTSA) (Figure 6b), the products mixture presented a two-phase system with a light brown color. This phenomenon indicated that the added ChCl might form a deep eutectic solvent with PTSA *in situ*, thus producing the phase separation effect. Comparing Figures 6a and 6b, there was little difference in the conversion of cetyl alcohol (96.4 and 97.3%). Both could cause the separation effect, but the color of the product mixture in Figure 6a was light yellow, suggesting that less PTSA dissolved in the wax ester phase by using DES-7 as catalyst (Figure 6a). Therefore, the acidic DES composed of ChCl and PTSA (1:4, mol/mol) was selected as the best recyclable catalyst for the synthesis of wax ester and exhibited excellent phase separation ability.

Table 3. Different catalysts used in the esterification*

Catalyst	Conversions of cetyl alcohol (%)
DES-7 (ChCl: PTSA=1:4) (**a**)	96.4±0.14^{b}
ChCl&PTSA (**b**)	97.3±0.28^{a}
PTSA (**c**)	97.8±0.28^{a}

Different lower-case letters were significantly different ($p<0.05$)
*Values are means ± SD of conversions of cetyl alcohol individually in three replicates. Duncan's test was used for comparisons between groups using Statistical Product and Service Solutions (SPSS). Where DES-deep eutectic solvent; ChCl-Choline chloride; PTSA-*p*-toluenesulfonic acid.

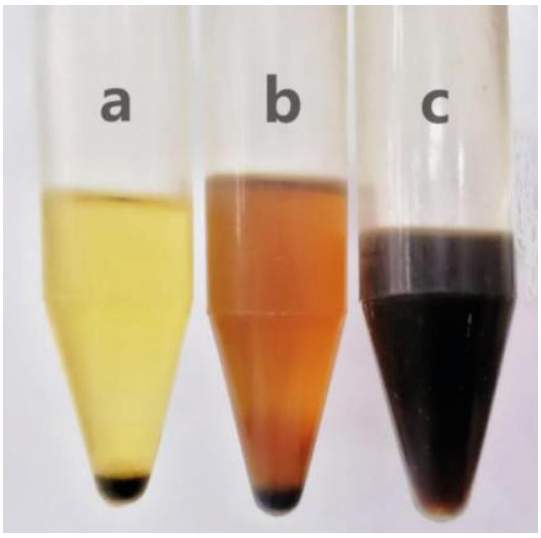

Figure 6. Phase separation of three acidic catalysts after esterification. (a): DES-7, (b): ChCl&PTSA, (c): PTSA. Reaction conditions: temperature 70 ºC, catalyst 5% (w/w) alcohol, molar ratio of oleic acid to alcohol was 1.3:1, reaction time was 3h. Values are means ± SD of conversions of cetyl alcohol individually in three replicates. Duncan's test was used for comparisons between groups using Statistical Product and Service Solutions (SPSS). Different lower-case letters were significantly different ($p<0.05$)

3.9. Analysis and separation of cetyl oleate

The gas chromatogram (GC) of the liquid wax ester (cetyl oleate) synthesized under the optimal conditions is shown in Figure 7. The unreacted fatty acid (oleic acid with purity of 99% was used here) and ce-

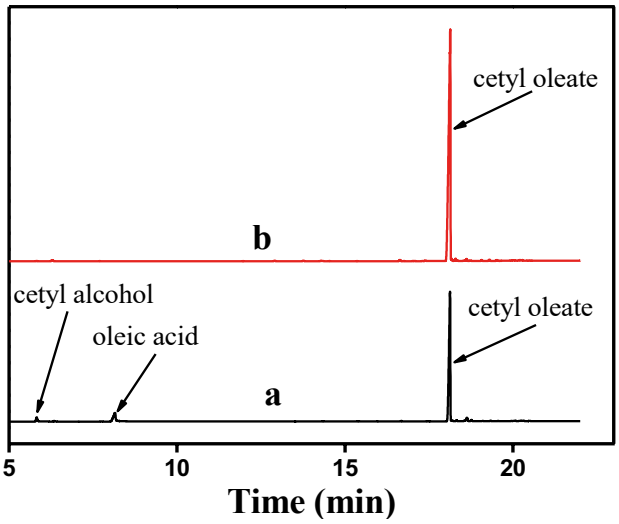

Figure 7. Gas chromatogram of cetyl oleate after esterification. (a) before purification, (b) purified with column chromatography. Purification condition: silica gel (200–300 mesh). Elution was carried using hexane/diethylether mixtures (80:20, v/v)

tyl alcohol, and the product wax ester were identified at 5.82 min, 8.14 min and 18.14 min, respectively (Figure 7a**)**, indicating that no side reaction occurred in the esterification reaction between oleic acid and cetyl alcohol catalyzed by the DES catalyst. After purified by silica gel column chromatography (Figure 7b), the cetyl oleate was obtained as a light-yellow liquid with a purity of 99%, which was identified by ^{1}HNMR and ^{13}CNMR.^{1}H NMR (500MHz, $CDCl_3$): δ=0.86 (br t, 6H), 1.26 (br d, 46H),1.62(br t, 4H), 2.00 (br t, 4H), 2.28 (br t, 2H), 4.05 (tr, J=10Hz, 2H), 5.33-5.35 (br m, 2H). ^{13}CNMR (125MHz, $CDCl_3$): δ=14.25,22.84, 25.17, 26.09, 27.32, 27.37, 28.82, 29.26, 29.29, 29.32, 29.41, 29.47, 29.51, 29.68, 29.73, 29.81, 29.84, 29.92, 32.08, 34.55, 64.54, 129.89, 130.13, 174.10.

3.10. Preparation of different oleic acid-based wax esters

Under the optimized reaction conditions, the substrate scope was examined as well (Figure 8). The esterification reactions between oleic acid (99% purity) and various long-chain fatty alcohols were conducted. To our delight, the solvent-free esterification reactions catalyzed by DES-7 could afford excellent conversions for various long-chain fatty alcohols, including lauryl alcohol, myristyl alcohol and stearyl alcohol, and the conversions reached 96.4-99.1%.

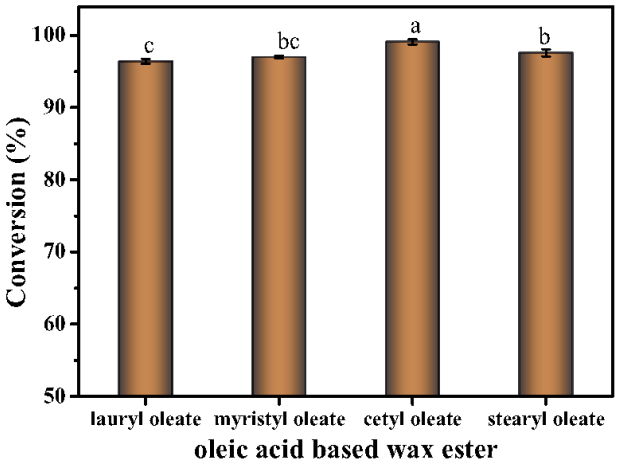

FIGURE 8. Esterification of oleic acid with long-chain alcohols. Reaction conditions: temperature 70 ºC, catalyst 5% (w/w) alcohol, molar ratio of oleic acid to alcohol was 1.3:1, reaction time was 3h. Values are means ± SD of conversions of cetyl alcohol individually in three replicates. Duncan's test was used for comparisons between groups using Statistical Product and Service Solutions (SPSS). Different lower-case letters were significantly different ($p<0.05$)

After purified by silica gel column chromatography, the liquid wax esters, including lauryl oleate, myristyl oleate and stearyl oleate were obtained with a purity of 99%. It was concluded that this acidic DES-catalyzed esterification reaction was an efficient and sustainable method for the preparation of liquid wax esters.

4. CONCLUSIONS

In this work, the acidic DES composed of ChCl and PTSA (1:4, mol/mol) could be used as an efficient and recyclable catalyst for the synthesis of oleic acid-based liquid wax esters through esterification reaction. The esterification conversion of cetyl alcohol reached 99.1% under optimal reaction conditions as follows: 5% DES as catalyst, molar ratio of fatty acid to alcohol of 1.3:1, reaction temperature of 70 ºC for 3h. The catalyst recovery experiments showed that this low-cost DES catalyst could be reused 5 times with uniform activity. Moreover, this solvent-free esterification could be used to the preparation of other oleic acid-based wax esters (lauryl oleate, myristyl oleate and stearyl oleate) and excellent conversions (> 96%) were obtained under such mild conditions.

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Physico-chemical characteristics and oxidative stability of oils from different Peruvian castor bean ecotypes

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SUMMARY: The aim of this research was to assess the physico-chemical properties and shelf-life of oils press-extracted at two temperatures (60 °C and 80 °C) from five Peruvian castor bean ecotypes. A wide variation for all traits was observed. Low acidity index, low peroxide index and absence of *p*-anisidine were recorded. The total tocopherol contents ranged from 798 to 1040 mg/kg. A higher antioxidant capacity was detected in methanolic extracts than in hexane extract. From the Rancimat performed at 150-170 °C, the predicted shelf-life at 25 °C ranged from 0.15 to 8.93 years; the higher extraction temperature led to a longer shelf-life, probably because of enzyme inactivation.

KEYWORDS: *Antioxidant capacity; Fatty acids; Rancimat;* Ricinus communis; *Tocols.*

RESUMEN: ***Características físico-químicas y estabilidad oxidativa de aceites de diferentes ecotipos de ricino peruano.*** El objetivo de esta investigación fue estudiar las propiedades fisicoquímicas y la vida útil de aceite de ricino extraido a presión a dos temperaturas (60 y 80 °C) de cinco ecotipos peruanos. Se notó una amplia variación para todas las características. Se observaron bajos índice de acidez, bajo índice de peróxido y ausencia de *p*-anisidina. El contenido total de tocoferoles osciló entre 798 y 1040 mg/kg. Se detectó una mayor capacidad antioxidante en los extractos en metanol que en los extractos en hexano. A partir del Rancimat realizado a 150-170 °C, la vida útil prevista a 25 °C osciló entre 0.15 y 8.93 años; la mayor temperatura de extracción condujo a una vida útil más larga, probablemente debido a inactivación de las enzimas.

PALABRAS CLAVE: *Ácidos grasos; Capacidad antioxidante; Rancimat;* Ricinus communis; *Tocoles.*

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1. INTRODUCTION

The castor bean (*Ricinus communis* L.), a perennial flowering plant from the Euphorbiaceae family of probable African origin, is currently firmly rooted in all the warm areas of the world. Castor beans present a wide variation in many characteristics, including size and precocity of the plant, color of stems, leaves and petioles, dehiscent or indehiscent fruit, as well as color, shape, size and chemical composition of the seeds (Wang *et al*., 2010). The seeds, highly toxic for the presence of an easily-extractable protein, ricin, which is a deadly natural poison (Patel *et al*., 2016), are rich in a prized viscous oil of relevant interest for the industry, utilized in the manufacturing of paints, plastics, lubricants, aeronautical fluids, biodiesel, cosmetics and pharmaceutical products (Mutlu and Meier, 2010). The unique characteristics of castor bean oil are a consequence of its high content in mono-unsaturated ricinoleic acid (12-hydroxy-9-octadecenoic acid), whose molecule has three functional reactive groups: a carboxyl group (COOH), an unsaturation point in carbon nine and a hydroxyl group in carbon 12 (Mutlu and Meier, 2010). The carboxyl group consents different esterification reactions; the single unsaturation point may be altered by epoxylation, hydrogenation or vulcanization while the hydroxyl group can be acetylated, alcoxylated or removed by dehydration, increasing the unsaturation and generating a semi-dry acid. The presence of the hydroxyl group in carbon 12 promotes a high and stable viscosity index, and great lubricity (Scholz and da Silva, 2008).

In recent years, concerns over global climate change and knowledge of the limited fossil oil reserves has stimulated the search for alternative and renewable sources which are apt for the industrial production of bio-based polymers, such as castor oil. Because of its great economic value and ever-increasing demand, the castor bean is widely cultivated in tropical, sub-tropical and temperate countries; its diffusion is favored by its short generation time, good drought and salt tolerance, and adaptation to marginal soils (Timko *et al*., 2014). India (1,198,000 Mg) was by far the biggest producer in 2018, followed by Mozambique (85,436 Mg), China (27,000 Mg), Brazil (14,224 Mg), Myanmar (12,068 Mg) and Ethiopia (10,930 Mg) (www.fao.org/faostat, last accessed 26/03/2020). An evaluation of the worldwide castor bean germplasm has evidenced a relatively low genetic variation (Allan *et al*., 2008), but only scattered information is available about differences among accessions for morpho-agronomic traits and oil composition (e.g. Akande *et al.*, 2012; Armendáriz *et al*., 2015; da Silva Ramos *et al*., 1984; Lavanya *et al*., 2012;).

Ricinus communis is not cropped in Peru, nor has the recent importation of varieties been recorded, except for some adaptation trials in the hot and humid lowlands of the Amazon (Villalobos *et al*., 2008). Castor bean plants, regarded as unwanted weeds, are often eradicated by farmers. Hence, local ecotypes are likely the survivors of old, perhaps unintentional, introduction episodes and have adapted to specific growing environments.

Castor oil is obtained from castor beans by mechanical pressing, solvent extraction or their combination (Mutlu and Meier, 2010; Perdomo *et al*., 2013). A solvent extraction under thermo-sonication at 70 and 60 °C was proposed by López-Ordaz *et al*. (2019) and Palconite *et al*. (2018), respectively. The mechanical process has lower costs and is more environmentally-friendly but gives inferior yields to the solvent extraction method (Mutlu and Meyer, 2010). Cold pressing is sometimes performed (Ananth *et al*., 2019), but heating to around 60 °C is often applied (Perdomo *et al*., 2013) to harden the interior of the beans and to improve the extraction efficiency (Patel *et al*., 2016). Nevertheless, specific studies investigating the best thermal conditions are still lacking.

The aim of the research was to study the differences among ecotypes in physico-chemical characteristics and oxidative stability of press-extracted oil from five Peruvian castor beans.

2. MATERIALS AND METHODS

2.1. Castor bean seeds

Five castor bean seed samples (5 kg each) were collected from four different eco-geographical regions in the Central-North part of Peru, i.e. Huarmey (coast, 14 m a.s.l.), Carhuaz (highlands, 2688 m a.s.l.), Casma (coast, 207 m a.s.l.) and La Carbonera (coast, 140 m a.s.l.). The seeds were dried to 3-5% humidity. Their dimensions (length,

width and thickness) were measured with a caliper on 200 seeds per accession, while their weight was assessed with a 220R lab balance (Precisa Gravimetrics AG, Dietikon, Switzerland) on three 1000-seed batches per accession. Moisture, ash, lipids and protein contents were determined according to methods 934.06, 942.05, 922.06 and 953.01 (AOAC International, 2019), respectively; total carbohydrate content was computed by difference, by subtracting the measured protein, fat, ash, fiber and water from the total weight. The analyses were repeated twice.

2.2. Oil extraction

Two different subsamples of each ecotype were heated at 60 or 80 °C for 20 and 30 min, respectively in an UN55 oven (Memmert, Schwabach, Germany). The oil was extracted using a PH1020 hydraulic press (Neo & Neo Next, China) under a 20-t pressure, centrifugated at 5000 rpm for 30 min with a Sigma 2-16P centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) to remove any impurities, and stored at 4 °C in dark glass containers until analysis.

2.3. Oil characteristics

Oil density was measured in accordance to the Codex Alimentarius (1999) and oil viscosity was assessed following method D2983 (ASTM International; www.astm.org). The free fatty acids (FFA, % oleic acid) were determined according to the official method Ca 5a-40 (AOCS, 1998). The refractive index was measured according to method 921.08 (AOAC International, 2019) working at 25 °C and using a digital A 24051 refractometer (Rudolph Research Analytical, NJ, USA) kept at 20 °C. The acidity index was determined by direct titration as described in method Cd 3d-63 (AOCS, 1998); the peroxide index was measured according to method 965.33 (AOAC International, 2019); the iodine index was determined by the Wijs method, in accordance with method 993.20 (AOAC International, 2019); the *p*-anisidine value was monitored by method Cd 18-90 (AOCS, 1998).

Oil color was determined by the CIELAB method using a Chroma Meter II tristimulus colorimeter (Minolta Italia SpA, Milan, Italy), using a standard white reflector plate and the illuminating C; three parameters were assessed: *L** (luminosity), *a** (red-green), *b** (yellow-blue).

The fatty acid composition was assessed only on the 60 °C-extracted samples by gas-chromatography following the method described by Simonetti *et al*. (2002). Tocopherol content and composition was evaluated by normal phase HPLC as outlined by Rodríguez *et al.* (2021). To measure the antioxidant capacity, the 2-2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical cation scavenging capacity tests were performed on hydrophilic (HF) and lipophilic (LF) fractions (Varas Condori *et al*., 2020) as follows: exactly 2 g of oil and 2 mL of n-hexane were vortexed together until complete dissolution. Subsequently, 2 mL of 80:20 methanol-water were added and vortexed. The mixture was centrifuged at 9870 *g* for 10 min. Finally, the methanolic phase (located in the lower part of the centrifuge tube) and the n-hexane phase (found in the upper part of the tube) were separated, and their volumes measured. ABTS and DPPH tests were performed as described by Yilmaz *et al*. (2015). The results are reported as mmol Trolox equivalent (TE)/kg oil.

The oxidation stability index (OSI; h) was evaluated using a Rancimat equipment (743 Rancimat Metrohm Co., Switzerland) following Official Method Cd 12b-92 (AOCS, 1998) at 150, 160 and 170 °C with an air flow of 20 L/h; the shelf-life (years) at 20, 25 and 20 °C was extrapolated from the OSI results.

All the chemical analyses were performed twice, while color and oxidative stability thrice, on independent samples.

2.4. Statistical analysis

The analysis of variance (ANOVA) was performed considering castor oil ecotypes (E) and extraction temperature (T) as main factors. Within each extraction temperature, a one-way ANOVA was also carried out and when significant differences were observed the Fisher`s Least Significant Difference test (LSD) at $p < 0.05$ was performed. Means and standard errors were computed using Excel 2013 (Microsoft, Redmond, WA, USA), while the ANOVAs and LSD analyses were computed with the software Statgraphics® Centurion XVI (Statpoint Technologies Inc., Warrenton VA, USA).

3. RESULTS AND DISCUSSION

3.1. Seed characteristics

Figure 1 shows the beans of the five ecotypes (Huarmey grande, Huarmey chico, Carhuaz, Casma and La Carbonera); the dimension parameters length, width, thickness and 1000-kernel weight are summarized in Table 1. The ANOVA (not shown) evidenced significant differences ($p < 0.05$) among the samples for all four traits. The Huarmey grande ecotype had the biggest seeds, followed by La Carbonera, and Casma; Huarmey chico and Carhuaz had the smallest seeds, with comparable size and weight. With the exception of Huarmey grande, the dimensions were within the ranges (length: 0.865-1.710 cm, width: 0.546-1.000 cm, thickness: 0.300-709 cm, 1000 seeds weight: 101.0-735.7 g) reported by Perdomo *et al*. (2013), Velasco *et al*. (2015) and Wang *et al*. (2010). Additionally, the two Huarmey ecotypes differed from the others in the color of the teguments, i.e. black vs. brown-grey with black lines.

The chemical composition of the seeds is reported in Table 1. The ANOVA (not shown) highlighted the existence of significant differences ($p < 0.05$) for

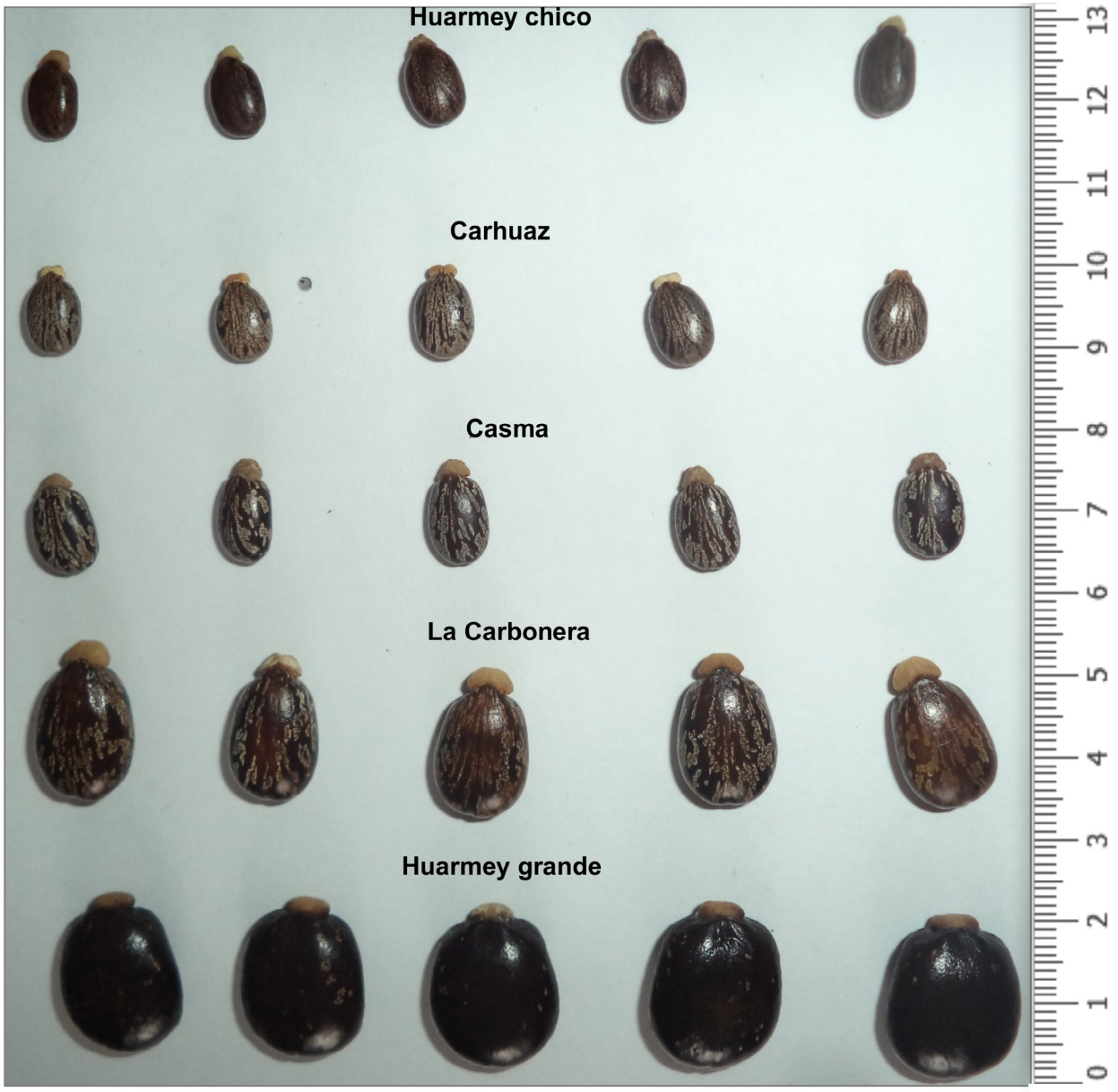

Figure 1. Castor beans of the five Peruvian ecotypes analyzed.

Table 1. Morphological characteristics and chemical composition (mean±standard error) of the seeds of five Peruvian *Ricinus communis* ecotypes. Length, width and thickness were measured on 200 seeds, 100-kernel weight on three 1000-kernel samples, moisture, ash, lipids, proteins and fiber on two independent samples for each ecotype. Carbohydrate content was computed by difference, subtracting the measured protein, fat, ash, fiber and water from the total weight.

	Huarmey grande	Huarmey chico	Casma	Carhuaz	La Carbonera
Length (cm)	1.77±0.05^{a}	0.95±0.03^{d}	1.17±0.04^{c}	0.94±0.03^{d}	1.61±0.05^{b}
Width (cm)	1.43±0.05^{a}	0.59±0.02^{d}	0.66±0.03^{c}	0.60±0.02^{d}	0.99±0.04^{b}
Thickness (cm)	0.78±0.02^{a}	0.44±0.01^{d}	0.50±0.02^{c}	0.44±0.02^{d}	0.67±0.02^{b}
1000-kernel weight (g)	877.91±3.11^{a}	122.58±0.14^{d}	184.06±1.21^{c}	122.62±0.84^{d}	464.42±3.24^{b}
Moisture (g/100 g)	3.26±0.03^{c}	3.82±0.06^{a}	3.33±0.07^{c}	3.14±0.07^{d}	3.55±0.04^{b}
Ash (g/100 g DM)	3.52±0.11^{c}	4.09±0.10^{a}	2.69±0.07^{e}	2.92±0.05^{d}	3.87±0.09^{b}
Lipids (g/100 g DM)	60.54±0.36^{a}	53.99±0.06^{d}	57.79±0.07^{c}	58.85±0.60^{b}	57.66±0.47^{c}
Proteins (g/100 g DM)	15.22±0.56^{d}	19.95±0.14^{a}	19.42±0.27^{a}	18.62±0.41^{b}	16.83±0.14^{c}
Fiber (g/100 g DM)	10.53±0.04^{c}	14.68±0.08^{a}	14.38±0.03^{b}	14.73±0.07^{a}	10.33±0.06^{d}
Carbohydrates (g/100 g DM)	10.19±0.24^{b}	7.29±0.07^{c}	5.72±0.33^{d}	4.89±0.21^{e}	11.31±0.25^{a}

Different letters in a row indicate significant differences ($p \leq 0.05$) among ecotypes according to the LSD test.

moisture, ash, lipids, protein, fiber and total carbohydrate contents. The seed moisture, always low, ranged from 3.1 to 3.8 g/100 g. The ash content was highest in Huarmey chico and lowest in Casma. Similar values (2.24 to 3.41 g/100 g DM) were reported by Vasco Leal *et al*. (2017) for 12 castor bean accessions from Mexico, which are largely inferior to the value (6.44 g/100 g) described for one Pakistani variety (Panhwar *et al*., 2016).

Huarmey chico showed the lowest lipid content, while Huarmey grande had the highest; the other three ecotypes were similar (on average, 58.1 g/100 g DM). The values are within the ranges (39.6-59.5, 34.6-56.6, 12.2-64.8, 37.2-60.6 g/100 g DM) reported, respectively, by da Silva Ramos *et al.* (1984) for 36 varieties, by Severino *et al.* (2015) for 40 breeding lines and commercial genotypes, by Goytia Jiménez *et al*. (2011) for 151 accessions collected in the state of Chiapas, Mexico, and by Wang *et al*. (2010) for the USDA world collection. However, these results exceed those reported by Vasco Leal *et al*. (2017) (41.5-51.0 g/100 g DM), Armendáris *et al*. (2015; 42.0-48.5 DM), and are partially higher than the values (44.6-54.8 g/100 g) reported by Velasco *et al*. (2015) in 121 accessions from southern Spain. Different extraction methods influence lipid recovery; for example, values ranging from 57.0 (Soxhlet) to 48.0-61.1% (thermosonication) were observed by López-Ordaz *et al*. (2019).

Protein concentration was maximum in Huarmey chico and Casma and minimum in Huarmey grande, all results comparable to those (12.61-16.02 g/100 g DM) described by Vasco Leal *et al*. (2017). Nevertheless, Perea-Flores *et al*. (2011) found a higher protein content (28.48 g/100 g DM) in the Mexican variety Tiripiteo.

The fiber content was low (10.3 and 10.5 g/100 g DM) in the big-seeded ecotypes La Carbonera and Huarmey grande, and significantly higher (14.4-14.7 g/100 g DM) in the other three castor oil accessions. Conversely, the carbohydrate concentration was superior in the former two ecotypes (11.3 and 10.2 g/100 g DM, respectively), and lower in the latter three (4.9-7.3 g/100 g DM).

The composition of castor oil seeds may be influenced not only by genetic causes (e.g. seed size, Severino *et al*., 2015; Velasco *et al*., 2015), but also by environmental factors (Ramanjaneyulu *et al*., 2013). The climatic conditions in Huarmey, Casma and La Carbonera are generally warm, sunny, with little rain; while Carhuaz has a cold climate with seasonal rains.

3.2. Oil characteristics

3.2.1 Color

The ANOVA (not presented) carried out on the color parameters *L**, *a**, *b** evidenced significant effects for ecotype and for ecotype x temperature

Table 2. Color coordinates *L**: luminosity, *a**: red-green and *b**: yellow-blue (mean±standard error; n=3) of castor oil extracted at two temperatures (60 °C and 80 °C) from five Peruvian ecotypes.

	°C	Huarmey grande	Huarmey chico	Casma	Carhuaz	La Carbonera
*L**	60	32.4±0.8^{a}	33.7±0.1^{a}	32.9±0.1^{a}	32.8±0.9^{a}	28.3±0.1^{b}
	80	35.8±0.4^{a}	32.3±0.1^{c}	33.0±0.2^{b}	28.9±0.1^{e}	30.5±0.2^{d}
*a**	60	1.4±0.2^{b}	1.1±0.1^{b}	1.5±0.1^{b}	1.1±0.3^{b}	2.5±0.1^{a}
	80	0.4±0.0^{c}	1.4±0.0^{b}	1.3±0.2^{b}	2.2±0.1^{a}	2.2±0.1^{a}
*b**	60	5.1±0.6^{b}	6.6±0.2^{a}	6.8±0.3^{a}	5.9±0.7^{ab}	2.4±0.3^{c}
	80	7.7±0.4^{a}	6.0±0.2^{c}	6.6±0.3^{b}	2.9±0.1^{e}	4.6±0.3^{d}

Different letters in a row indicate significant differences ($p \leq 0.05$) among ecotypes according to the LSD test.

interaction, but not for oil extraction temperature *per se*. Because the interaction was relevant, Table 2 reports the results separately for 60 and 80 °C. At 60 °C the luminosity was similar except for La Carbonera, which was slightly darker; while at 80 °C Huarmey grande was more luminous than the others, and La Carbonera was still the darkest. The other two parameters showed that *a** was slightly higher (i.e. redder) for La Carbonera oil, while *b** was greater (i.e. yellower) for Huarmey chico, Casma and Carhuaz (at 60 °C) and for Huarmey grande (at 80 °C). The low luminosity of the samples is partially a consequence of the extraction method utilized. Mechanical pressing tends to drag different components present in the seeds, such as gums: these impurities hinder the penetration of the light, thus reducing the luminosity. In fact, solvent-extracted oil is markedly clearer, as noticed by Conceição *et al*. (2007) and by Palconite *et al*. (2018).

3.2.2. Physicochemical parameters

Figure 2 presents the physicochemical characteristics of the castor oil extracted at two different temperatures (60 and 80 °C). The ANOVAs (not presented) showed significant effects due to the ecotype as well as the extraction temperature; the ecotype always had the largest effect, except for oil density when the temperature was preeminent. The interaction between the two factors was also significant but generally of minor relevance except for the peroxide and iodine indices.

Compared to 60 °C, the 80 °C treatment led to better oil yield, superior oil density and kinematic viscosity, slightly higher refraction index, and inferior acidity index and free fatty acid content. For the peroxide and iodine indices, the interaction between temperature and ecotype was more relevant than the temperature *per se*, as each genotype behaved differently. The *p*-anisidine was always undetectable.

Heating influences the process of rupture of lipid bodies, thus favoring oil extraction; the better yields at the superior temperature were probably linked to more drastic shattering of lipid bodies and superior oil fluidity. Interestingly, the yield was significantly correlated to the weight of the seeds ($r = 0.78$ and $r = 0.83$ at 60 and 80 °C, respectively). At 60 °C the oil yield varied from 33.4 (Huarmey chico) to 39.0% (Huarmey grande), and at 80 °C from 33.5 to 42.0% (same ecotypes). These values match those reported by Perdomo *et al*. (2013), who obtained a yield of 36.6% using a mechanical press at 60 °C on a Mexican variety. Similarly, our results are within the variation (31.99-48.39%) summarized in their review by Ahmad *et al*. (2020). In general, yield seems to be influenced by the size of the seed (Huarmey grande seeds were exceptionally large).

At 60 °C, the oil density of Carhuaz and La Carbonera was the highest (0.949 and 0.949 g/cm^3), while Casma had the lowest (0.947 g/cm^3); at 80 °C Carhuaz and Huarmey grande showed superior results (0.949 and 0.949 g/cm^3). The differences between the results of the two extraction temperatures may be related to the fact that higher temperatures favor the migration of compounds such as gums, proteins, fiber, carbohydrates, etc. into the oil, thus increasing its density (Vasco Leal *et al*., 2017). Our results are within the variation (0.946-0.958 g/cm^3) described by Conceição *et al*. (2007), Panhwar *et al*. (2016) and Vasco Leal *et al*. (2017) for Brazilian, Pakistani and Mexican accessions.

At 60 °C the kinematic viscosity of Huarmey grande was the highest (227.1 mm^2/s), followed by

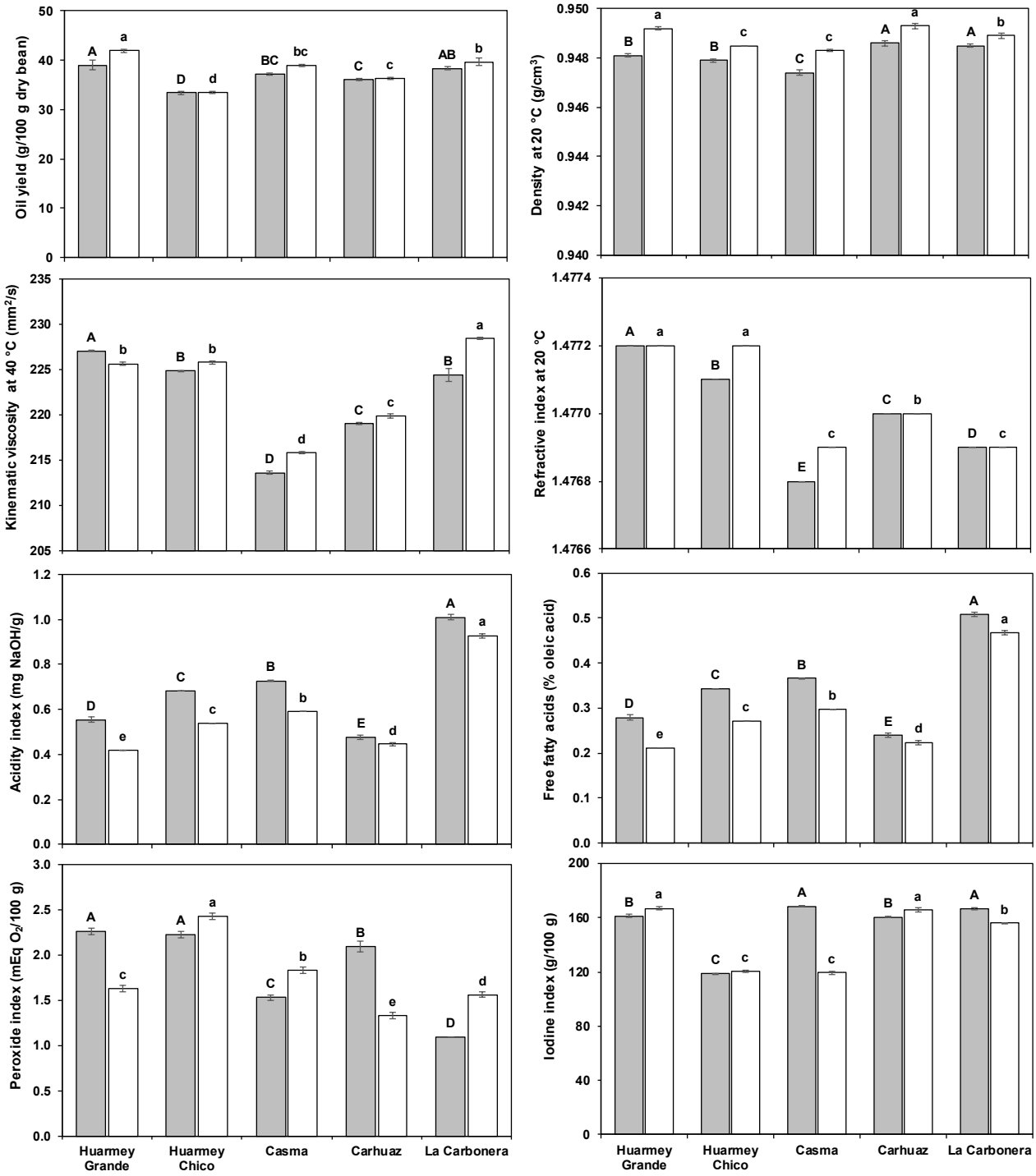

Figure 2. Physico-chemical characteristics of the oil obtained by pressing extraction at 60 °C (grey bars) and 80 °C (white bars). Error bars represent the standard error (n=2). Different letters indicate significant differences ($p \leq 0.05$) among ecotypes for each extraction temperature (capital letters: 60 °C; small letters: 80 °C) according to the LSD test.

those of Huarmey chico and La Carbonera, while Casma presented a relatively low value (213.6 mm^2/s, respectively). At 80 °C La Carbonera showed the maximum value (228.4 mm^2/s) and Casma, again, the minimum (215.8 mm^2/s). These results are marginally lower than those (239.39 mm^2/s) reported by Conceição *et al*. (2007) but are far inferior to those (250.04 to 265.84 mm^2/s) by Vasco Leal *et al*. (2017), although these last researchers used different analytical equipment.

The refraction index at 60 °C was greatest in La Carbonera (1.477), and at 80 °C in Huarmey grande and Huarmey chico (1.477 in both samples) but the differences among ecotypes were generally minimal. A similar value (1.479) was reported by Canoira *et al*. (2010); while an inferior result (1.431) was observed by Panhwar *et al*. (2016).

Concerning the acidity index and the free fatty acid content, only La Carbonera evidenced high values, while all the other ecotypes showed 0.420-0.727 mg NaOH/g and 0.211-0.366 g/100 g, respectively; the minimum values were those of Carhuaz at 60 °C and of Huarmey grande at 80 °C. In general, lower values were observed at 80 °C extraction. Perdomo *et al*. (2013) stated that the temperature affects the free fatty acid content and thus the acidity index. In fact, all seed oils have lipolytic enzymes which release free fatty acids. However, if the enzymes are inactivated by high temperatures, hydrolysis is limited, free fatty acids are scarce and acidity is low. The results of this research are comparable to those (0.542-1.218 mg KOH/g) reported by Vasco Leal *et al*. (2017) and to those (0.823 to 1.390 mg KOH/g) determined by Perdomo *et al*. (2013). Therefore, the acidity indices of the five Peruvian ecotypes are ideal and in general the oil has a low content in free fatty acids. Poor post-harvest handling of the seeds and poor storage of the oil often affect this quality indicator, hence low acidity values are achieved when the raw material are damaged or exposed to extreme conditions (high temperatures, humid environments, etc.).

The lowest peroxide index scores were reached by La Carbonera and Casma at 60 °C, and by Carhuaz and La Carbonera at 80 °C. A similar situation was evident also for the iodine index: Casma and La Carbonera had the lowest values at 80 °C while the two Huarmey and Carhuaz had the smallest at 60 °C. The peroxide values were lower than those (4.63 to 4.90 meq O_2/kg oil) reported by López-Ordaz *et al*. (2019) but similar to the one (2.25 meq O_2/kg) described by Panhwar *et al*. (2016), while the iodine values (Figure 2) were higher than those scored by López-Ordaz *et al*. (2019), Torrentes-Espinoza *et al*. (2017), and Panhwar *et al*. (2016), i.e. 84.9-85.9, 91.0 and 83.6 g I_2/100 g lipid, respectively, probably because of different ecotypes and origins of the seeds as well as different extraction methods

3.2.3. Fatty acids

The castor oil contained mainly ricinoleic acid (on average, 86.8%), followed by linoleic acid (4.2%), oleic acid (3.1%) and other minor fatty acids (Table 3). The one-way ANOVA (not presented) did not show significant differences for ricinoleic or linoleic acids among samples; the variation of the other fatty acids, even if significant, was very limited.

Table 3. Fatty acids composition (%; mean±standard error; n=2) of castor oil extracted at 60 °C from five Peruvian ecotypes.

Fatty acid	Huarmey grande	Huarmey chico	Casma	Carhuaz	La Carbonera
C16:0	0.83 ± 0.02^{c}	1.22 ± 0.04^{b}	1.58 ± 0.01^{a}	0.89 ± 0.02^{c}	1.17 ± 0.02^{b}
C18:0	0.69 ± 0.05^{b}	0.97 ± 0.03^{a}	1.07 ± 0.03^{a}	1.07 ± 0.05^{a}	1.03 ± 0.11^{a}
C18:1n9	$3.04 \pm 0.19b^{c}$	2.54 ± 0.05^{c}	3.54 ± 0.13^{a}	3.19 ± 0.20^{ab}	3.07 ± 0.03^{ab}
C18:1n7	0.43 ± 0.03^{c}	0.46 ± 0.01^{bc}	0.55 ± 0.03^{ab}	0.42 ± 0.02^{c}	0.60 ± 0.04^{a}
C18:2n6	4.21 ± 0.19	4.29 ± 0.18	4.72 ± 0.18	3.84 ± 0.09	4.17 ± 0.23
C18:3n3	0.54 ± 0.02^{a}	0.52 ± 0.01^{ab}	0.48 ± 0.02^{bc}	0.50 ± 0.01^{ab}	0.44 ± 0.01^{c}
C20:1n9	0.29 ± 0.01^{a}	0.23 ± 0.01^{b}	0.30 ± 0.00^{a}	0.32 ± 0.02^{a}	0.18 ± 0.02^{b}
C18:1OH	87.09 ± 0.54	87.31 ± 0.52	85.68 ± 0.49	86.92 ± 0.49	86.89 ± 0.52
Others	2.88 ± 0.08^{a}	2.47 ± 0.19^{ab}	2.06 ± 0.09^{b}	2.85 ± 0.09^{a}	2.45 ± 0.12^{ab}

Different letters in a row indicate significant differences ($p \leq 0.05$) among ecotypes according to the LSD test.

Overall, the oil of our ecotypes was constituted by about 91% mono-unsaturated fatty acids (MUFA), 7% poly-unsaturated fatty acids and 2% saturated fatty acids. Similar percentages for ricinoleic acid (from 75.0 to 90.0%), linoleic acid (4.1-9.7%) and oleic acid (3.0%-7.7%) are reported in the literature (Ahmad *et al*., 2020; da Silva Ramos *et al.,* 1984; Harhar *et al*., 2016; Torrentes-Espinoza *et al*., 2017; Velasco *et al*., 2015; Wang *et al*., 2011).

3.2.4. Tocopherols

The ANOVA (not shown) demonstrated the existence of significant differences merely among ecotypes. No differences were observed between the two extraction temperatures, suggesting good thermal stability of the compounds. Only tocopherols were detected (Table 4); the most abundant homologue was δ-tocopherol (on average, 520.2 mg/kg), followed by γ-tocopherol (366.8 mg/kg), α-tocopherol (9.5 mg/kg) and β-tocopherol (7.5 mg/kg), for an average total tocopherol content of 904.1 mg/kg. Interestingly, and unlike the other ecotypes, Huarmey grande had more γ-tocopherol than δ-tocopherol (506.8 vs. 448.7 mg/kg). The Huarmey chico samples showed the highest total tocopherol content (1038.4 mg/kg) and Carhuaz the lowest (780.1 mg/kg). Very little information is available in the literature on the presence of tocopherols in castor oil. Total tocopherol content of all ecotypes was higher than the values reported by Ananth *et al*. (2019), i.e. 461.3 mg/kg, and by Harhar *et al*. (2016), i.e. 183 mg/kg but lower than the levels recorded by Velasco *et al*. (2005) in a natural high-oleic acid mutant (2617 mg/kg) and in a standard castor oil line (1345 mg/kg). Velasco *et al*. (2015) observed a very large variation (99.6-282.2 mg/kg) for total tocopherol content in the seeds of 121 accessions from southern Spain.

On average, α- and β-tocopherol showed a minimal percentage (1.1 and 0.8%, respectively) of total tocopherols, while γ-tocopherol represented 40.2%

Table 4. Mean (±standard error; n=2) tocopherol content (mg/kg DM) and antioxidant capacity (ABTS and DPPH methods; mmol TE/kg oil) of the hydrophilic (methanol 80%) and lipophilic (hexane) fractions recovered from castor oil extracted at two temperatures (60 °C and 80 °C) from five Peruvian ecotypes.

	°C	Huarmey grande	Huarmey chico	Casma	Carhuaz	La Carbonera
α-tocopherol	60	13.1±0.3^{a}	9.8±0.5^{b}	9.8±0.5^{b}	8.2 ±0.8^{bc}	6.2±0.7^{c}
	80	13.0±1.2^{a}	9.6±0.1^{b}	9.5±0.7^{b}	8.5±0.5^{b}	7.1±1.0^{b}
β-tocopherol	60	10.9±0.2^{a}	7.9±0.5^{b}	6.7±0.3^{b}	5.7±0.3^{b}	7.6±1.1^{b}
	80	11.6±1.0^{a}	6.5±0.3^{bc}	6.6±0.4^{b}	5.3±0.3^{c}	7.4±0.4^{b}
γ-tocopherol	60	506.8±4.7^{a}	390.0±0.3^{b}	382.8±11.6^{b}	251.5±6.2^{d}	308.7±0.1^{c}
	80	500.3±4.7^{a}	389.9±2.4^{b}	379.4±9.5^{b}	253.1±3.6^{d}	305.6±23.0^{c}
δ-tocopherol	60	448.7±2.5^{c}	632.6±6.8^{a}	523.1±1.3^{b}	513.0±1.2^{b}	475.5±18.7^{c}
	80	457.7±6.4^{d}	631.5±12.9^{a}	527.8±1.7^{b}	514.8±1.8^{bc}	477.8±23.0^{c}
Total tocopherols	60	979.5±7.3^{b}	1040.3±8.1^{a}	922.5±12.1^{c}	778.5±8.5^{d}	797.9±19.0^{d}
	80	982.6±13.3^{b}	1037.5±15.1^{a}	923.4±10.9^{c}	781.7±6.1^{d}	797.8±8.6^{d}
$ABTS_{MeOH\ 80\%}$	60	34.0±1.9^{c}	39.6±1.2^{ab}	33.3±1.4^{c}	41.1±0.7^{a}	35.4±1.1^{bc}
	80	38.9±1.4^{a}	32.7±0.9^{c}	33.9±0.6^{bc}	31.4±0.6^{c}	36.9±0.7^{ab}
$ABTS_{Hexane}$	60	9.3±0.7^{a}	9.8±0.2^{a}	8.6±0.1^{ab}	6.9±0.4^{b}	7.6±0.6^{b}
	80	10.3±0.4^{a}	9.4±0.1^{b}	9.1±0.1^{b}	7.5±0.1^{c}	7.8±0.1^{c}
$DPPH_{MeOH\ 80\%}$	60	30.1±0.1^{a}	26.2±0.3^{b}	26.7±0.3^{b}	21.7±0.4^{d}	24.1±0.1^{c}
	80	26.4±1.1^{a}	27.1±0.0^{a}	26.6±0.2^{a}	22.7±0.4^{b}	23.3±0.1^{b}
$DPPH_{Hexane}$	60	5.4±0.4^{a}	5.1±0.2^{a}	3.8±0.1^{b}	1.7±0.2^{c}	5.9±0.3^{a}
	80	5.3±0.2^{c}	7.1±0.4^{a}	5.8±0.2^{bc}	2.8±0.3^{d}	6.3±0.0^{ab}

Different letters in a row indicate significant differences ($p \leq 0.05$) among ecotypes according to the LSD test.
ABTS: 2-2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical cation: MeOH 80%: 80:20 methanol-water extraction solvent; Hexane: n-hexane extraction solvent.

and δ-tocopherol 59.7%. Similarly, Velasco *et al.* (2005) and Velasco *et al.* (2015) identified γ-tocopherol and δ-tocopherol as the two most relevant homologues. Nevertheless, Ananth *et al*. (2019) and Harhar *et al.* (2016) identified γ-tocopherol as the most abundant homologue.

3.2.5. Antioxidant capacity

The antioxidant capacities of the hydrophilic and the lipophilic extracts are presented in Table 4. The ANOVA (not presented) highlighted a relevant ecotype influence and a significant temperature effect only for the DPPH results of hexane extracts. The interaction between ecotype and extraction temperature was predominant in the 80% methanol extracts for the ABTS test, although it was less important for DPPH. At first glance, it is easy to see that most of the antioxidant capacity was due to the hydrophilic compounds, extracted by the 80% methanol solution. We tested the total polyphenol content by the Folin-Ciocalteu method as described by Varas Condori *et al*. (2020), but we did not observe detectable values (not reported). Therefore, the high antioxidant capacity of this fraction is probably attributable to the ricinoleic acid. The antioxidant capacity in lipophilic extract is likely due to the tocopherols.

In general, Huarmey grande showed the highest antioxidant capacities in the methanolic phase at both extraction temperatures. For the ABTS test, Huarmey chico and Carhuaz also had top results at 60 °C and La Carbonera at 80 °C. For DPPH, instead, the other best performers (along with Huarmey Grande) were Huarmey chico and Casma, at 80 °C. For the lipophilic extracts, Huarmey grande, Huarmey chico and Casma had the best ABTS antioxidant capacity, in accordance with their tocopherol contents. The situation was somewhat different for DPPH because of La Carbonera's outstanding values.

It is difficult to compare our results with the scarce data in literature because of the different variables involved. Ananth *et al*. (2019) observed that the ABTS radical scavenging capacity of hydrophilic castor oil extract was superior to that of the lipophilic extract (0.63±0.02 vs. 0.13±0.04 μM TEAC/g oil DM), but the opposite was true for the DPPH assay (0.18±0.02 vs. 0.61 ± 0.03 μM TEAC/g oil DM). Santos *et al*. (2018), working with methanolic extracts, observed a free radical inhibitory capacity of 15.31±0.13 mg ascorbic acid equivalent/100 mL for ABTS, while for DPPH a 1.87±0.18% inhibition was found.

3.2.6. Oxidative stability index (OSI) and shelf-life

The OSI values of the oils analyzed by Rancimat at 150, 160 and 170 °C, with a constant 20 L/h airflow, are presented in Table 5 while the extrapolated shelf life at 20, 25 and 30 °C is reported in Table 6. The ANOVA (not presented) recorded a significant effect of the ecotype and, to a lesser degree, of the extraction temperature; the interaction was significant only for OSI and of minor relevance. The effect of the extraction temperature was higher for shelf-life than for OSI.

Huarmey grande generally showed the highest oxidative stability, and La Carbonera the lowest (Table 5). The huge difference (with a ratio around 2.0) between these two ecotypes at 150 °C decreased with increasing temperatures, reaching the lowest ratio (1.38) at 170 °C. To allow comparison with the values reported in the literature, the extrapolations at 110 and at 120 °C were performed. The OSI of the oils extracted at 80 °C ranged from 66.2 h to 145.6 h at 110 °C, and from 32.4 h to 69.2 h at 120 °C. Over-

Table 5. Oxidative stability index (h; mean±standard error; n=3) of castor oil extracted at two temperatures (60 °C and 80 °C) from five Peruvian ecotypes, and tested by Rancimat at 150, 160 and 170 °C.

	°C	Huarmey grande	Huarmey chico	Casma	Carhuaz	La Carbonera
150 °C	60	6.88±0.06^{a}	6.34±0.16^{b}	5.86±0.02^{c}	6.70±0.10^{a}	3.72±0.02^{d}
	80	7.41±0.07^{a}	6.24±0.05^{c}	5.79±0.08^{d}	6.99±0.14^{b}	3.69±0.09^{e}
160 °C	60	3.42±0.01^{a}	3.22±0.05^{b}	2.93±0.07^{c}	3.26±0.02^{b}	2.38±0.02^{d}
	80	3.55±0.02^{a}	3.37±0.03^{b}	2.87±0.07^{d}	3.18±0.06^{c}	1.95±0.05^{e}
170 °C	60	1.57±0.06^{ab}	1.63±0.02^{a}	1.44±0.02^{c}	1.52±0.02^{bc}	1.46±0.02b^{c}
	80	1.68±0.05^{a}	1.61±0.02^{a}	1.34±0.04^{b}	1.57±0.04^{a}	0.88±0.02^{c}

Different letters in a row indicate significant differences ($p \leq 0.05$) among ecotypes according to the LSD test.

Table 6. Shelf-life (years; mean±standard error; n=3) at 20, 25 and 30 °C of castor oil extracted at two temperatures (60 °C and 80 °C) from five Peruvian ecotypes.

	°C	Huarmey grande	Huarmey chico	Casma	Carhuaz	La Carbonera
20 °C	60	9.58±0.21^{b}	4.91±0.53^{c}	6.18±0.41^{c}	11.67±0.64^{a}	0.19±0.01^{d}
	80	10.67±0.62^{b}	4.93±0.20^{d}	9.00±0.70^{c}	12.98±0.18^{a}	4.77±0.52^{d}
25 °C	60	6.62±0.18^{b}	3.50±0.36^{c}	4.35±0.28^{c}	8.06±0.43^{ab}	0.15±0.01^{d}
	80	7.37±0.44^{b}	3.51±0.13^{d}	6.24±0.46^{c}	8.93±0.12^{a}	3.34±0.35^{d}
30 °C	60	4.58±0.16^{b}	2.49±0.25^{c}	3.06±0.19^{c}	5.56±0.28^{a}	0.12±0.01^{d}
	80	5.09±0.32^{b}	2.50±0.09^{d}	4.33±0.31^{c}	6.15±0.08^{a}	2.33±0.24^{d}

Different letters in a row indicate significant differences ($p \leq 0.05$) among ecotypes according to the LSD test.

all, the oxidative stability of the Peruvian accessions was high, in line with or often better than those reported by other authors. For example, Harhar *et al*. (2016) found an OSI of 35.5±4.0 h at 110 °C in Moroccan castor oil, while Adam Ali *et al*. (2016) obtained a value of 48.0 h at 120 °C in solvent-extracted castor oil. The oxidative stability of castor oil is therefore superior to that of other oils, such as chia (1.49 h at 110 °C; Villanueva *et al*., 2017), sacha inchi (0.493 h at 110 °C; Rodríguez *et al*., 2015), argan, olive, sesame and nigella (31.0, 27.0, 28.5 and 17.0 h at 110 °C, respectively (Harhar *et al*., 2016). Furthermore, all the samples exceeded the minimum 8 h oxidation stability at 110 °C requirement prescribed in the EN 14214 specification for biodiesel.

The shelf-life of all castor oils was enhanced when the seeds were extracted at 80 °C, indicating that the temperature played an important role in stability, probably through enzyme inactivation. Carhuaz and Huarmey grande consistently had the longest shelf-life, whereas La Carbonera had the shortest, particularly when extracted at 60 °C. The oxidation of polyunsaturated fatty acids generates volatile compounds that impart undesirable flavors and aromas, endangering the nutritional quality of the oil and limiting its shelf-life (Ixtaina *et al*., 2012). However, the hydroxyl behavior of the unsaturated ricinoleic acid makes castor oil a natural polyol, boosting oxidative stability and shelf-life compared to other vegetable oils. For example, the shelf-life of sacha inchi oil is 3.29, 1.79 and 0.79 years at 20, 25 and 30 °C, respectively (Rodríguez *et al*., 2015), and that of cottonseed oil is 46 days at 25 °C (Kurtulbaş *et al*., 2018). At 25 °C the shelf-life as determined by the Rancimat extrapolation of the biodiesel oil obtained from the transesterification of cooking oil waste was lower (3.1 months), and even after the addition of different strong antioxidants (BHA, BHT, PY, PG, and TBHQ), it only reached a maximum of 37.9 months (3.2 years; Zhou *et al*., 2016). Therefore, the excellent shelf-life of castor oil guarantees a protracted storage without appreciable deterioration.

4. CONCLUSIONS

A wide variation was observed in all the traits analyzed. The abundant ricinoleic acid, coupled with high total tocopherol contents and antioxidant capacity (mainly due to the hydrophilic extracts) promoted good oxidative stability and long shelf-life.

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The effects of electrical and ultrasonic pretreatments on the moisture, oil content, color, texture, sensory properties and energy consumption of microwave-fried zucchini slices

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SUMMARY: In this study, the effects of a moderate electrical field application and two different blanching methods (conventional and ultrasound) on the frying (deep-frying in oil at 180 °C for 6 minutes and compared to the microwave (400W)) of zucchini slices were investigated. Microwave-fried samples presented a lower moisture content than deep fried ones. The moderate electrical field significantly reduced the oil content before the microwave frying. Greenness (–a*), which is important for the zucchini samples, was found at its best (-3.25) in the combination group of moderate electrical field pre-treated, ultrasound blanched, and microwave fried. Gumminess, cohesiveness, and fracturability of the zucchini slices decreased while chewiness, springiness, and resilience increased after microwave frying. The scores of the sensory test were higher for the ultrasonic blanching and microwave fried sample groups. Besides, these electrical methods were found more advantageous in terms of energy consumption.

***KEYWORDS*:** *Frying; Microwave; Moderate electrical field; Texture; Zucchini.*

RESUMEN: ***Efecto de pretratamientos eléctricos y ultrasónicos sobre la humedad, el contenido de aceite, color, textura, propiedades sensoriales y consumo de energía de rodajas de calabacín frito en microondas*.** En este estudio se investigaron los efectos de una aplicación moderada en el campo eléctrico y dos métodos diferentes de escaldado (convencional y ultrasonido) en la fritura (fritura en aceite a 180 °C durante 6 minutos y comparada con el microondas (400W)) de virutas de calabacín. Las muestras fritas en microondas presentaron un menor contenido de humedad que las de fritura clásica. El campo eléctrico moderado redujo significativamente el contenido de aceite antes de la fritura en microondas. El color verde (–a*) que es importante para las muestras de calabacín se encontró como máximo (-3.25) en el grupo combinado de campo eléctrico moderado pretratado, ultrasonido blanqueado, y frito con microondas. La gomosidad, la cohesividad y la fragilidad de las rodajas de calabacín disminuyeron, mientras que la masticación, elasticidad y resiliencia aumentaron después de freír con microondas. Las valoraciones sensoriales fueron más altas en los grupos de muestras de blanqueo ultrasónico y frito con microondas. Además, estos métodos eléctricos fueron más ventajosos desde el punto de vista del consumo de energía.

PALABRAS CLAVE: *Calabacín; Campo eléctrico moderado; Freir; Microondas; Textura.*

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1. INTRODUCTION

Frying technology has a wide range of application in the food industry, such as the production of french fries, vegetable chips, especially potato, meat products, including meatballs, chicken products like fingers or nuggets, mozzarella and onion rings, some seafood products such as fish fillets and it is also applied to bakery products. These products used to be consumed a lot due to their attractive textural properties, but conscientious consumers reduced their daily intake because these types of products are rich in oil (Barutçu *et al.,* 2009; Shaker, 2015; Su *et al.,* 2016) and have a high risk of acrylamide formation (Jung *et al.,* 2003; Gökmen *et al.,* 2006; Tuta *et al.,* 2010). For these reasons, the studies were focused on the novel methods to decrease the oil absorption or processing time by frying under vacuum (Fan *et al*., 2005; Moreira *et al*. 2009; Troncoso *et al*. 2009; Dueik *et al.,* 2010) or frying with microwave (Öztop *et al.,* 2007; Su *et al.,* 2016; Aydınkaptan and Mazı, 2017) and or pulsed electric field (PEF) pre-treatments (Liu *et al.* 2007; Jonasitz *et al.,* 2011; Fauster *et al.,* 2018).

Electric field application was advantageous to fried potato production in terms of structure and oil intake (Jonasitz *et al.,* 2011). Liu *et al.* (2017) investigated the pre-treatment of the pulsed electric field (PEF) on frying and reported a membrane permeabilization effect on the vegetables and enhancing effect on the preferable textural properties, along with a beneficial impact on enhanced mass transfer (Ignat *et al.,* 2015). Electric field procures cell wall permeability, the softening of tissues, and pore formation (Ngadi *et al.,* 2003; Lebovka *et al.,* 2004; Rayman *et al.,* 2011). It helps to transfer water to the surface, so the drying becomes faster in vegetable slices (Çakmak *et al.,* 2016) or moves the nutritive components during processing (Bazhal and Vorobiev, 2000). Moderate electric field (MEF) is an electroporation technique. Electroporation occurs through the formation of pores and, which increases the permeability of biological membranes; therefore, the natural osmotic balance inside and outside the plant cells is disturbed. That affects the electrical, thermal, diffusion and, rheological properties of plant tissues and increases internal diffusion processes and moisture removal from cell vacuoles (Ngadi *et al.,* 2003; Rayman *et al.,* 2011).

In recent studies, microwave frying was often used alternatively to deep frying, as Gharachorloo *et al.* (2010) mentioned. In general, previous research determined that the microwave process has the advantages of increasing the moisture evaporation rate and uniform heating, decreasing the oil content, improving crispness and protecting the color of fried foods (Sham *et al.,* 2001; Su *et al.*, 2016; Al Faruq *et al.,* 2019; Sun *et al.,* 2019). Removal of water requires less time than traditional frying because the internal heat generation occurs after microwave energy, causing water to boil within the food (Barutçu *et al.,* 2009).

Ultrasound also produces the same results by cavitation and sponge effects; moisture removal improves during dehydration (Rodrigues *et al.,* 2008). Al Faruq *et al.* (2019), who studied ultrasound and microwave combined with the frying of apple slices, found a significant reduction in the oil uptake after ultrasound treatment compared to microwave frying. Improvements in crispiness and color were also detected. Similar results were found by Su *et al.* (2018) for potato chips. They found that ultrasound reduced the oil uptake and improved the color after ultrasound-assisted microwave frying. In another research, the ultrasound and microwave -assisted vacuum frying of mushroom chips were studied, which accelerated the frying rate; oil uptake was reduced, and texture and color properties were improved using this method (Devi *et al.,* 2018). Similarly, Huang *et al*. (2018) applied microwave and ultrasound in the frying process of pumpkin chips, and reported a synergistic effect in promoting the quality parameters. In addition to these studies, Sunsano *et al.* (2018) researched the acrylamide reduction during the microwave frying of French fries. They determined that the acrylamide content was less than 100 μg/kg on a weight basis (wb) after microwave frying.

Blanching before frying also improves the process. Blanching affects plant material cells and increases moisture removal at the beginning stage of the frying process, thus maintaining color and inactivating enzymes. Blanching also removes air from the sample, which facilitates heat transfer afterward. Ignat *et al.* (2015) reported the blanching treatment as a critical stage in the frying process. Troncoso *et al.* (2009) specified the effect of blanching on protecting the color and texture by preventing oil absorption. Fan *et al*. (2005) blanched the carrots prior to frying to protect their color. Similarly, pumpkin slices were blanched before ultrasound-assisted mi-

crowave vacuum frying (Huang *et al.,* 2018). It was also reported by Belkova *et al*. (2018) that pretreatment such as blanching reduces acrylamide. Apple slices were also blanched before microwave frying (Al Faruq *et al.,* 2019). The importance of a blanching pre-treatment in zucchini was well described by Neves *et al.* (2019). They mentioned the effectiveness of blanching on microbial reduction and improving product quality such as zucchini squash.

Zucchini (*Cucurbita pepo L.)* is a green squash (Neves *et al.,* 2019), low in calories (Iswaldi *et al.,* 2013), but rich in vitamins and minerals (Bagheri *et al.,* 2019), consumed generally in the fried form in summer. This squash can be found in many shapes and different skin colors (Iswaldi *et al.,* 2013). This vegetable was generally processed for freezing and drying (Paciulli *et al.,* 2015; Cuccurullo *et al.,* 2017), and limited studies researched zucchini frying (Abtahi *et al.,* 2016). There is no study about the ultrasound blanching effect on frying to the knowledge of the authors. Moreover, there is a lack of studies about investigating the pre-treating effect of electrical methods combined with blanching and the impact on the quality of fried products.

Therefore, the purpose of this study was to evaluate the quality characteristics (color, texture, oil, and moisture contents) of microwave-fried zucchini slices after electrical pre-treatment (MEF) and two different blanching methods (ultrasound and traditional) by comparison to the deep-frying method using sun flower oil at 180 °C for 6 minutes.

2. MATERIALS AND METHODS

2.1. Materials

Zucchini (*Cucurbita pepo L.*) was obtained from a local market (İzmir, Turkey). Samples were sliced with a slicer (Berkel, Germany), at a thickness of 0.3 cm and a diameter of 4.5 cm. Sunflower oil (Yudum Sun flower oil, Istanbul, Turkey) was used for frying. Chemicals (hexane, guaiacol, hydrogen peroxide, and sodium phosphate) were of analytical grade and obtained from Merck (Darmstad, Germany).

The raw materials were washed and peeled, divided into two groups as electrical treatment group (MEF) and the control. Each group was divided into two more groups for blanching as ultrasound blanching (US) and traditional blanching (TB). After that,

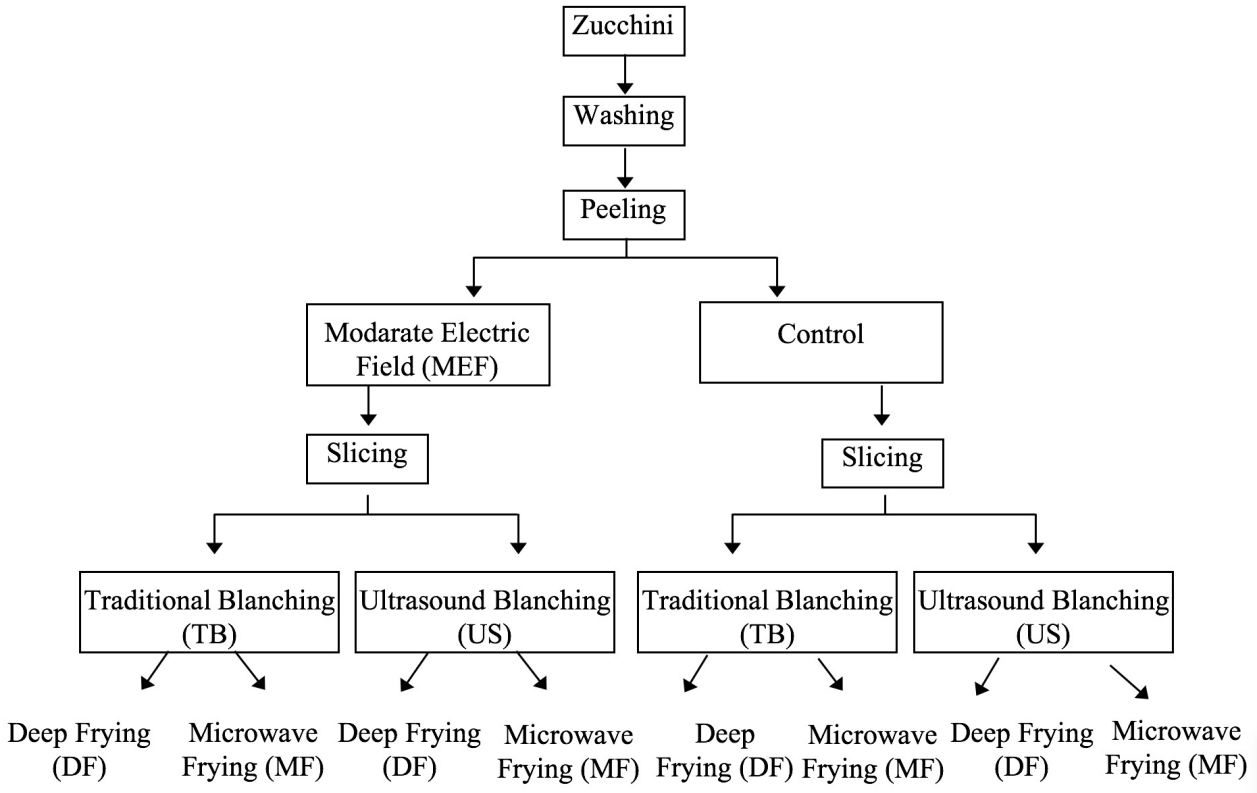

Figure 1. Flow chart of fried zucchini slices.

all four groups were separately fried by microwave (MF) and deep-frying (DF). The 8 sample groups (MEF+TB+MF), (MEF+US+MF), (MEF+TB+DF), (MEF+US+DF), (US+DF), (US+MF), (TB+MF), and (TB+DF) were processed as shown in Figure 1.

2.2. Moderate Electric Field (MEF)

MEF was applied by using a drum-type electroplasmolyzator designed by a research group in the Ege University, Food Engineering Department, with the cooperation of a Cermak Machine (Manisa, Tukey) from a previous research study (Baysal *et al.,* 2007). This equipment has two drums with stainless steel pins. A device was used to provide electric current to the system and a feed unit made contact between the pins and samples. The distance between the pins of the drum-type electroplasmolyzator was adjusted to 1.8 cm. MEF was applied before slicing. Electric current was provided to the system at between 0-400V.

The voltage gradients, and times (40, 50, 60, 70, 80V for 30, 60 and 90 s) were determined by pre-treatments. The process conditions were determined by pre-treatments for zucchini as 40V/60 s voltage gradient. The voltage was not effective for the cell poration under 40V/cm, although the structure, and color lost were seen on the surface of the samples when treated at over 40 V. 60 seconds were chosen because frying time over 60 s harmed the structure, whereas 30 s was found to be insufficient.

2.3. Blanching methods

Samples were blanched in an ultrasonic bath (35 kHz, Everest ultrasonic, Turkey) at 80 °C for 2 min. A conventional method was also carried out in the water bath (Nuve ST30, Turkey) (at 80 °C for 3 min) with a zucchini-to-water ratio of 1:8 (w/w). These parameters were selected with pre-treatments for enzyme (peroxidase) inactivation. After blanching, the slices were cooled under running tap water. Then the surface water was dried with absorbent paper.

2.4. Frying methods

Microwave frying was performed in a household microwave oven (GW72N Samsung Electronics), which works in the range of 100 to 900 W. The oil was heated to the frying temperature of 180±1 °C by using three different microwave power levels of 300, 400, 600 W used in the pre-treatments to select the effective power. For each frying experiment, 250 mL of fresh oil were placed in a Pyrex beaker when the temperature reached 180 °C. Ten zucchini slices (25 g±1.0 g) were immersed in the oil (sample-oil ratio of 1:10 w/v) to conduct a frying run at a specified time and power. The temperature was controlled by fiber optic sensors (Neoptix Qualitrol Campany, USA). All runs were conducted with fresh oil. The lowest power level (300 W) provided low temperatures and took longer times, increasing the oil intake. Therefore, 300 W was also eliminated. In the case of the highest power (600 W), the temperature increased suddenly, and this was eliminated also to provide an effective process. For this reason, samples were fried at 400 W. After selecting this power level, different times were treated, such as 4, 6, 7 minutes at 400 W to select the sufficient time. The samples fried for 7 minutes began to brown. This situation was undesirable. Samples fried for 4 minutes at 400 W did not fry enough, and the peroxidase enzyme was not inactivated. Therefore, the frying time was selected as 6 minutes at 400 W and the enzyme was inactivated.

Deep frying was carried out under the same conditions as microwave frying at 180 °C for 6 min, which inactivated the peroxidase enzyme, with a 3-L capacity Sinbo model fryer (SDF 3827, France) equipped with temperature adjustment in the range of 90 to 190 °C. The sample-oil ratio was 1:10, (w/v) the same as the microwave method. Before a new run, the basket cooling period was observed closely, and fresh oil was used for each sample. After reaching the intended frying temperature, samples were placed in the basket, and the same procedure was followed.

The excess oil on the surface of the samples was removed with dry tissue paper for 20 s after both frying techniques. The frying process was performed in 2 replicates and, analyses were replicated 3 times.

2.5. Analytical methods

Peroxidase activity for determining the blanching time was conducted qualitatively by adding 1 mL guaiacol as the substrate and 1 mL H_2O_2 (0.5%) as the hydrogen donor to the mixture of 5 mL demineralized water and 5 g sample. The test was evaluated as positive when a reddish-brown color was observed and negative if there was no color. A neg-

ative test indicated the inactivation of the enzyme (Cemeroğlu, 2010).

The samples were analyzed for moisture contents with infrared moisture equipment (MOC63u, Shimadzu Inc. Japan) (Anon, 1990). Three samples were taken at random, and a shredded 1.5 g sample was tested in triplicate.

The total oil content was determined by solvent extraction using the Soxhlet method with hexane for 6 hours with the samples dried and ground into small particles (Ignat *et al.,* 2015).

Color values (L*, a*, b*) were measured with a Minolta chromameter (CR-400 Konica Minolta Inc, Japan). The total color difference (ΔE) was calculated (Eq. 1) by taking the TB+DF as a reference. Three different samples were scanned at three different positions, and the average values of three replicate measurements were reported.

$$\Delta E=\sqrt{(L^*-L^*_{ref})^2+(a^*-a^*_{ref})^2+(b^*-b^*_{ref})^2} \quad (Eq.1)$$

Textural properties were measured with a TA-XT plus texture analyzer (Stable Micro System Co. Ltd., Surrey, UK). A spherical stainless-steel test probe (P/25) of 25 mm in diameter in compression test mode was used to determine the hardness of the product by placing the one slice of the sample over the end of a hollow cylinder against the probe (Su *et al.,* 2016). The test parameters were: 0.80 mm/s pre-speed, followed by 0.80 mm/s test-speed and 4 mm/s post-test speed. The test distance was set at 3 mm by preliminary tests. Breaking force (N), Hardness (g-force), Fracturability (g-force), Springiness (m), Cohesiveness (N cm), Gumminess (N), Chewiness (J), and Resilience (N) were measured. Three slices were tested in triplicate for each group. The parameters were defined as the peak force observed at the maximum compression.

The sensory analysis was performed in line with international standards (Norma UNE, 2020) The tests were developed in a standard room equipped with 10 individual tasting areas. The sensory test was performed with 20 untrained panelists. All samples were given at room temperature and coded with three-digit random numbers. Water and bread pieces were served to panelists for oral rinsing. An acceptance test was used by applying a hedonic scale structured in 9 points, 1 being "I dislike extremely" and 9 being "I like extremely", indicating an increasing general appeal level in the 0.05 significance scale. Characteristics such as color, texture, flavor, and general appearance were evaluated (Altuğ and Elmaci, 2005; Tejada *et al.,* 2020).

The energy consumption of each process was measured using a digital energy meter. The energy meter recorded the energy during each treatment process (Su *et al.,* 2018). The calculation of power consumption for the sample groups was made by the cumulative sum of power for each treatment.

Results of the analyses were statistically analyzed by one-way analysis of variance (ANOVA) software SPSS 18 (SPSS Inc., Chicago, IL, U.S.A.) with the Duncan test to evaluate differences between treatments at a level of significance of $p < 0.05$.

3. RESULTS AND DISCUSSION

The zucchini slices were fried with the selected parameters (power level and time), which provided the peroxidase enzyme inactivation and the samples were analyzed for comparing quality properties.

3.1. Influence of moisture and oil contents after the frying process

The quality properties of the fried zucchini slices are given in Table 1. Moisture contents significantly differed at the end of the applications ($p < 0.05$). The results showed that ultrasound blanching reduced the moisture content considerably for the same frying time as the traditonal method ($p < 0.05$). This is an advantageous result considering that the time for US blanching was 1 min less than the traditional method. The combination group of MEF+US+MF had the least moisture content at 26.67%. The zucchini slices, blanched in the traditional method, had a 93.65% moisture content before frying, whereas the moisture content found in the samples blanched with ultrasound was 93.09%. These values were 91.12 and 91.82% for the MEF+US and MEF+TB, respectively (Table 1). The electrical treatment destroyed the cell and made the water transfer easier. The microscopic channels were increased by ultrasound, and this accelerated the water pathway. The synergistic effect of these two applications helped to reduce the moisture in both the deep oil and microwave frying. Similarly, Bagheri and Tinani (2019) maintained that ultrasonic pre-treatment for 20 and 30 min before drying zucchini slices resulted in the

Table 1. Moisture, oil and color contents in zucchini slices.

Sample	Moisture (%)	Oil (%)	L*	a*	b*	ΔE
TB	93.65±0.20[a]					
US	93.09±0.10[b]					
MEF+TB	91.82±0.05[c]					
MEF+US	91.12±0.10[d]					
MEF+TB+MF	31.49±0.10[f]	25.92±0.60[e]	64.14±0.02[g]	-2.20±0.00[d]	35.80 ±002[b]	7.60±0.04[e]
MEF+US+MF	26.67±0.20[k]	38.10±0.35[b]	60.71±0.03[h]	-3.25±0.00[h]	33.55 ±0.05[c]	5.94±0.01[f]
MEF+TB+DF	62.37±1.00[g]	19.02±0.30[f]	72.84 ±0.01[b]	-2.42±0.03[e]	38.49 ±0.50[a]	14.50±0.00[a]
MEF+US+DF	59.10±0.55[h]	27.81±0.55[d]	71.27±0.00[c]	-3.15±0.01[g]	37.29 ±0.01[a]	13.58±0.06[b]
TB+MF	44.98±0.58[i]	30.00±0.60[c]	70.68 ±0.01[e]	-2.51±0.02[f]	32.42±0.01[c]	3.36±0.00[g]
TB+DF	72.70±0.60[e]	9.09±0.25[h]	70.78±0.05[d]	-0.14±0.01[a]	29.10±0.03[d]	
US+MF	35.24±0.40[j]	40.62±0.45[a]	66.73±0.05[f]	-1.58±0.00[c]	39.37±0.04[a]	8.23±0.01[d]
US+DF	63.74±0.02[f]	16.81±0.50[g]	74.65±0.04[a]	-0.33±0.04[b]	32.26±0.10[c]	10.39 ±0.04[c]

[a]Mean value ± standard deviation, and the number of samples analyzed (n= 3).
[b]a,b,c,.. Different letters within columns are significantly different according to Duncan´s test (p<0.05).
[c]Abbreviations: (MEF+TB+MF)=moderate electric field+traditonal blanching+microwave frying; (MEF+US+MF)=moderate electric field+ultrasound blanching+microwave frying; (MEF+TB+DF)= moderate electric field+traditonal blanching+deep-oil frying;(MEF+US+DF)=moderate electric field+ultasound blanching+deep-oil frying ;(TB+MF)=traditonal blanching+microwave frying and (TB+DF)=traditonal blanching+deep-oil frying; (US+MF)=ultasound blanching+microwave frying; (US+DF)=ultasound blanching+deep-oil frying. L*: Lightness, +a*/-a*: Redness/Greenness; +b*/-b*: Yellowness/Blueness; ΔE: Total Color Difference

cavitation phenomenon, causing the breakdown of cells, with microscopic channels becoming more prolonged and more profound. The water evaporated efficiently by the formation of microscopic channels due to an increase in water diffusivity.

In the US treated deep oil frying process, the moisture was 63.74%, although this value was reduced to 35.24% when the same sample was fried with the microwave technique. Su *et al.* (2018) studied with microwave vacuum frying of potato chips and explained the effect of microwave by finding a marked increase in the moisture evaporation kinetics and effective moisture diffusivity compared to fried samples without the microwave. They suggested that the higher microwave setting used in the process achieved a higher moisture evaporation rate and higher effective moisture diffusivity. Similarly, it was pointed out for apple chips that the moisture removal became easier after the microwaves, which penetrated the food and led to water boiling within the food, which increased the vapor pressure differential between the center and the surface of the product (Sham *et al.,* 2001). It was also found that the microwaved ones had more moisture loss than the deep oil-fried potatoes, similar to our study (Oztop *et al.,* 2007). Huang *et al.* (2018) stated that the ultrasound application lowered the moisture content significantly when comparing the microwave-assisted vacuum frying and vacuum frying samples. Hosseinzadeh and Shaheed (2015) also determined that the moisture content in zucchini slices was between 31.6-39.7% after frying at 150, 170, 190 °C. They said that the moisture loss was high, and a high amount of oil absorption was recorded. The oil uptake was in line with our study with the lower moisture contents. Moisture loss and oil absorption are the two most important mass transfer processes taking place during the frying of food (Quan *et al*., 2014). Fat uptake reduced from 7.5 to 6.8 by applying PEF at 1.0 kV/cm electric field (Fauster *et al.,* 2018), and elasticity increased, while firmness decreased.

The oil content was found at its highest in the US+MF group and lowest in the TB+DF group (Table 1). MEF significantly affected the oil content before microwave frying compared to the groups of MEF+TB+MF with TB+MF (p < 0.05). MEF pre-treatment increases the oil absorption of the sample in deep-oil frying rather than MF with both blanching methods. This situation was due to the synergistic effect of MEF and deep-oil frying. During deep-oil frying, the transfer of water and intracellular substances to the surface of the fries

could be increased due to the MEF-induced electroporation. Thus, the water at the surface of the samples improves the rate of mass transfer and increases moisture removal and oil uptake. In addition, Quan *et al.* (2014) indicated that in the early stage of frying, the rate of moisture evaporation was high, which facilitated the formation of large pores at faster rates. The formation of larger pores at faster rates facilitated the fast absorption of oil into these pores. Therefore, it can be said that MEF provided a faster early stage in deep-oil frying than MW frying and the oil absorption was high in this stage in deep-oil fried samples.

Ultrasound caused more rapid moisture removal and higher oil absorption compared to the groups without ultrasound. Janositz *et al.* (2011) mentioned that PEF decreased oil uptake by the mechanisms of permeabilized cell membranes. A higher vapor pressure difference which reduces dehydration also removes substrates, reducing sugars, such as saccharides for the Maillard reaction. Ignat *et al.* (2015) confirmed this condition by using potato cubes submitted to PEF treatments and par-fried for 1 min with a 74.2% moisture content. In another study, the oil uptake of fried apple slices was reduced after ultrasonic application in microwave frying (Al Faruq *et al.,* 2019).

It was found that the deep-oil frying groups had less oil than the microwave frying group ($p < 0.05$). Similarly, Sunsano *et al.* (2018) stated that the oil uptake was more significant in potatoes with microwave frying than deep frying. The increase in temperature and the internal pressure were faster in microwave frying than in conventional frying due to the volumetric heating in the presence of microwaves. Therefore, the moisture escape became easier. The creation of structural channels through the sample tissue during MW frying favored the oil uptake significantly compared to deep-oil frying (Sansano *et al.,* 2018). Similar to our study, previous researchers suggested that a large amount of oil is absorbed when the moisture loss is high. This condition was explained by Quan *et al.* (2014) in the dry areas of chips which were previously occupied by water and become dryer and less hydrophilic. These made an easier interaction between the oil and pores and even the non-porous areas of the chips. However, in contrast to this, Aydınkaptan and Mazı (2017), determined that microwave- fried French fries had a lower oil content and moisture content than those fried conventionally. They explained a slight decrease in the oil content of all samples an increasing level of oil degradation. In line with this, Devi *et al.* (2018) determined that microwave-fried samples had lower oil values than deep-oil fried ones. The authors argued that the diffusion of oil into the product was limited by a high evaporation rate of water during microwave frying; thus, the oil content was lower in MW.

3.2. Changes in color properties

The color values L*, a*, and b* of the samples were measured and then the total color differences (ΔE) were calculated for each group and are presented in Table 1. There was a significant difference between the ΔE values ($p < 0.05$). The highest L* (lightness) was found in the US+DF group and the lowest in the MEF+US+MF group. Traditional methods showed higher L* values. There were no significant differences among the L* values of the TB+DF and TB+MF groups ($p > 0.05$). The L* value decreased after TB+MF when electrically pre-treated, and the same effect was found in the US+MF group. This may be because the higher oil content showed less light in the samples. MEF application with the poration effect caused more oil absorption and a lower lightness value. Also, the accumulation of moisture could lead the sample to be less bright. In addition, traditional blanching protected the brightness better than US when comparing the groups (MEF+TB+MF), (MEF+US+MF), (MEF+TB+DF), and (MEF+US+DF). This could be due to the cavitation effect of US, and the brightness was affected by the treatment.

There are different remarks about lightness in the literature. For example, it was mentioned that when brightness is reduced, the product obtains the desired golden color. Additionally, the reason for lowering the brightness or darkness of fried zucchini was explained by the nonenzymatic browning reactions due to increased frying temperatures (Hosseinzadeh and Shaheed, 2015). In contrast to this, as previously stated, a higher L* value is needed to give better consumer acceptance. The L* values for the fried samples decreased with increasing frying temperature, and it was significantly ($p < 0.05$) highest in the ultrasound-assisted microwave frying group at the same frying temperature (Devi *et al.,* 2018). In an-

other study, the lightness parameter (L*) decreased to a lesser extent in microwave frying than in conventional frying. The color values for microwave fried French fries were lighter and more yellow at higher temperatures compared to the conventional fries. During the microwave frying of potatoes, the L* value for microwave fried was found higher than that for conventional fried under the same conditions (Parikh and Takhar, 2016). Huang *et al.,* (2018) published that ultrasound produces a slight increase in microwave frying lightness.

The -a* value, which shows the green color, was found mostly higher in MEF+US+MF but lower in the group TB+DF; therefore, the process US and MF protects the specific color of zucchini slices. The green color was found higher in the MF-treated samples when US+MF was compared to the US+DF groups ($p < 0.05$). This was because of the microwave heating effect that began from the inside. In line with this opinion in previous research, it was found that the moisture ratio in the frying process was associated with browning. Reduction in the moisture content in potato chips during microwave-assisted frying preserved the color better. But low temperature did not significantly affect the a* value in the microwave-assisted vacuum frying of fried potato chips (Su *et al.,* 2016). The microwave used in the frying process is a kind of assisted dehydration technology which is based on the thermal effect of microwaves. In another study, they stated that the microwave energy travels through the material and is absorbed more in the wet region than in the dry region of the product (Sun *et al.,* 2019). At 193 **°C,** the MF French fries had significantly larger a* values for all frying times than deep-oil frying (60, 90, and 120 s) (Parikh and Takhar, 2016). However, Ignat *et al.* (2015) found the a* values to be lower for the blanched potato than in the low PEF at 9000 pulses at 75kV/cm treatment.

There were no significant differences between the b* values of MEF+TB+DF and MEF+US+DF and US+MF groups ($p > 0.05$). In addition, the b* values did not significantly differ between the groups of MEF+US+MF, US+DF, and TB+MF ($p > 0.05$). The effect of TB and US was the same on the yellowness, but MF was significantly and positively affected. When b* values were high for fried products, this showed a more yellow product, which was preferred (Krokida *et al.,* 2001). In another study, ultrasound decreased the non-enzymatic browning in the vacuum frying of mushroom chips. They also determined a significant increase in the b* value after ultrasound (Devi *et al.,* 2018).

Total color differences significantly differed between the groups due to the L* and a* values ($p < 0.05$). The chemical browning reactions in food, oil absorbed by food, time and temperature of the frying process, etc. affect the color of fried products (Aydinkaptan and Mazi, 2017).

3.3. Changes in the textural characteristics

During frying, due to the removal of water from the slices, some textural changes occurred. Textural properties such as hardness (g-force), fracturability (g-force), cohesiveness (N cm) and chewiness (J) are shown in Table 2. The breaking forces were also evaluated, and significant differences were found between the forces ($p < 0.05$). As mentioned previously (Fan *et al.,* 2005), when the breaking force was found to be lower, the crispiness value was higher. After the MEF application, the breaking force was significantly lower than the other groups ($p < 0.05$).

In parallel with this study, Ignat *et al*. (2015) noted that the PEF treatment for the potato crisps needed a lower force for breaking, which was accomplished by making the plant tissue firmer through the effect of electroporation. Su *et al*. (2017) suggested that breaking force and crispiness were inversely proportional. They found the moisture content in the potato chips fried in microwave-assisted vacuum frying directly proportional to breaking force and reversely proportional to crispiness.

Microwave frying increased the breaking force rather more than deep oil. Deep-oil frying gave a softer textural property to the samples than the microwave method. The structure could be improved using the application of MEF before microwave and deep-oil frying. Similar to our study, ultrasound-assisted microwave frying showed a higher moisture removal rate, which may have led to the formation of a crust and mad the product crispier (Devi *et al*., 2018). The breaking force decreased with increased frying time and microwave power due to reduced water content and increased oil content (Al Faruq *et al.,* 2019).

The US significantly decreased the hardness compared to traditional blanching ($p < 0.05$). The ultrasound, together with the effect of sponge and

Table 2. Textural properties of zucchini slices

Sample Group	Breaking force (N)	Hardness (g-force)	Fracturability (g-force)	Springiness (m)	Cohesiveness (N cm)	Gumminess (N)	Chewiness (J)	Resilience (N)
Raw zucchini	19492.30±20.22[a]	26525.647±85.17[a]	9.071±2.33[c]	0.67±0.09[b]	0.79±0.01[a]	21066.51±18.10[a]	13607.096±15.10[a]	0.65±0.02[a]
MEF+TB+MF	19.33±26.45[h]	7.37±10.12[e]	0.98±1.22[i]	0.54±0.01[c]	0.60±0.01[c]	0.71±10.45[g]	6.37±2.50[b]	0.16±0.02[d]
MEF+US+MF	99.85±30.50[d]	19.43±10.20[b]	5.48±3.11[e]	0.90±0.02[a]	0.75±0.00[a]	0.61±0.90[g]	4.98±1.21[c]	0.12±0.01[e]
MEF+TB+DF	16.14±9.27[i]	16.44±6.66[c]	11.96±4.16[b]	0.51±0.02[c]	0.55±0.01[c]	9.19±3.88[c]	4.68±1.49[c]	0.11±0.01[f]
MEF+US+DF	68.98±15.08[e]	1.79±3.55[g]	3.68±1.45[g]	0.13±0.04[e]	0.53±0.02[c]	3.71±1.40[d]	2.62±2.55[d]	0.17±0.00[d]
TB+MF	22.54±12.52[g]	7.47±1.45[d]	6.28±1.17[d]	0.02±0.02[f]	0.43±0.01[d]	3.23±0.80[e]	0.09±1.42[h]	0.20±0.01[c]
TB+DF	1249.16±40.41[b]	1.09±2.31[h]	18.54±0.50[a]	0.49±0.01[c]	0.56±0.06[c]	11.65±2.48[b]	0.30±0.77[f]	0.21±0.03[c]
US+MF	60.78±10.12[f]	3.78±2.00[f]	1.59±0.60[h]	0.57±0.02[c]	0.65±0.02[b]	0.67±1.65[h]	1.43±0.01[e]	0.26±0.01[b]
US+DF	278.62±50.52[c]	0.69±1.52[i]	3.88±1.93[f]	0.30±0.06[d]	0.62±0.02[c]	2.48±0.45[f]	0.16±1.90[g]	0.23±0.03[c]

[a]Mean value ± standard deviation, and the number of samples analyzed (n= 3).
[b]a,b,c,.. Different letters within columns are significantly different according to Duncan´s test (p<0.05).
[c]Abbreviations: (MEF+TB+MF)=moderate electric field+traditonal blanching+microwave frying; (MEF+US+MF)=moderate electric field+ultrasound blanching+microwave frying; (MEF+TB+DF)=moderate electric field+traditonal blanching+deep-oil frying;(MEF+US+-DF)=moderate electric field+ultasound blanching+deep-oil frying ;(TB+MF)=traditonal blanching+microwave frying and (TB+DF)=traditonal blanching+deep-oil frying; (US+MF)=ultasound blanching+microwave frying; (US+DF)=ultasound blanching+deep-oil frying. N: Newton; g-force: gram force; m: metre; J: joule; N cm: Newton centimeter.

cavitation, provided more crispiness compared to conventional blanching. Hhowever, when compared to MEF, it was less effective. The deep-oil fried samples had a softer structure than the microwave fried ones. More fracturability was found in the MEF+TB+DF and TB+DF groups. This was due to the effect of the frying process which reduced the breaking force of the zucchini slices through the removal of water. Due to the removal of water from the structure, the fried slices gained brittleness and hardness. In other words, the breaking force decreased significantly (Karacabey *et al.,* 2016). Sansano *et al*. (2018) confirmed these results by stating that microwave-fried samples were harder than conventional ones, mainly because of the significant water loss in microwave frying.

Cohesiveness, which was not desired in high levels, was found to be lower in the TB+MF samples, and also gumminess was low in the MEF+US+DF samples. Traditinonal blanching and frying increased gumminess. The moisture content of the US blanched groups was lower than the other in line with the gumminess. Chewiness was found highest in the group of MEF+TB+MF as 6.376. Resilience, which indicates flexibility, was found highest after the applications of US+MF.

The oil content and moisture were also effective on the textural properties of the fried samples. Microwave frying reduced the time compared with conventional frying (Sahin *et al*., 2007). They also mentioned that the moisture content decreased while color developed during both conventional and microwave frying. This situation was due to the decrease

Table 3. Energy consumption for processing groups.

Sample	Energy consumption (kWh)
MEF+TB+MF	0.42±0.01[c]
MEF+US+MF	0.36±0.01[d]
MEF+TB+DF	0.56±0.02[a]
MEF+US+DF	0.50±0.05[b]
TB+MF	0.18±0.08[g]
TB+DF	0.32±0.06[e]
US+MF	0.12±0.04[h]
US+DF	0.26±0.02[f]

[a]Mean value ± standard deviation, and the number of samples analyzed (n= 3)
[b]a,b,c,.. Different letters within columns are significantly different according to Duncan´s test (p<0.05).
[c]Abbreviations:(MEF+TB+MF)=moderate electric field+traditonal blanching+microwave frying; (MEF+US+MF)=moderate electric field+ultrasound blanching+microwave frying; (MEF+TB+DF)= moderate electric field+traditonal blanching+deep-oil frying;(MEF+US+DF)=moderate electric field+ultasound blanching+deep-oil frying; (TB+MF)=traditonal blanching+microwave frying and (TB+DF)=traditonal blanching+deep-oil frying; (US+MF)=ultasound blanching+microwave frying; (US+DF)=ultasound blanching+deep-oil frying. (kWh): Kilowatthours.

Table 4. Average scores of panelists for hedenoic test

Sample	Color	Texture	Odor	Flavor	General appearance
MEF+TB+MF	5.50±0.05[d]	5.11±0.02[f]	6.00±0.15[d]	6.89±0.14[c]	7.70±0.25[a]
MEF+US+MF	7.67±1.01[a]	8.10±0.09[b]	5.33±0.42[f]	6.20±0.48[e]	6.89±0.78[b]
MEF+US+DF	6.00±0.08[b]	8.70±0.15[a]	5.33±0.48[f]	6.20±0.36[e]	6.89±1.12[b]
MEF+TB+DF	5.55±0.04[d]	7.80±0.13[c]	6.67±0.69[c]	7.78±0.98[a]	6.88±0.14[b]
US+DF	5.60±0.06[d]	6.00±0.74[d]	5.78±0.52[e]	7.22±0.65[b]	5.77±0.65[d]
US+MF	5.40±0.05[d]	8.11±0.90[b]	6.67±0.66[c]	6.10±0.25[f]	5.89±0.24[d]
TB+MF	5.90±0.07[c]	5.67±1.05[e]	7.30±1.00[a]	6.11±0.36[f]	3.11±0.98[e]
TB+DF	4.40±0.06[e]	3.67±0.82[g]	6.88±0.85[b]	6.44±0.76[d]	6.67±0.45[c]

[a]Mean value ± standard deviation, test was carried with 20 panelists in 2 sessions.
[b]a,b,c,.. Different letters within columns are significantly different according to Duncan´s test (p<0.05)
[c]Abbreviations: (MEF+TB+MF) = moderate electric field+traditonal blanching+microwave frying; (MEF+US+MF) = moderate electric field+ultrasound blanching+microwave frying; (MEF+TB+DF) = moderate electric field+traditonal blanching+deep-oil frying; (MEF+US+DF) = moderate electric field+ultasound blanching+deep-oil frying; (TB+MF) = traditonal blanching+microwave frying; (TB+DF) = traditonal blanching+deep-oil frying; (US+MF) = ultasound blanching+microwave frying; (US+DF) = ultasound blanching+deep-oil frying.

in moisture content as frying time increased, which resulted in harder products, and microwave energy caused the fast water evaporation rates and created higher pore density of larger pores in the chips. Such a porous texture was expected to increase the crispness in chips. Based on the results of analysis in this study, it fell in line with the study of Quan *et al.* (2014), who found that it was possible to produce crunchier, more visually appealing fried products in a short time by the microwave-assisted frying.

3.4. Evaluation of sensory properties

The sensory quality of zucchini slices is shown in Table 4. The results showed that zucchini slices processed by MEF and US treatments had high scores for texture and color. The MEF +TB+DF groups were preferred due to the flavor and electrical applications, and ultrasonic blanching affected the samples' odor. The ultrasonic blanching mentioned before in the textural properties made the slices crispier, more attractive and preferable for the panelists. The combined effect of ultrasound and microwave showed better sensory quality. The impact of ultrasound combined with microwave on the sensorial properties such as odor and color was reported in a previous study. They informed significant differences between ultrasound-assisted microwave frying and microwave frying (Al Faruq *et al.,* 2019).

The sensory results were found to be in agreement with the color and texture measurements. The scores in the panel were higher for the color of samples, which had higher greenness values. Just the contrary, Troncoso *et al*. (2019) explained that the instrumental and sensory properties of color and texture could be irreversible. For example, panelists can prefer the sample with low lightness values. Electrically treated, traditionally blanched, and deep-fried slices were preferred by the panelists in terms of flavor, which was found to be more delicate in the texture analysis. However, the panelist gave high scores to MEF+TB+MF for general appeal.

3.5. Energy consumption

Power consumption was an important parameter to calculate the cost. In the industrial process, the most important target was to use a low amount of energy. The energy consumption of all the groups was calculated and given in Table 3. The energy saving was found significant after the ultrasonic treatment and microwave frying rather than the other techniques. The results showed that the power consumption of processing zucchini slices with the microwave was significantly lower than deep frying.

Ultrasound provided less energy consumption when compared to the sample groups of TB+DF and US+DF. The same effect was also determined in the microwave-fried samples due to the blanching methods. It is worth mentioning that the frying method effectively maximized energy saving with the same processing time.

Energy efficiency is a critical factor that restricts the

development of fried fruit and vegetable slices. Consequently, the application of the combined technique may maximize energy savings and reduce expenses.

Similar results were found in previous research. Ultrasound was pointed out to require lower energy consumption. Su *et al.* (2018) reported that the combination of ultrasound could decrease the frying time and the energy consumption compared to the microwave. Fauster *et al*. (2018) suggested that PEF showed lower energy consumption than thermal pre-heating in French fry production. Similarly, Ignat *et al.* (2015) discussed the effect of an electroporation pre-treatment on processing time and energy saving. They determined that the low electric field application needed the lowest energy compared to deep-fat frying, blanching, and high-pulsed electric field application.

4. CONCLUSIONS

The single and synergistic effects of ultrasound blanching and moderate electrical pre-treatment on frying were investigated. The deep-oil and microwave frying of zucchini slices at the same temperature and during the same frying time were compared in terms of the optimal moisture and oil contents for color and textural properties. Electrical pre-treatments such as moderate electric field were found to be significantly effective on the frying of zucchini slices by making water transfer from the samples' surface easier. The electric field's synergistic effect, ultrasound and microwave treatments showed the least moisture content and the highest greenness. In addition to this, the electric field reduced oil intake before microwave frying. The breaking force of the final products was significantly affected by different frying methods and pre-treatments. Textural properties improved after microwave frying, so they were more chewy, resilient and springy but less fracturable, gummy and cohesive. Also, hard edible fried zucchini slices were produced. These alternative methods could be implemented in frying technology, considering these positive aspects with savings in energy and time.

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Biodiesel production enhanced by ultrasound-assisted esterification and transesterification of inedible olive oil

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SUMMARY: In the first phase of this study, inedible olive oil with different initial free fatty acid concentrations (2.5, 5.0, and 10.0%) was processed through acid-catalyzed esterification. Various heating methods were used for this purpose. The ultrasound-assisted esterification and traditional magnetic stirrer-assisted esterification methods were similar to each other in terms of their effects on free fatty acid reduction. However, the ultrasound reaction time was significantly shorter than that of the traditional magnetic stirrer. In the second phase of this study, biodiesel production was carried out through the ultrasound-assisted transesterification of inedible olive oil. Independent variables were, namely, ultrasound power level (30, 90, and 150 W), methanol/oil mole ratio (3, 9, and 15), catalyst concentration (0.5, 1.0, and 1.5%), ultrasound time (15, 30, and 45 min), and reaction temperature (45, 55, and 65 °C), which affected the yield indices and physicochemical constants of the produced biodiesel. The purest biodiesel (98.95%) and the highest amount of yield (92.69%) were observed when using an ultrasound power level of 90 W, a methanol/oil mole ratio of 9, a catalyst concentration of 1.0%, an ultrasound time of 30 min, and a reaction temperature of 55 °C. Optimizing the reaction conditions of the ultrasound operation can effectively increase the biodiesel yield (92.69%), while reducing the energy consumption (4.775 kWh/kg) and shortening the reaction time (30 min), compared to the traditional magnetic stirrer (77.28%, 2.17 kWh/kg, and 120 min, respectively). Therefore, ultrasound-assisted transesterification can serve as an effective alternative because of its fast and economic operation for making biodiesel out of inedible olive oil.

KEYWORDS: *Biodiesel; Esterification; Olive oil; Transesterification; Ultrasound*

RESUMEN: ***Producción de biodiésel mejorada por esterificación y transesterificación asistida por ultrasonidos de aceite de oliva no comestible.*** En la primera fase de este estudio, el aceite de oliva no comestible tenía diferentes concentraciones iniciales de ácidos grasos libres (2,5, 5,0 y 10,0%) y se procesó mediante esterificación catalizada por ácido. Se utilizaron varios métodos de calentamiento para este propósito. La esterificación asistida por ultrasonido y los métodos tradicionales de esterificación asistida por agitador magnético fueron similares entre sí en términos de sus efectos sobre la reducción de ácidos grasos libres. Sin embargo, el tiempo de reacción usando ultrasonidos fue significativamente más corto que el de agitador magnético tradicional. En la segunda fase de este estudio, la producción de biodiesel, a partir de aceite de oliva no comestible, se llevó a cabo mediante transesterificación asistida por ultrasonidos. Las variables independientes fueron, nivel de potencia de ultrasonido (30, 90 y 150 W), relación molar metanol/aceite (3, 9 y 15), concentración de catalizador (0,5, 1,0 y 1,5%), tiempo de ultrasonido (15, 30 y 45 min) y temperatura de reacción (45, 55 y 65 °C) que afectaron al rendimiento y a las constantes fisicoquímicas del biodiesel producido. El biodiésel más puro (98,95%) y el mayor rendimiento (92,69%) se observaron cuando se utilizó un nivel de potencia de ultrasonido de 90 W, una relación molar de metanol / aceite de 9, una concentración de catalizador del 1,0%, un tiempo de ultrasonido de 30 min, y una temperatura de reacción de 55 °C. La optimización de las condiciones de reacción de la operación de ultrasonido puede aumentar efectivamente el rendimiento de biodiésel (92,69%), al tiempo que reduce la cantidad de consumo de energía (4,775 kWh/kg) y acorta el tiempo de reacción (30 min), en comparación con el agitador magnético tradicional (77,28 %, 2,17 kWh/kg y 120 min, respectivamente). Por lo tanto, la transesterificación asistida por ultrasonido puede servir como una alternativa eficaz debido a su operación rápida y económica en la producción de biodiesel a partir de aceite de oliva no comestible.

PALABRAS CLAVE: *Aceite de oliva; Biodiesel; Esterificación; Transesterificación; Ultrasonido*

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1. INTRODUCTION

In the twenty-first century, there are numerous non-fossil fuels available such as hydrogen, solar energy, wind power, etc. Among them, biodiesel (fatty acid methyl ester) is one of the most well-known fuels which can be used as an environmentally clean energy (Maneerung *et al*., 2016; Tan *et al*., 2015). The transesterification of vegetable oils is a popular method for biodiesel production (Badday *et al*., 2013).

Previous research has dealt with several heating methods for the transesterification of vegetable oils in order to produce biodiesel (Talebian-Kiakalaieh *et al.*, 2013). Nowadays, new heating methods such as microwave, ohmic, and ultrasound are beginning to be recognized as effective approaches for biodiesel production. Employing new heating methods can result in a reduction in reaction time, energy consumption, and the costs of biodiesel production (Ho *et al*., 2016). Ultrasound provides physical mixing as well as the activation energy required for initiating the transesterification reaction that results in temperature increase at the phase boundary of the reactants which further enhances the reaction rate (Sharma *et al.,* 2020). This effect contributes to cavitation bubbles, whereby the solution is effectively mixed, immiscible liquid layers are disrupted and mass transfer is promoted in interfacial areas (Badday *et al*., 2013). In a relevant study, Badday *et al*. (2013) showed that ultrasound-assisted transesterification (UAT) requires a maximum reaction time of 50 min in order to achieve 85% biodiesel yield from crude Jatropha oil. Traditional-assisted transesterification (TAT) entails a longer duration of reaction time (i.e. 300 min).

When olive oil is processed and produced, the presence of moisture and hydrolytic enzymes can potentially cause the free fatty acids in the olive oil to reach non-standard high amounts. This occurrence can make the olive oil inedible. Some types of inedible olive oil have high amounts of free fatty acids, meaning that high rates of loss become an inevitable part of their refining process. In fact, this makes the refining process a costly endeavor for the commercial oil industry (Kanitkar *et al.*, 2011). While biodiesel can be made out of inedible olive oil economically and valuably, the alkaline-catalyzed transesterification process turns out to be less efficient when processing olive oil samples, especially those that have substantial contents in free fatty acids. The reason for this is that soap forms between the free fatty acids and the alkaline catalyst. To complete alkaline-catalyzed transesterification, it is necessary to have free fatty acid values which are lower than 3% (Meher *et al*., 2006; Vicente *et al*., 2004). Accordingly, acid-catalyzed esterification can enable a decrease in free fatty acid content in the inedible olive oil. While acid-catalyzed esterification is regarded as a pre-treatment, biodiesel can then be made out of inedible olive oil by means of transesterification.

This study consists of two phases. In the first phase, the ultrasound-assisted esterification (UAE) of inedible olive oil is carried out with various free fatty acid concentrations in the beginning of the process. This is to be compared with traditional-assisted esterification (TAE). In the second phase, the UAT factors (i.e. ultrasound power level, methanol/oil mole ratio, catalyst concentration, reaction time, and reaction temperature) are examined which can have different effects on the purity, yield, physicochemical constants, energy consumption, and heating constants of the biodiesel which is produced from inedible olive oil. The findings are then compared to those obtained by TAT.

2. MATERIALS AND METHODS

2.1. Materials

All experimental chemicals were analytical grade and supplied from Sigma–Aldrich (St. Louis, USA) and Merck (Darmstadt, Germany). The olive oil (Molecular weight of 871.0656 g/mol, Kinematic viscosity of 36.574 ± 0.015 mm^2/s, density of 882.1854 ± 0.9636 kg/m^3, and refractive index of 1.4685 ± 0.007) was purchased from Etka Company (Rudbar, Iran). The major fatty acids in the olive oil were oleic acid (69.40 ± 2.86 %), Linoleic acid (11.09 ± 1.76 %), palmitic acid (13.98 ± 1.17 %), and stearic acid (3.38 ± 0.29 %).

2.2. Biodiesel production methods

2.2.1. Esterification procedure

According to Chai *et al*. (2014), UAE was carried out with the help of sulfuric acid (10%, w/w H_2SO_4/ free fatty acid) as the catalyst, which was dissolved in methanol (methanol/free fatty acid molar ratio

of 40). The said ingredients were mixed for 5 minutes. In conforming with the American Oil Chemists' Society (AOCS) Official Method (Ca 5a-40), the free fatty acid of olive oil is 2.36%, which can increase to 2.5, 5.0, and 10.0% by pure oleic acid (AOCS, 2000). The sulfuric acid-methanol solution was mixed with 100 g of inedible olive oil which contained high amounts of free fatty acid. The reaction lasted for 30 minutes using an ultrasonic probe (Bandelin HD 3200, Bandelin Electronics, Berlin, Germany). Distilled water was used as a temperature regulator by circulating in the jacketed vessel. A high grade titanium tip (TT13, 13 mm diameter) assisted in sonicating the substrates with an unchanging horn depth of 2 cm. Also, the hot plate mixer operated at 60 ºC, for 120 minutes, and at 600 rpm as the traditional-assisted esterification method. At the end of the reaction time, the reaction mixture was immediately cooled down to room temperature. The water-soluble components were separated from the product in a separatory funnel. Esterification yield was calculated in conformity with a method used previously by Chai *et al*. (2014).

2.2.2. Transesterification procedure

UAT was optimized including the methanol/oil mole ratio (3, 9, and 15), KOH catalyst concentration (0.5, 1.0, and 1.5 %), power level (30, 90, and 150 W), reaction time (15, 30, and, 45 min), and reaction temperature (45, 55, and 65 ºC). Each factor was optimized by considering an intermediate value (central point) of other factors.

The catalyst-methanol solution was mixed with 100 g esterified inedible olive oil. Then, the reaction took place with the help of an ultrasonic probe. A hot plate mixer operated at 60 ºC for 120 minutes and at 600 rpm as TAT.

At the end of the reaction, fatty acid methyl esters were refined chemically according to a method used previously by Dehghan *et al*., 2019. Methyl margarate was added to the crude biodiesel as an internal standard (Atapour and Kariminia, 2011). The purity of biodiesel was determined using the GC/FID in conformity with the method reported by Golmakani *et al*., 2012a, 2012b. The weight of yield, purity, and final yield were determined according to methods reported by Atapour and Kariminia, 2011; Hsiao *et al*., 2010.

2.3. Physicochemical constants of biodiesel

The kinematic viscosity, refractive index and density of biodiesel were measured in conformity with the guidelines of the American Society for Testing Materials (ASTM; D445), the AOCS Cc7-25 Official Method, and the AOCS 1a-64 Official Method, respectively (AOCS, 2000; ASTM, 2013). The profile of fatty acids in the biodiesel was measured in conformity with a method used by Golmakani *et al.* (2012a) and, Golmakani *et al.* (2012b). Also, the color attributes (L*, lightness; a*, greenness-redness; b*, blueness-yellowness) of biodiesel were measured according to Habibi *et al*. (2016).

2.4. Examination of biodiesel under optimal states

An optimal state under which UAT operated efficiently was developed to a point where the purity, weight of yield and final yield of the biodiesel reached their highest values. The heating constants of the biodiesel (i.e. cloud, flash, fire, and pour points) were measured under optimal conditions in conformity with the ASTM Official Methods (D2500, D92, and D97) (ASTM, 2013). The amount of energy consumed along with the processes of alcohol separation, washing, and drying, along with the specific consumptions of energy for the production of 1 kg of biodiesel, were measured using a method reported by Motasemi and Ani (2012).

2.5. Statistical analysis

Experiments were repeated at least three times and the data were calculated as mean values of the measurements. Standard deviations are reported in the tables and figures. A general linear model (GLM) analysis from SAS (version 9.2; SAS Institute Inc. Cary, USA) was used to compare among the means.

3. RESULTS

3.1. Esterification yield

The initial free fatty acid concentration and the esterification method caused various effects on the esterification yield of inedible olive oil (Table 1). The highest esterification yield was obtained in the presence of 2.5% initial free fatty acid. By the end of the operation, this corresponded with the free fatty acid concentration being lower than 0.5%. Even

Table 1. Effect of traditional- and ultrasound-assisted esterification on the yield of high free fatty acid olive oil.

Initial free fatty acid concentration	Esterification method	Final free fatty acid concentration	Esterification yield (%)
2.5	Traditional	0.46±0.02^{c}*	81.60±0.09^{a}
2.5	Ultrasound	0.45±0.08^{c}	82.00±0.07^{a}
5	Traditional	1.00±0.00^{b}	80.00±0.02^{b}
5	Ultrasound	0.98±0.09^{b}	80.40±0.19^{b}
10	Traditional	2.05±0.04^{a}	79.50±0.50^{b}
10	Ultrasound	1.99±0.02^{a}	80.10±0.01^{b}

* Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's multiple range test; Degree of significance: $P < 0.05$; In each column, means with different letters are significantly different.

though no significant differences were observed between the esterification yield of inedible olive oil in the presence of 5.0% free fatty acid and 10.0% free fatty acid, their esterification yields were lower than that of the sample with 2.5% free fatty acid. In spite of the fact that no significant differences were observed between the UAE and TAE methods, including their final free fatty acid concentration and esterification yield, the UAE operated at a higher speed (i.e. 4 times faster) compared to the TAE. During sonication, fluids tend to undergo rapid distortion due to the compression and rarefaction movements initiated by the sonic waves, which is followed by a cavitation effect on the reaction stream formed by the rapid creation and collapse of micro bubbles. This process significantly affects the mechanism of the reaction, thereby enhancing the reaction rate. Hence, the impact of ultrasound can increase the catalyst activity by its least contribution to the reaction. Also, it has the tendency to increase the mass transfer rate, thereby accelerating free fatty acid esterification (Yasvanthrajan *et al.,* 2020). These findings are in accordance with previous results (Trinh *et al*., 2018) in which the esterification of rubber seed oil was evaluated. The mentioned research showed that UAE and TAE methods reduced the free fatty acid concentration after being subjected to 30 and 90 min of reaction time, respectively.

3.2. Effects of UAT on biodiesel yield

3.2.1. Ultrasound power level

The power level of the ultrasound affected the yield indices of the produced biodiesel (Figure 1a). By increasing the ultrasound power level from 30 to 90 W, the final yield was enhanced from 76.10 to 92.69%. This positive effect can be attributed to broader acoustic streaming and more cavitation bubbles. The increase in the mentioned factors can cause enhancements in the dispersion and mass transferal effects of the ultrasound, while allowing the two immiscible reaction layers to be thoroughly emulsified (Joshi *et al*., 2018; Korkut and Bayramoglu, 2018). However, the final yield decreased from 92.69 to 80.62% when the ultrasound power was further increased from 90 to 150 W. In fact, high levels of power can make the tip of the horn overpopulated with bubbles, thereby resulting in bubble coalescence and acoustic decoupling. These criteria act to reduce the number of active cavitation bubbles (Korkut and Bayramoglu, 2018). The ultrasound power of 90 W proved to be high enough to obtain a purity of 98.95% and a final yield of 92.69%, under the studied conditions. These findings are in accordance with the results of Korkut and Bayramoglu (2018) who evaluated the UAT of canola oil. They reported that by raising the ultrasound power to 40 W an increase in the reaction yield by up to 99.4% was observed; whereas higher levels of ultrasound power reduced the reaction yield.

3.2.2. Methanol/oil mole ratio

As shown in Figure 1b, the methanol/oil mole ratio affected the yield indices. Rising the mole ratio from 3 to 9, significantly increased the yield indices. However, as the mole ratio increased from 9 to 15, a downward trend was recorded for the yield indices. When the methanol/oil mole ratio measured 9, the results corresponded with the highest weight of yield, purity, and final yield (93.68, 98.95, and 92.69%, respectively). Since transesterification is

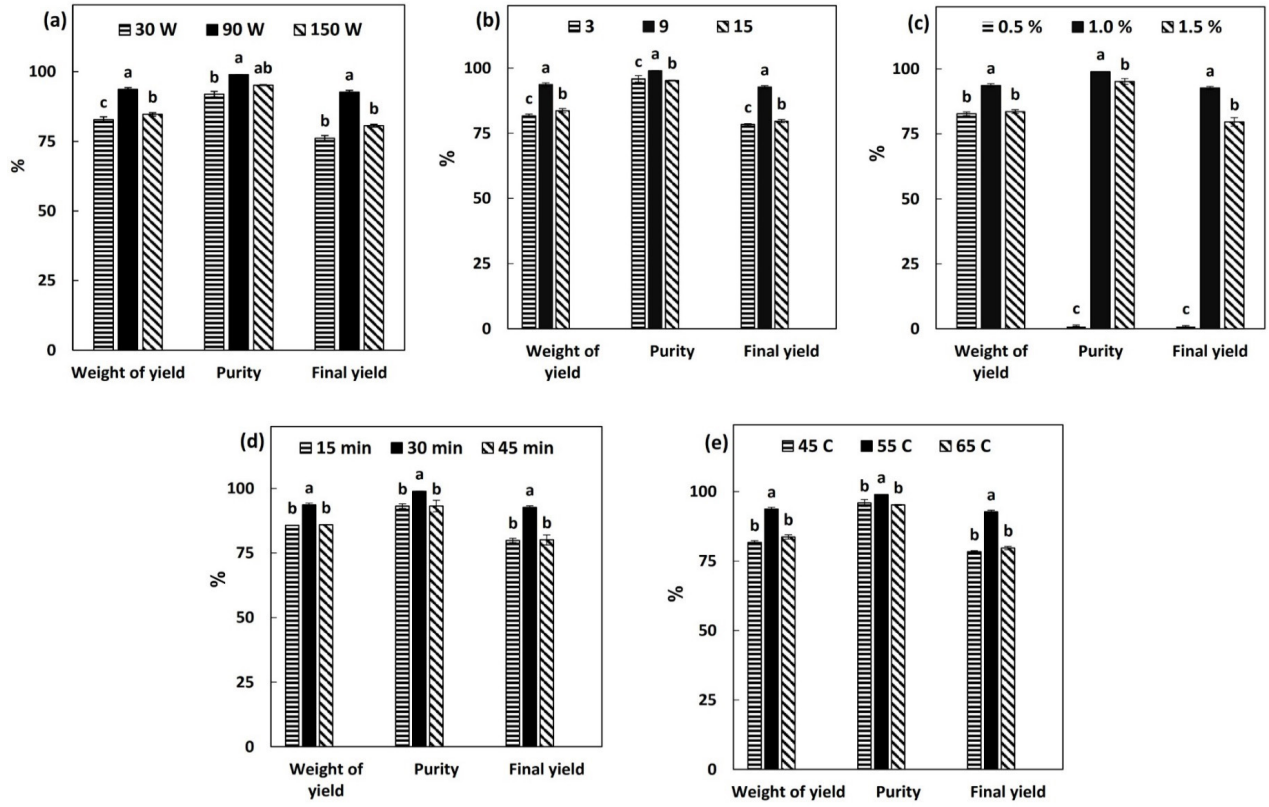

FIGURE 1. Effects of (a) microwave power level, (b) methanol/oil mole ratio, (c) catalyst concentration, (d) reaction time, and (e) reaction temperature on ultrasound-assisted transesterification of inedible olive oil. Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's multiple range test; Degree of significance: $P < 0.05$; In each figure and for each response, means with different letters are significantly different.

an equilibrium reaction, larger amounts of methanol can modify the reaction to make the equilibrium favorable to the formation of fatty acid methyl esters, The stoichiometric mole ratio of triacylglycerol to methanol for the occurrence of transesterification is 1:3 and, in fact, an excess amount of methanol is preferable for the completion of the reaction (Ho *et al*., 2016). At high methanol/oil mole ratios, the lower concentration of oil causes a decrease in the interaction between triacylglycerol molecules and catalytic active sites, thereby reducing the reaction kinetics and the reaction yield. Moreover, freeing the reaction mixture from unnecessary methanol requires more energy (Korkut and Bayramoglu, 2018). Ultrasound radiation rapidly generates tiny methanol droplets and, as a result, the emulsion forms easily and quickly in the oil phase. When the solution is rich in methanol, the droplets tend to become larger in the emulsion. In such circumstances, larger droplets are created as a result of the frequent encounters among small-size droplets of methanol, compared to when methanol is not abundantly present in the solution. As large droplets become more common, the transesterification reaction could then take on a slower rate (Thanh *et al*., 2010). Similarly, Yin *et al*. (2017) studied how different mole ratios of methanol to soybean oil (4-12) can affect UAT yield. They showed that the UAT yield significantly increased by rising the mole ratio from 4 to 8. Then, by rising the mole ratio from 8 to 10, the yield did not increase significantly. By its further increase to 12, the UAT yield decreased to an extent that was lower than the amount obtained when there was a mole ratio of 8.

3.2.3. Catalyst concentration

The transesterification reaction is commonly affected by the concentration of the catalyst. This factor was observed to have significant effects on the yield indices (Figure 1c). The lowest values for biodiesel purity (1.17%) and its final yield (0.97%) were recorded when the catalyst concentration was

0.5%. An insufficient catalyst concentration could not contribute adequately to the conversion of inedible olive oil to biodiesel. The yield indices of biodiesel increased as the catalyst concentration rose to 1.0% in terms of adequacy. This correlation is natural since a higher concentration of catalysts is associated with more of their active sites, and thus the conversion of reactants is better facilitated for the reaction to be pushed forward. The transesterification reaction begins with the carbonyl carbon atoms of monoacylglycerol, diacylglycerol, and triacylglycerol reacting with methoxide ions (CH_3O^-). Dissolving KOH in methanol produces CH_3O^- according to the following reaction: $OH^- + CH_3OH \rightarrow CH_3O^- + H_2O$. Since KOH is a strong alkaline, it has a large "dissociation constant" in methanol. Therefore, the concentration of CH_3O^- depends only on the concentration of the KOH catalyst. The result is that a higher concentration of KOH encourages a faster procession in the reaction (Ho *et al*., 2016; Thanh *et al*., 2010). Raising the catalyst concentration from 1.0 to 1.5% reduced the production of biodiesel. Several disadvantages usually discourage researchers from using catalysts excessively when experimenting with transesterification reactions. Large amounts of catalyst tend to increase the extent of how soluble fatty acid methyl esters can be in glycerol, thereby causing residual levels of fatty acid methyl ester after glycerol has been separated. Decreasing the fatty acid methyl ester content would reduce the purity and yield of biodiesel as well, (Thanh *et al*., 2010). Furthermore, higher concentrations of catalysts usually increase the viscosity of the reaction mixture, and this acts as a hindrance to glycerol separation (Korkut and Bayramoglu, 2018). The high concentrations of catalysts can lead to prolific levels of soap formation which then cause difficulties in the separating step of emulsification. This chain of events ultimately concludes with a lower biodiesel yield (Yin *et al*., 2017). As a consequence, the optimal amount of KOH in this study was 1.0%, as far as the relevant treatments are concerned. Similarly, Deshmane and Adewuyi (2013), produced biodiesel from soybean oil using UAT at different catalyst concentrations (0.6–1.4%). They reported that the biodiesel yield was improved by raising the catalyst concentration from 0.6 to 1%. However, further increases in the concentration amounted to almost no change or even a slight decrease in the reaction rate.

3.2.4. Reaction time

The duration of the reaction time had notable effects on the yield indices of biodiesel (Figure 1d). Prolonging the reaction time from 15 to 30 min caused an increase in the yield indices of biodiesel. At the early stages of the reaction, the provision of an insufficient contact time between the reaction mixtures led to lower levels of purity and yield. The highest values pertaining to the weight of yield, purity, and the final yield were obtained during a total reaction time of 30 min (93.68, 98.95, and 92.69%, respectively). The reversible nature of the transesterification reaction is a reason for the decrease in yield indices when reactions take longer than 30 min. This reduction could be due to the solubility of fatty acid methyl ester in the glycerol and also because of other unwanted reactions that can negatively affect the yield and purity (Badday *et al*., 2013). Similar observations were also reported (Badday *et al*., 2013) when the ultrasound method was used for transesterified crude Jatropha oil. In the mentioned research, an increase in biodiesel yield was observed when the reaction time was prolonged from 10 to 60 min, but it started to decrease if the reaction took more than 60 min.

3.2.5. Reaction temperature

The yield indices of biodiesel were affected by the temperature at which the ultrasound operated (Figure 1e). Rising the reaction temperature from 45 to 55 °C caused significant increases in biodiesel yield indices. These findings are logical to obtain because KOH has an enhanced solubility, while there is a thorough contact between the components of the reaction mixture, leading to a higher biodiesel yield (Maddikeri *et al*., 2013). However, an extra increase in the reaction temperature from 55 to 65 °C can begin to reduce the yield indices which can be attributed to the overheating of the reaction mixture. This could result in the loss of methanol and energy, along with a lower level of cavitational effects. Also, due to the lower thermal stability of fatty acid methyl esters under extreme conditions and the decomposition of the fatty acid methyl esters (especially the polyunsaturated ones) which can further produce by-products, temperatures higher than 55 °C have adverse effects on fatty acid methyl esters (Maddikeri *et al*., 2013). Therefore,

the reaction temperature of 55 °C is considered the most suitable temperature for the production of biodiesel from inedible olive oil. Similarly, Maddikeri *et al*., 2013 showed how different temperatures (i.e. 30-50 °C) affected the UAT yield of waste cooking oil. They stated that an increase in temperature from 30 to 40 °C caused an increase of 10% (from 78 to 88%) in the biodiesel yield, whereas another 10 °C increase (from 40 to 50 °C) in the reaction temperature only made a negligible improvement (from 88 to 93%) in the biodiesel yield (Maddikeri *et al*., 2013).

3.3. Biodiesel examination

Different UAT factors affected the physical constants of inedible olive oil methyl esters (Table 2). The biodiesel kinematic viscosity is one of the most important constants that can be affected by the storage condition, transportation, and the operation aimed at fatty acid methyl esters. Even though there was a high kinematic viscosity of inedible olive oil at 40 ºC (36.806 mm^2/s), the value decreased significantly after transesterification. The kinematic viscosity reached its lowest value (4.201 mm^2/s) in the presence of an optimal state of UAT operation. In conformity with the ASTM D6751 Official Method, the normal range of kinematic viscosity for biodiesel is 1.9-6 mm^2/s. Therefore, all fatty acid methyl esters fall within the standard range indicated by ASTM.

A negative correlation was observed between the kinematic viscosity and the final yield. The values pertaining to the power level, methanol mole ratio, catalyst concentration, reaction time and reaction temperature were R^2=0.65, 0.44, 0.98, 0.29, and 0.66, respectively. Being an ester, triacylglycerol consists of three fatty acids in association with glycerol. It can be converted to three fatty acid methyl esters with lower molecular weights after transes-

Table 2. Effect of ultrasound-assisted transesterification on physical constants of olive oil methyl esters.

Transesterification factor	Kinematic viscosity (mm^2/s)	Density (kg/m^3)	Refractive index	Color attribute		
				L*	a*	b*
Power level (W)						
30	5.132±0.002^{b}**	888.9797±0.9811^{b}	1.4544±0.0001^{a}	52.32±2.92^{c}	-4.27±1.59^{a}	48.11±2.15^{b}
90	4.201±0.000^{c}	892.9963±0.3292^{a}	1.4530±0.0000^{c}	57.66±5.13^{a}	-6.33±2.08^{b}	26.33±3.05^{bc}
150	5.651±0.000^{a}	886.6598±0.6398^{c}	1.4541±0.0001^{b}	48.63±3.98^{bc}	-2.31±1.11^{bc}	39.16±1.92^{a}
Methanol/oil mole ratio						
3	6.746±0.003^{a}	885.6811±0.8676^{b}	1.4669±0.0000^{a}	54.86±1.76^{c}	-4.11±1.54^{a}	52.22±6.65^{a}
9	4.201±0.000^{c}	892.9963±0.3292^{a}	1.4530±0.0000^{c}	57.66±5.13^{a}	-6.33±2.08^{b}	26.33±3.05^{b}
15	6.552±0.001^{b}	885.0180±0.6793^{c}	1.4534±0.0001^{b}	47.67±1.93^{b}	-3.22±1.35^{a}	52.45±2.61^{a}
Catalyst concentration (%)						
0.5	35.755±0.007^{a}	886.6569±0.6651^{c}	1.4664±0.0001^{a}	41.66±2.51^{c}	-1.45±0.57^{a}	51.76±1.67^{a}
1.0	4.201±0.000^{c}	892.9963±0.3292^{a}	1.4530±0.0000^{b}	57.66±5.13^{a}	-6.33±2.08^{c}	26.33±3.05^{b}
1.5	4.321±0.000^{b}	891.0325±0.2759^{b}	1.4533±0.0001^{b}	46.45±4.04^{ab}	2.54±1.64^{b}	45.74±4.26^{c}
Reaction time (min)						
15	4.252±0.001^{b}	890.0486±0.3308^{b}	1.4539±0.0001^{a}	49.33±7.23^{ab}	-4.33±1.52^{ab}	49.33±3.51^{b}
30	4.201±0.000^{c}	892.9963±0.3292^{a}	1.4530±0.0000^{c}	57.66±5.13^{a}	-6.33±2.08^{b}	26.33±3.05^{ab}
45	5.016±0.001^{a}	886.7189±0.1692^{c}	1.4535±0.0002^{b}	51.33±3.51^{b}	-3.60±2.51^{a}	50.66±4.04^{a}
Temperature (ºC)						
45	5.237±0.006^{a}	886.6705±0.3366^{b}	1.4548±0.0001^{a}	52.33±5.50^{ab}	-4.66±3.78^{b}	45.50±3.21^{b}
55	4.201±0.000^{c}	892.9963±0.3292^{a}	1.4530±0.0000^{c}	57.66±5.13^{a}	-6.33±2.08^{ab}	23.50±3.03^{ab}
65	4.556±0.004^{b}	873.7199±0.5203^{c}	1.4540±0.0001^{b}	49.66±1.52^{b}	-5.33±2.51^{a}	44.50±6.11^{a}

** Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's multiple range test; Degree of significance: $P < 0.05$; In each column and for each factor, means with different letters are significantly different.

terification (Talebian-Kiakalaieh *et al*., 2013). An increase in the final yield can be accompanied by a decrease in the molecular weight and, subsequently, a decrease in the kinematic viscosity.

Inedible olive oil showed a density of 880.1510 kg/m^3, which increased following the process of transesterification. The biodiesel increased in density and reached its highest value when there were optimal UAT conditions, resulting in the maximum final yield. Since the saturation degree of the biodiesel was not significantly affected by transesterification, triacylglycerols are not thought to cause the density value for the biodiesel and, instead, the density is defined by the molecular weight of the biodiesel (Habibi *et al*., 2016). A positive correlation was observed between the density and the final yield. The values pertaining to the power level, methanol mole ratio, catalyst concentration, reaction time and reaction temperature were R^2=0.65, 0.30, 0.97, 0.71, and 0.48, respectively. So, the highest amount of final yield (i.e. the lowest molecular weight) and the highest density were recorded under optimal states of UAT.

Inedible olive oil had a refractive index of 1.4685, which decreased because of transesterification. Optimal conditions of UAT caused the lowest value for refractive index (1.4530), thereby making the final yield increase maximally. Furthermore, a strong negative correlation existed between the refractive index and the final yield (R^2=0.86-0.99). Accordingly, a consistent feature of the physical constant is the refractive index when researchers aim to predict progress in transesterification.

Biodiesel can be evaluated qualitatively by its color, and it can be used in assessments of the transesterification process. The highest value for L* (57.66) and the lowest for a* (-6.33) and b* (26.33) were observed under optimal states of UAT. An increase in the L* value, along with a higher value for the final yield, were associated with lower a* and b* values. As the physical constants change, the transesterification yield changes significantly.

3.4. Comparison between UAT and TAT

3.4.1. Weight of yield, purity, and final yield

According to the findings, the optimal state for the operation of UAT can be described as a set of circumstances which include a power level of 90 W, a methanol/oil mole ratio of 9, a catalyst concentration of 1.0%, a reaction time of 30 min, and a reaction temperature of 55 °C. Figure 2 compares the optimum conditions of UAT and TAT. The weight of yield, purity, and final yield of UAT were higher (93.68, 98.95, and 92.69%, respectively) compared to those obtained by TAT (88.66, 87.17, and 77.28%, respectively). The cavitation and its physical effects can lead to this improved performance. Furthermore, there are related features such as the liquid micro-circulation (acoustic streaming), local turbulence and micro-emulsion formation. These features increase the interactions that are necessary for the reaction. An external, mass transfer barrier existed between the oil and the methanol phase. Since micro-emulsions joined the two phases that participated as reactants, an enormous increase was observed in the areas where the reactants collide, thereby accelerating the reaction rates. In the case of TAT, the reactants receive energy exclusively as heat, and this occurs by the mechanisms of conduction and convention. These two mechanisms do not contribute to the establishment of a sufficient contact between the reactants. Accordingly, the UAT is capable of reducing the reaction time significantly, as compared to the duration required by the TAT. The UAT can achieve higher levels of purity and more yield. These findings are in accordance with the results of Maddikeri *et al*. (2013), who produced biodiesel from waste cooking oil by using the UAT operation. A higher biodiesel yield was reported (90%) when using UAT compared to TAT (70%).

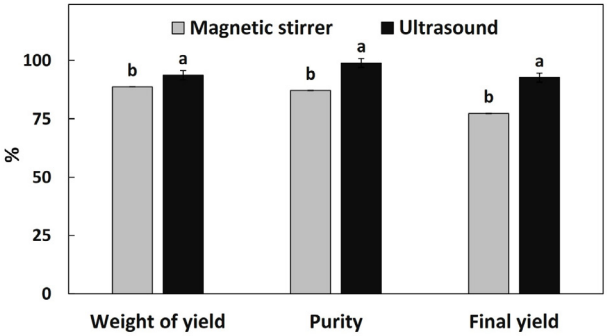

Figure 2. Effects of different transesterification methods on fatty acid methyl ester yield of inedible olive oil. Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's multiple range test; Degree of significance: $P < 0.05$; For each response, means with different letters are significantly different.

3.4.2. Physicochemical constants of biodiesel

No considerable differences were observed between the profiles of fatty acids when using the UAT and TAT. Accordingly, different transesterification methods had no selectivity on the transesterification of different fatty acids (i.e. with various saturation degrees and chain length). Nonetheless, the UAT created a biodiesel that showed higher values for density and L* value, but lower values for a* value, b* value, kinematic viscosity, and refractive index. These differences can be due to the higher amount of final yield obtained by transesterification when using the UAT compared to the TAT. The UAT produced a biodiesel that showed higher levels of purity than those produced by TAT. Accordingly, the application of UAT can yield a biodiesel which exhibits a higher brightness value (L* value), and thus the purification steps can be reduced.

As shown in Table 3, no significant differences were observed between the UAT and TAT, including their flash point, fire point, and cloud point. In conformity with the ASTM D6751 Official Method, the flash point, cloud point, and pour point of biodiesel must be at least 130 °C, -3 to 12 °C, and -15 to 10 °C, respectively. In this research, the biodiesel became a product that fell into the standard ASTM range. In conformity with the findings of the pour point, the

Table 3. Effects of traditional- and ultrasound-assisted transesterification on energy consumption and physicochemical constants of inedible olive oil methyl esters.

Constant	Transesterification method	
	Traditional	Ultrasound
Physical constants		
Kinematic viscosity (mm^2/s)	5.385±0.000[a]**	4.201±0.000[b]
Density (kg/m^3)	886.9347±0.0135[b]	892.9963±0.3292[a]
Refractive index	1.4553±0.0004[a]	1.4530±0.0000[b]
L*	58.13±0.85[b]	57.66±5.13[a]
a*	-8.43±0.43[a]	-6.33±2.08[b]
b*	46.40±1.14[a]	23.50±3.03[b]
Heating constants (ºC)		
Flash point	180.5±3.5[a]	181.5±2.1[a]
Fire point	190.5±4.9[a]	193.5±0.6[a]
Cloud point	3.0±1.4[a]	4.0±0.0[a]
Pour point	-3.0±1.4[a]	-8.0±0.0[b]
Fatty acid profile (%)		
Palmitic acid	15.66±0.60**	16.47±0.12
Palmitoleic acid	1.42±0.03	0.95±0.12
Stearic acid	1.39±0.40	2.30±2.60
Oleic acid	71.07±0.08	71.36±2.60
Linoleic acid	9.95±0.14	8.73±1.30
α-Linolenic acid	0.51±0.16	0.19±0.00
Energy consumption		
Transesterification reaction (kWh)	0.227	0.045
Separation of alcohol (kWh)	0.063	0.063
Separation of catalyst (kWh)	0.008	0.008
Drying (kWh)	0.071	0.071
Total energy consumption (kWh)	0.369	0.217
Specific energy consumption (kWh/kg)	4.775	2.17

** Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's multiple range test; Degree of significance: $P < 0.05$; In each row, means with different letters are significantly different.

biodiesel that had been produced by UAT eventually showed less viscosity which allowed it to be pumped at more efficient rates.

3.4.3. Energy consumption

Table 3 shows energy consumptions by UAT and TAT during the transesterification step and further through all of the steps of biodiesel purification. The percentages of energy consumption during the transesterification step were 20.74 and 61.52% for UAT and TAT, respectively. The amount of energy that was consumed during the transesterification step in TAT was 0.227 kWh, which is at least five-fold compared to that of the UAT (0.045 kWh). However, during their purification steps, the energy consumptions for both UAT and TAT were equal. TAT requires 4.775 kWh/kg to produce 1 kg biodiesel. This amount was at least 2 times higher than that required by the UAT (2.170 kWh/kg). It can be concluded that UAT can significantly increase the final yield, shorten the reaction time and reduce the energy consumption (Motasemi and Ani, 2012). The main production costs of biodiesel strongly depend on energy consumption during the transesterification step in addition to the cost of feedstock. Processing inedible olive oil by UAT has many advantages for biodiesel production. These advantages are primarily a lower energy consumption and a lower feedstock cost.

4. CONCLUSION

A comprehensive purpose of this research involved finding descriptions of how the ultrasound method can affect the esterification and transesterification of inedible olive oil compared to the traditional hot plate mixer method. No significant differences were observed between the yield obtained by UAE and TAE, but the duration of the reaction time required by UAE was significantly 4 times shorter than that of TAE.

Processing inedible olive oil by UAT showed an optimal performance, compared to that of the TAT method. The conditions in which UAT can operate optimally are an ultrasound power level of 90 W, a methanol/oil mole ratio of 9, a catalyst concentration of 1.0%, a reaction time of 30 min, and a reaction temperature of 55 °C. The UAT can significantly increase the weight of yield, purity, and final yield of the produced biodiesel (93.68, 98.95, and 92.69%, respectively) compared to TAT (88.66, 87.17, and 77.28%, respectively).

The UAT can operate within 4 times shorter amount of time and can consume lower amounts of energy compared to the TAT method. Therefore, the UAT evidently has advantages as a method for producing biodiesel by processing inedible olive oil which would be otherwise considered as a waste if it is not used in this regard and as such.

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Fatty acid composition of phospholipids and triacylglycerols in the flesh of the thick-lipped grey mullet *(Chelon labrosus)* living in Tunisian geothermal water and seawater: A comparative study

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SUMMARY: This study was conducted to elucidate the effects of rearing conditions on the composition of different phospholipid (PLs) classes and triacylglycerols (TAG) of the thick-lipped grey mullet (*Chelon labrosus*), a muscle originating from seawater and geothermal water. The major fatty acids in the examined lipid classes of the two fish groups were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6)**,** arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3), and docosahexaenoic acid (C22:6n-3). The analyses demonstrated that the fatty acid profiles of the PL classes in the seawater fish group were characterized by the predominance of n-3 polyunsaturated fatty acids (PUFA). By contrast, in geothermal fish, the distribution of PUFA series proportions differed between the phospholipid fractions. It was found PUFA n-3 was particularly abundant in PS and PI, while the n-6 series dominated the PC and PE PUFA group. Nonetheless, it was found that neutral lipid fatty acids were characterized by saturated fatty acids (SFA) followed by monounsaturated fatty acids (MUFA) in the seawater fish and by PUFA in the geothermal fish. The results presented here give useful information on the role of lipid classes in the physiological adaptation of *C. labrosus* which can serve for the optiminzation of these aquaculture systems.

KEYWORDS: *Fatty acid composition; Geothermal water, Phospholipids; Seawater thick-lipped grey mullet* (Chelon labrosus)*; Triacylglycerols.*

RESUMEN: ***Composición en ácidos grasos de fosfolípidos y triacilgliceroles de la carne del salmonete gris de labios gruesos*** **(Chelon labrosus)** ***que vive en agua geotérmica y agua de mar tunecina: un estudio comparativo.*** Este estudio se llevó a cabo para dilucidar los efectos de las condiciones de cría sobre la composición de diferentes clases de fosfolípidos (PL) y triacilgliceroles (TAG) del músculo de salmonetes de labios gruesos (*Chelon labrosus*) procedentes de agua de mar y de agua geotérmica. Los principales ácidos grasos en las clases de lípidos examinados de los dos grupos de peces fueron, palmítico (C16:0), esteárico (C18:0), oleico (C18:1n-9), linoleico (C18:2n-6), araquidónico (C20:4n-6), eicosapentaenoico (C20:5n-3) y ácido docosahexaenoico (C22:6n-3). Las determinaciones mostraron que los perfiles de ácidos grasos de los PL, en el grupo de peces de agua de mar, se caracterizaron por el predominio de ácidos grasos poliinsaturados n-3 (PUFA). Por el contrario, en los peces geotérmicos, la distribución de las proporciones de las series de PUFA difirió entre las fracciones de fosfolípidos. Se encontró que los PUFA n-3 eran particularmente abundantes en PS y PI, mientras que la serie n-6 dominaba el grupo de PUFA PC y PE. No obstante, se encontró que en lipidos neutros, los mayoritarios son los ácidos grasos saturados (SFA) seguidos de los ácidos grasos monoinsaturados (MUFA) en el pescado de agua de mar y los PUFA en el pescado geotérmico. Los resultados actuales brindan información útil sobre el papel de las clases de lípidos en la adaptación fisiológica de *C. labrosus* que puede servir para la optimización de estos sistemas de acuicultura.

PALABRAS CLAVE: *Agua geotérmica; Composición de ácidos grasos; Fosfolípidos; Salmonete gris de labios gruesos* (Chelon labrosus); *Triacilgliceroles.*

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1. INTRODUCTION

In order to meet the increased demand for seafood, aquaculture has experienced significant progress over recent decades. According to the latest FAO report, the world aquaculture production reached 114.5 million tonnes and was valued at 263.6 billion USD in 2018 (FAO, 2020). According to the same statistics, fish farming accounted for almost half (47%) of the global aquaculture production. Mullets are members of the Mugilidae family and are among the most ubiquitous fish resources worldwide. Their farming has been practiced for centuries (FAO,2015a). In the Mediterranean basin, the thick-lipped grey mullet *Chelon labrosus* is cultured in natural earthen ponds under extensive or semi-intensive regimes (De las Heras *et al*., 2015). It has been described as an easily cultivable species and constitutes a promising species for aquaculture diversification (Zouiten *et al.,* 2008; Ben Khemis *et al.,* 2013). Indeed, as other mullet species, *C. labrosus* is a low trophic level feeder, obtaining its energy directly from the first trophic level (Brusle, 1981). In addition, the eurytherm and euryhaline species, *C. labrosus* is able to tolerate wide ranges of temperature and salinity (Cardona, 2006; Rabeh *et al*., 2013). Furthermore, several authors have reported that *C. labrosus'* osmoregulation abilities appear early during in their development, allowing them to maintain elevated growth rates even under hyposaline conditions (Nordlie *et al.,* 1982; Cardona*,* 2006). In most North African regions, the production of *C. labrosus* may be carried out in a variety of ecosystems, such as coastal lagoons with brackish to hyper saline waters (Crosetti*,* 2016) or reservoirs and ponds with fresh waters (Losse *et al.,* 1991). For many years, Tunisia has opted to breed fish in reservoirs and artificial lakes as a strategy for suppling aquatic products to the interior regions and for providing work opportunities for local communities (Besbes *et al*., 2020). For instance, natural water flow from artesian sources is used directly as a culture medium in the south of Tunisia (Béchima locality) to facilitate in the raising of some freshwater and marine fish species. In this respect, recent investigation has shown the aptitude of *Oreochromis niloticus* and *C. labrosus* to live in geothermal waters (26 to 30°C) and to display good growth rates (Azaza *et al.,* 2008a; Azaza *et al.,* 2008b).

It is well established that the capacity of an organism to efficiently adapt environmental changes is predominantly dependent on its metabolic flexibility (Smith *et al.,* 2018). This phenomenan includes a series of metabolic reorganizations and biochemical adjustments allowing the animal to meet increases in energy requirements and to maintain homeostasis under new environmental condition (Soengas *et al.,* 2007). As the densest form of energy in marine ecosystems and fundamental components of the cell membrane, lipids and their key components, fatty acids (FA), play key roles in the adaptation of aquatic organisms to new environmental conditions (Fokina *et al.,* 2017). In this regard, several studies have reported high variability in the FA composition of fish depending on different abiotic and biotic factors such as the type and amount of food available, water temperature, pH, salinity, and reproduction cycle (Shirai *et al.,* 2002; Kaushik *et al.,* 2006). Changes in FA composition are likely to induce conformational remodeling of membrane proteins, including receptors and channels, and ultimately affect cell responses to extracellular challenges (Brown, 1994). FA are distributed into two major sub-classes which represent an essential and integral part of these compounds. Lipid classes can be broadly divided into neutral, mainly triacylglycerol (TAGs), and polar lipids (such as phospholipids). Phospholipids (PLs), are the major structural constituents of biological membranes. They are involved in the maintenance of membrane integrity and permeability and provide the matrix for the function of a large variety of catalytic processes (Dowhan *et al*., 2008). The most biologically important phospholipids of organisms are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). These lipid classes are particularly known for their role in the maintaining of membrane integrity and fluidity and in the ensuring of the fish acclimatization process to changes in environmental conditions (Murzina *et al.,* 2020). Neutral lipids which includeTAG, serve mainly as a depot for lipids and provide most of the energy consumed (Sargent *et al*., 1976). These molecules, which mainly come from food sources, tend to directly accumulate in fish muscle **(**Sushchik *et al*., 2020). The accumulation of these lipids can be differentiated by either external factors, such as fluctuations in environmental conditions, temperature, and food availability, or

by internal factors, such as metabolic and physiological activities.

Despite their commercial importance, there are few studies on the biochemical composition of grey mullets (Rabeh *et al*., 2015; Ben Khemis *et al*., 2019). According to our hypothesis, lipids could indicate the ability of *C. labrosus* to adapt to abiotic factors, including water temperature and salinity. Hence, the objective of the present study was to identify and compare the FA composition of individual PL (PC, PE, PI and PS) and NL (mainly TAG) classes in *C. labrosus* reared in geothermal and seawater conditions in order to gain further insight into the physiological fitness of mullets.

2. MATERIALS AND METHODS

2.1. Experimental material

Immature thick-lipped grey mullets *C. labrosus* (30 to 40 g body mass) were supplied by an experimental fish culturing center (National Institute of Marine Science and Technology (INSTM)- Tunisia). The specimens of *C. labrosus* provided by INSTM-Center de Monastir were reared in seawater conditions with a salinity of 35 ppt and an ambiant temperature ranging from18 to 20 °C. While the specimens supplied by INSTM-Center de Béchima were reared in geothermal water conditions with a salinity of 2 ppt and a temperature varying between a minimum of 23 °C and the maxima of 28 °C. During the period from June 2006 to May 2007, all fish were hand fed six times between 08.00 and 18.00 h every 2 h until satiation. To reduce the sampling differences or external influences, six individuals from each habit were randomly selected at the same age and all fed the same local diet. The diet was made of 45% fresh sardines, 40% soybean meal, 10% fish meal, 4% vegetable oil and 1% vitamin premix. The moisture, crude protein, crude lipid, crude ash and lipid composition of the diet are shown in Table 1. They were fasted for 24 h before sampling, and six specimens form each group were sacrificed and the dorsal muscles (without skin) were sampled and conserved at -30 °C until analysis.

Table 1. Ingredients of the experimental diet.

Ingredients	
Fatty acids composition %	
C14:0	4.59±0.19
C15:0	0.05±0.03
C16:0	28.80±0.90
C16:1	0.55±0.00
C16:2	1.25±0.01
C16:3	2.78±0.46
C16:4	0.39±0.01
C17:0	0.10±0.10
C18:1n-9	2.43±0.25
C18:2n-6	29.60±2.36
C18:3n-6	16.11±0.87
C18:3n-3	1.18±0.06
C18:4n-3	0.73±0.01
C20:0	0.83±0.01
C20:1n-9	25.32±2.07
C22:1n-11	0.71±0.07
C20:3n-6	0.18±0.02
C20:3n-3	0.44±0.03
C20:4n-3	0.07±0.05
C20:5n-3	0.37±0.02
C20:4n-6	0.55±0.07
C22:5n-3	2.74±0.55
C22:5n-6	1.06±0.18
C22:6n-3	1.25±0.01
C21:5	0.02±0.01
C24:1n-9	0.59±0.12
Proximate composition	
Humidity	35
Protein	37
Crude fat	13
Ash	2
Gross energy (kcal·g^{-1})	4.34

Note: Results are expressed as mole % of total FAME based on peak areas. Data are means ± standard deviations from triplicate estimations (n =3) using ANOVA (Tukey HSD test).

2.2. Lipid extraction

Lipids were extracted from fillet samples according to the method of Folch *et al.* (1957) with a mixture of chloroform:methanol (2:1, v/v) containing buthylated-hydroxy-toluene (BHT), which was added to the solvent mixture as an antioxidant. For 1 g of fresh sample (individually analyzed), 30 mL of the solvent mixture were used. After evaporation of the solvent mixture under nitrogen, the extracts were dried overnight in a vacuum desiccator and quantified gravimetrically. Once weighed, the lipids were re-dissolved

in the organic solvent and the obtained lipid extracts were conserved in -30 °C until analysis.

2.3. Lipid class separation

The lipid classes from total lipid samples were separated using thin-layer chromatography (TLC) with one dimensional double development following the method of Olsen and Henderson (1989). Concisely, 500 µL of lipid extracts from each sample were separated on silica gel plates (20 × 20 cm, Merck, Germany) into neutral and polar fractions. The plates were activated by heating at 105 °C for 1 h and developed with hexane/diethyl-ether/glacial-acetic-acid (80:20:2, v/v) for the neutral lipids (NL), and methyl acetate/isopropanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v) for the polar lipids (PL). The individual lipid categories were detected under UV light after being sprayed with 0.1% 2'-7'dichloro-fluorescein in absolute methanol. Lipid fractions were identified by corresponding standards and scraped from the plate into separate tubes and their constitutive fatty acids were transmethylated.

2.4 Analysis of fatty acids

After evaporation to dryness, the lipid extracts and fractions were trans-esterified for fatty acid analysis according to the method of Cecchi *et al.* (1985). The resulting fatty acid methyl esters (FAME) were extracted using sodium methylate ($NaOCH_3$) in the presence of hexane and sulfuric acid (H_2SO_4). Methyl nonadecanoate 19:0 (Sigma-Aldrich, St. Louis, MO, USA), which was absent from our samples, was added as an internal standard product.

Fatty acid methyl esters (FAMEs) were separated by a HP6890 gas chromatograph (Agilent Technologies; Santa Clara, CA) with a split/splitless injector. Nitrogen was the gas carrier at a flow rate of 1.5 mL·min^{-1} in an Innowax 250 capillary column (0.25 inside diameter × 30m length, 0.25 µm film; Agilent Technologies). The gradient temperature program was set as follows: from 50 °C to 180 °C at a rate of 4 °C·min^{-1}, from 180 °C to 220 °C at a rate of 1.33 °C·min^{-1}, and finally to stabilize at 220 °C for 7 min. The detector and injector were maintained at 250 °C. The identification of fatty acid methyl esters was based on the comparison of their retention times with those of authentic standards (C4 C24 by SUPELCO) and a well-characterized fish oil (Menhaden oil by SUPELCO). FA peaks were integrated and analyzed using the Agilent G2070BA GC Hewlett-Packard Chemstation Software. All fatty acid data were reported as percentage of total fatty acids.

2.5 Calculation of indices and statistical analysis

The equations of unsaturation index (UI) and unsaturated-to-saturated FA ratio (U/S) were calculated according to Snyder and Hennessey (2003) and Wallaert and Babin (1994).

The unsaturation index (UI):

$$UI = \Sigma\ (\%monoenes + 2 \times \%dienes + 3 \times \%trienes\ldots)\ /100$$

where monoenes, diened, and trienes were fatty acids containing 1, 2, 3 double bonds, respectively.

The unsaturated-to-saturated FA ratio (U/S):

$$U/S = \Sigma(\%UFA)/\Sigma(\%SFA)$$

where %: weight percentage; UFA: unsaturated fatty acids; SFA: saturated fatty acid.

The statistical analyses were performed using R software version 3.3.3 (R Core Team 2017).

The data were checked for normality using the Shapiro-Wilk's test (Shapiro and Wilk, 1965) and Levene's tests, respectively. One-way analysis of variance (ANOVA) and Tukey HSD's test (at $p < 0.05$) were performed so as to detect significant statistical differences. The results are presented as means ± standard deviation (SD). Graphs were plotted using Prism. The covariance matrix was computed, and the Principal Component Analysis (PCA) was applied using the FactoMiner R Package (Lê *et al*., 2008) to evaluate the differences in the compositions of the samples under different conditions.

3. RESULTS

Four major classes of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) in the muscle of *C. labrosus* with a dominant neutral lipid (TAG) in different rearing conditions were identified. Indeed, *C. labrosus* from geothermal water was richer in PLs and TAG than those from seawater. A detailed distribution of the fat-

Table 2. Percentages of individual fatty acids in the TAG, PE, PC, PI and PS from the muscle tissue of *Chelon labrosus* reared in seawater conditions.

	Seawater				
% Fattyacids	**PC**	**PE**	**PS**	**PI**	**TAG**
C14:0	0.53±0.07^{a}	0.19±0.02^{b}	0.61±0.07^{c}	0.48±0.01^{a}	6.95± 0.54^{d}
C15:0	0.03±0.01^{a}	0.09±0.05^{b}	0.11±0.03^{b}	0.13±0.02^{b}	0.25±0.02^{c}
C16:0	37.28±2.06a	4.38±0.09^{b}	33.22±0.28^{c}	18.12±0.58^{d}	22.30±1.26^{d}
C17:0	0.41±0.11^{a}	0.73±0.07^{b}	0.46±0.24^{c}	0.39±0.10^{a}	0.45±0.03^{c}
C18:0	7.00±2.80^{a}	35.21±0.91^{b}	2.34±0.15^{c}	4.69±0.57^{d}	3.94±0.42^{d}
C20:0	0.09±0.02^{a}	0.27±0.08^{b}	0.06±0.01^{c}	0.07±0.02^{c}	0.13±0.03^{a}
C22:0	0.02±0.01^{a}	0.04±0.01^{b}	0.04±0.01^{b}	0.05±0.01^{b}	0.04±0.01^{b}
C24:0	0.11±0.04^{a}	ND	ND	0.01±0.00^{b}	0.04±0.01^{c}
C15:1	0.34±0.05^{a}	0.06±0.02^{b}	0.34±0.08^{a}	0.24±0.05^{c}	0.76±0.02^{d}
C16:1n-9	1.12±0.16^{a}	0.49±0.16^{b}	1.51±0.35^{c}	1.40±0.02^{c}	9.28±0.17^{d}
C18:1n-9	18.00±1.03^{a}	7.84±0.11^{b}	12.85±1.89^{c}	10.10±0.26^{c}	21.14±0.66^{a}
C20:1n-9	0.68±0.09^{a}	0.89±0.05^{b}	0.42±0.07^{c}	0.49±0.08^{c}	1.16±0.05^{b}
C22:1n-11	0.19±0.02^{a}	0.18±0.03^{a}	0.22±0.02^{a}	0.30±0.03^{b}	0.40±0.02^{c}
C24:1n-9	0.10±0.01^{a}	0.32±0.07^{b}	0.21±0.07^{c}	0.32±0.03^{b}	0.12±0.01^{a}
C18:2n-6	5.94±0.20^{a}	1.92±0.23^{b}	6.08±0.39^{a}	5.87±0.15^{a}	10.06±0.02^{c}
C18:3n-6	0.13±0.02^{a}	0.12±0.03^{a}	0.14±0.01^{a}	0.17±0.06^{a}	0.50±0.12^{b}
C20:2n-6	0.44±0.06^{a}	0.28±0.03^{b}	0.26±0.03^{b}	0.22±0.00^{b}	0.40±0.03^{a}
C20:3n-6	0.15±0.02^{a}	0.20±0.03^{b}	0.19±0.00^{b}	0.26±0.03^{c}	0.05±0.01^{d}
C20:4n-6	2.14±0.45^{a}	8.02±0.15^{b}	2.81±0.09^{a}	3.47±0.14^{c}	0.91±0.16^{d}
C22:5n-6	0.68±0.07^{a}	1.07±0.24^{b}	0.96±0.18^{b}	1.20±0.16^{b}	0.16±0.01^{c}
C18:3n-3	0.50±0.10^{a}	0.40±0.28^{a}	0.95±0.35^{b}	1.17±0.07^{b}	2.35±0.06^{c}
C18:4n-3	0.16±0.03^{a}	0.54±0.05^{b}	0.35±0.08^{c}	0.73±0.17^{d}	0.71±0.02^{d}
C20:3n-3	0.06±0.01^{a}	0.11±0.07^{b}	0.08±0.00^{b}	0.08±0.02^{b}	0.18±0.01^{c}
C20:4n-3	0.20±0.02^{a}	0.25±0.03^{a}	0.48±0.18^{b}	0.70±0.05^{c}	0.52±0.13^{b}
C20:5n-3	7.46±0.27^{a}	8.57±0.68^{a}	15.10±0.47^{b}	21.40±0.70^{c}	5.85±0.33^{d}
C22:5n-3	1.68±0.17^{a}	3.71±0.27^{b}	2.41±0.37^{c}	2.74±0.04^{c}	1.14±0.07^{d}
C22:6n-3	13.56±0.47^{a}	23.40±0.86^{b}	16.62±0.16^{c}	23.42±0.10^{b}	5.48±0.01^{d}
C16:2	0.71±0.28^{a}	0.30±0.04^{b}	0.80±0.17^{a}	1.09±0.05^{c}	1.40±0.07^{d}
C16:3	0.17±0.19^{a}	0.16±0.08^{a}	0.23±0.07^{b}	0.42±0.14^{c}	1.59±0.08^{d}
C16:4	0.03±0.01^{a}	0.05±0.01^{b}	0.04±0.02^{a}	0.09±0.01^{b}	0.88±0.06^{c}
C21:5	0.11±0.01^{a}	0.20±0.04^{b}	0.14±0.01^{c}	0.16±0.01^{c}	0.28±0.01^{d}
n-3/n-6	2.4±0.03^{a}	1.44±0.05^{b}	3.45±0.08^{c}	4.5±0.07^{d}	1.25±0.12^{b}
U/S	1.4±0.1^{a}	2.8±0.02^{b}	1.3±0.11^{a}	3.18±0.04^{c}	1.09±0.02^{d}
UI	1.06±0.01^{a}	1.7±0.02^{b}	2.3±0.1^{b}	2.9±0.02^{c}	0.9±0.1^{d}
Total PL (mg·g^{-1} Fw)			0.52±0.05		
TAG (mg·g^{-1} Fw)			0.12±0.01		

Note: Results are expressed as mole % of total FAME based on peak areas. Data are means ± standard deviations from triplicate estimations (n =3). Means followed by different letters in the same line are significantly different ($p < 0.05$) using ANOVA (Tukey HSD test). SFA, MUFA, and PUFA mean saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid, respectively. PC, PE, PI, PS and TAG mean phosphatidylcholine; phosphatidylethanolamine; phosphatidylinositol; phosphatidylserine and triacylglycerol, respectively. ND = not detected.

ty acids within the classes is given in Tables 2 and 3. The results show that the amount of neutral and polar lipids of *C. labrosus* from geothermal water was more important than those from seawater. The predominant FAs in the two groups (seawater and geothermal) were C16:0, C18:0, C18:1, C18:2n-6, C20:4n-6, C20:5n-3 and C22:6n-3 in PL and TAG. Among saturates, palmitic acid (C16:0) was the major fatty acid in all lipid classes and exhibited the highest levels, mainly in the PC, PS, and TAG of the two studied fish groups (Tables 2 and 3). In addition, stearic acid (C18:0) appeared to be particularly abundant in both polar and neutral lipid fractions. The main monounsaturated fatty acid (MUFA) was oleic acid (C18:1) and was found in high amounts in PC as well as in TAG (in both seawater and freshwater groups). Regarding polyunsaturated fatty acids (PUFA), we noted a significant increase in C18:2n-6

in the major polar lipid classes of the geothermal group. C22:6n-3 (DHA) and C20:5n-3 (EPA) were particularly abundant in PI. Indeed, 23 to 25% of PI were made of DHA for both fish groups.

To better visualize the differences in FA in the lipid classes, five-line charts referring to the major fatty acid groups, namely satured fatty acids (SFA), MUFAs and PUFAs, are presented in Figure 1. The results showed that in almost all lipid fractions of the seawater fishes, PUFA (representing up to 50% of the total FA of PS species) was by far the major FA group followed by SFA with corresponding increases in UI and U/S. By contrast, in geothermal fish the distribution proportions of the PUFA series substantially differed between polar lipid fractions. Indeed, PUFA n-3 was particularly abundant in PS and PI fractions while the n-6 series dominated the PC and PE subclass. Additionally, it was found that

Table 3. Percentages of individual fatty acids in the TAG, PE, PC, PI and PS from the muscle tissue of *Chelon labrosus* reared in geothermal water.

	Geothermal water				
% Fattyacids	**PC**	**PE**	**PS**	**PI**	**TAG**
C14:0	0.51±0.19^{a}	0.29±0.05^{b}	1.91±0.09^{c}	1.15±0.06^{d}	3.23±0.68^{e}
C15:0	0.65±0.03^{a}	0.68±0.28^{a}	0.44±0.07^{b}	0.40±0.06^{b}	7.92±0.65^{c}
C16:0	33.09±0.90^{a}	10.99±0.06^{b}	7.81±0.58^{b}	6.54±0.29^{b}	24.08±1.31^{a}
C17:0	0.74±0.10^{a}	0.19±0.07^{b}	0.46±0.07^{c}	1.19±0.04^{d}	2.60±0.27^{e}
C18:0	5.67±1.66^{a}	12.84±0.23^{b}	31.74±1.76^{c}	13.78±0.59^{b}	10.38±1.37^{b}
C20:0	0.10±0.01^{a}	0.47±0.01^{b}	0.07±0.01^{c}	0.22±0.04^{d}	0.10±0.01^{a}
C22:0	0.12±0.00^{a}	0.61±0.07^{b}	0.28±0.07^{c}	0.23±0.02^{c}	0,29±0.09^{c}
C15:1	0.55±0.00^{b}	0.29±0.02^{b}	0.31±0.05^{a}	0.64±0.14^{b}	0.75±0.03^{a}
C16:1n-9	2.43±0.25^{b}	1.94±0.06^{b}	0.93±0.06^{b}	1.41±0.08^{a}	6.03±0.33^{b}
C18:1n-9	25.32±2.07^{b}	15.54±0.32^{b}	9.00±0.52^{b}	11.06±0.14^{b}	15.41±0.32^{b}
C20:1n-9	0.71±0.07^{a}	0.83±0.13^{a}	0.81±0.12^{b}	0.45±0.13^{a}	1.20±0.34^{a}
C22:1n-11	0.59±0.12^{b}	0.53±0.07^{b}	0.17±0.01^{b}	0.06±0.01^{b}	0.37±0.02^{ab}
C24:1n-9	ND	ND	ND	1.31±0.24^{b}	ND
C18:2n-6	16.11±0.87^{b}	28.34±0.01^{b}	3.44±0.25^{b}	11.29±1.12^{b}	8.80±0.29^{b}
C18:3n-6	ND	ND	0.11±0.02^{a}	0.13±0.03^{a}	ND
C20:2n-6	0.18±0.02^{b}	1.09±0.11^{b}	0.14±0.02^{b}	0.26±0.11^{a}	1.49±0.29^{b}
C20:3n-6	0.55±0.07^{b}	2.16±0.01^{b}	0.94±0.06^{b}	0.76±0.15^{b}	0.36±0.05^{b}
C20:4n-6	1.06±0.18^{b}	1.53±0.35^{b}	9.34±0.91^{b}	5.22±0.44^{b}	1.98±0.28^{b}
C22:5n-6	1.18±0.06^{b}	1.23±0.27^{a}	1.46±0.21^{b}	3.17±0.30^{b}	0.69±0.15^{b}
C18:3n-3	0.73±0.01^{b}	1.27±0.05^{b}	0.26±0.04^{b}	0.56±0.05^{b}	1.17±0.25^{b}
C18:4n-3	0.44±0.03^{b}	1.37±0.03^{b}	0.08±0.01^{b}	0.49±0.05^{b}	1.46±0.22^{b}
C20:3n-3	0.07±0.05^{a}	1.21±0.07^{b}	0.36±0.03^{b}	0.37±0.05^{b}	0.23±0.08^{a}
C20:4n-3	0.37±0.02^{b}	1.20±0.19^{b}	0.62±0.09^{a}	0.69±0.08^{a}	0.63±0.20^{a}
C20:5n-3	2.74±0.55^{b}	5.16±0.51^{b}	5.54±0.55^{b}	6.44±0.08^{b}	2.47±0.41^{b}
C22:5n-3	1.25±0.01^{b}	1.67±0.05^{b}	5.10±0.45^{b}	4.12±0.07^{b}	0.94±0.13^{a}
C22:6n-3	2.78±0.46^{b}	6.59±0.16^{b}	17.36±1.45^{a}	25.63±0.66^{b}	4.96±0.12^{b}
C16:2	0.39±0.01^{b}	0.14±0.00^{b}	0.70±0.11^{a}	0.94±0.02^{b}	0.85±0.03^{b}
C16:3	0.84±0.07^{b}	1.14±0.03^{b}	0.23±0.03^{a}	0.87±0.07^{b}	1.20±0.19^{b}
C16:4	0.02±0.01^{a}	0.32±0.02^{b}	0.12±0.02^{b}	0.04±0.01^{b}	0.13±0.01^{b}
C21:5	0.83±0.11^{b}	0.36±0.04^{b}	0.24±0.03^{b}	0.42±0.05^{b}	0.27±0.02^{a}
n-3/n-6	0.44±0.02^{a}	2.83±0.02^{b}	1.89±0.03^{c}	1.7±0.09^{c}	0.8±0.01^{d}
U/S	1.2±0.03^{a}	1.44±0.1^{b}	1.7±0.02^{c}	3.17±0.1^{d}	1.8±0.05^{c}
UI	1.5±0.05^{a}	2.5±0.06^{b}	2.6±0.04^{b}	3.1±0.1^{c}	1.47±0.08^{c}
Total PL (mg·g^{-1} Fw)			0.64±0.02		
TAG (mg·g^{-1} Fw)			0.27±0.05		

Note: Results are expressed as mole % of total FAME based on peak areas. Data are means ± standard deviations from triplicate estimations (n =3). Means followed by different letters in the same line are significantly different ($p < 0.05$) using ANOVA (Tukey HSD test). SFA, MUFA, and PUFA mean saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid, respectively. PC, PE, PI, PS and TAG mean phosphatidylcholine; phosphatidylethanolamine; phosphatidylinositol; phosphatidylserine and triacylglycerol, respectively. ND = not detected.

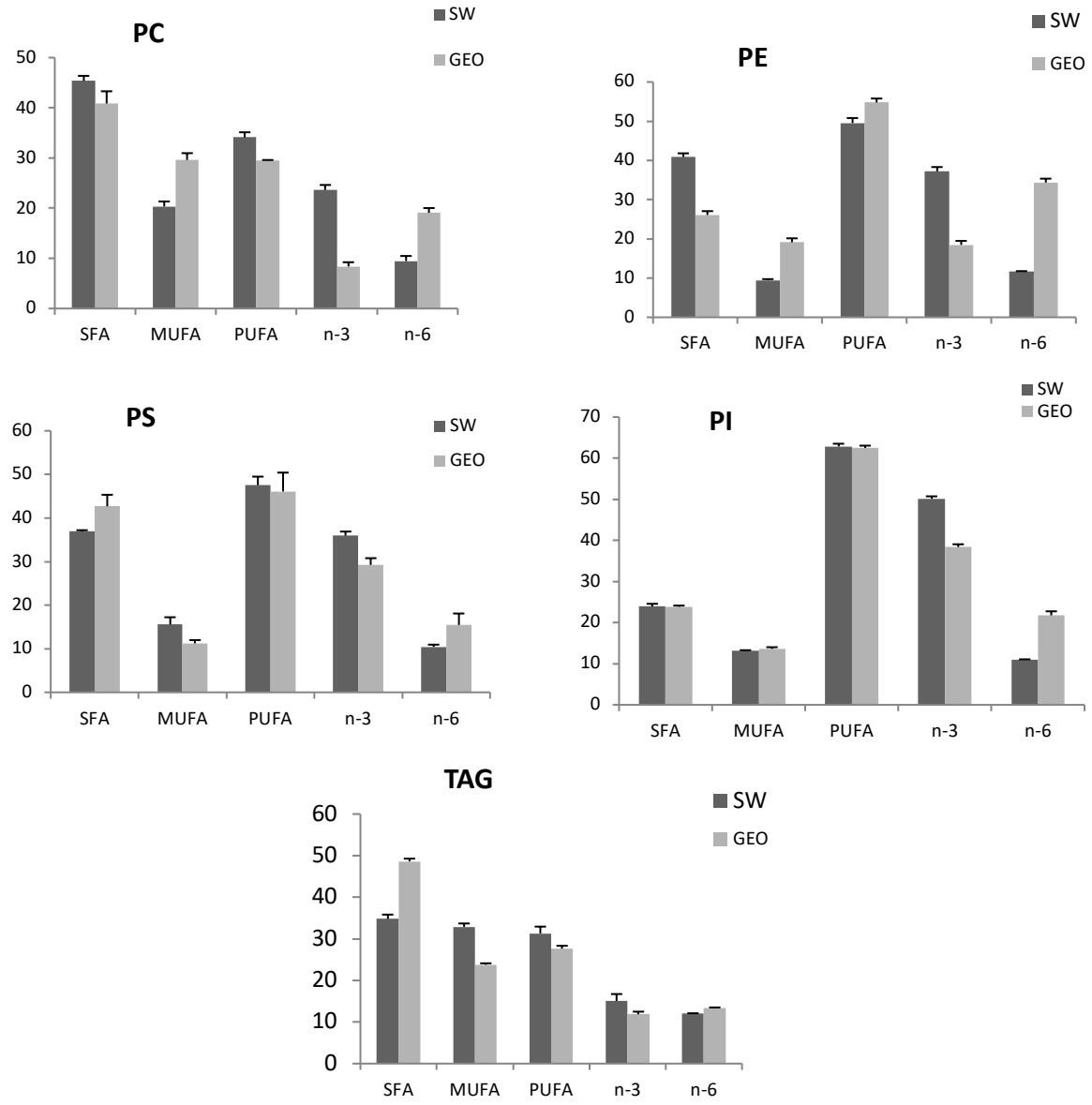

Figure 1. Changes in SFA, MUFA, PUFA, n-3 and n-6 of PC, PE, PS, PI, and TAG in the muscle of *Chelon labrosus* reared in seawater and geothermal water. Values reported are mean ± SD from triplicate estimations (n =3) using ANOVA (Tukey HSD test).

the level of n-3/n-6 ratio was markedly increased in the PE fraction of the geothermal group. It is also important to note that, SFA stood out as the most abundant fatty acid group in TAG mainly in the geothermal group.

The principal component analysis (PCA) was performed to gain better insight into the effects of rearing conditions on the fatty acid composition of lipid classes in the flesh of the thick-lipped grey mullet, *C. labrosus*. Figure 2 depicts the twoprincipal-components that described 57.72% of the total data variability (PC1 34.22% and PC2 23.5%). The PCA biplot of the overall data described a clear separation between samples from the seawater and the geothermal water. The projection of individuals (each fatty acid from each lipid class of the different fish groups) on the same factorial plan (1:2) showed that samples could be clustered into two groups (I and II). Group I, which consisted of individuals sampled from geothermal water, showed a positive contribution to the first component (PC1), which was characterized by the substantial PUFA n-6 in the PE class. Group II was, however, characterized by high PUFA, particularly n-3 PUFA, DHA and EPA of the phospholipid

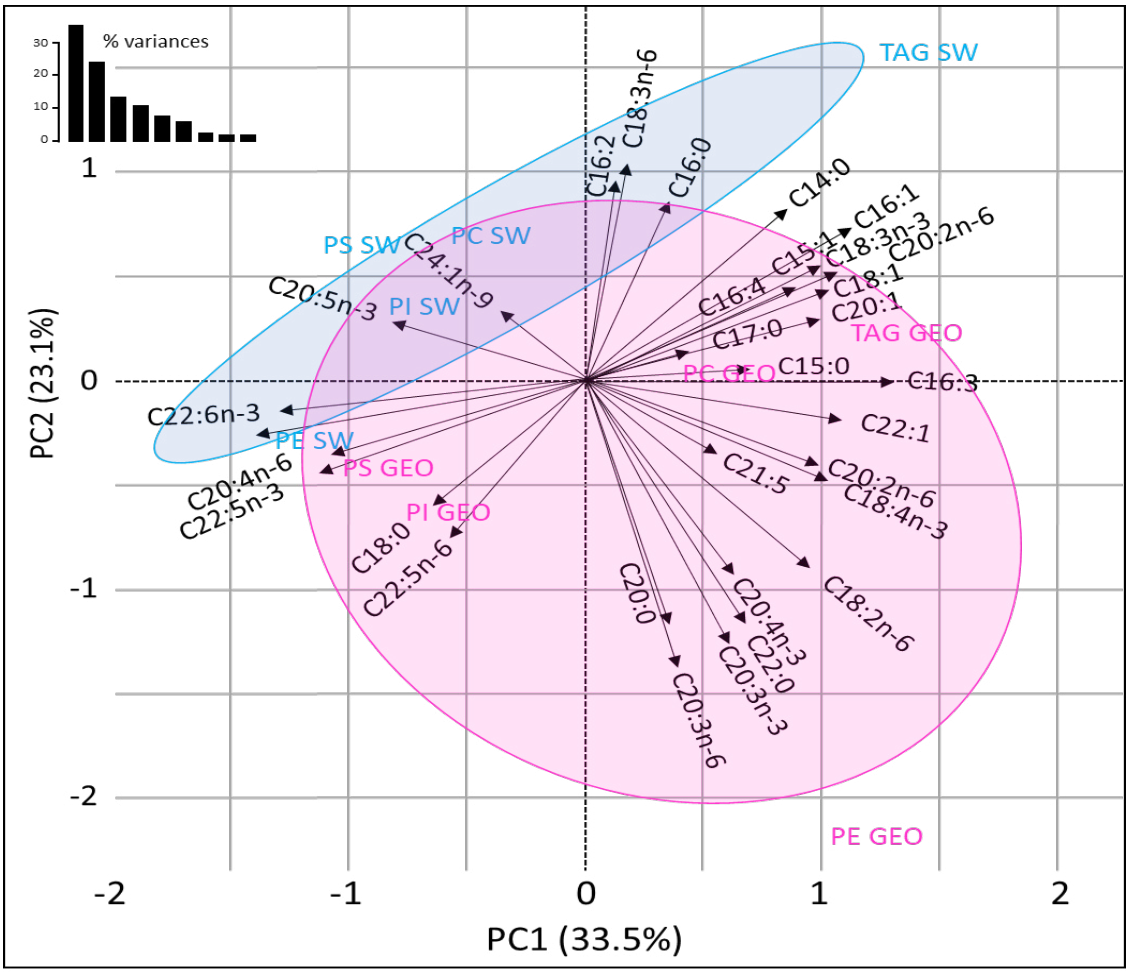

Figure 2. Principal analysis component (PCA) represented by two factors F1 and F2 and produced by lipid class and fatty acid composition in *Chelon labrosus* muscle reared in seawater and geothermal water.

class representing individuals sampled from seawater (Figure 2).

4. DISCUSSION

Teleosteen fish are exposed to varying and occasionally extreme environmental conditions that can produce potent effects on their physiology (Somero, 2004). The Thick-lipped grey mullet *C. labrosus*, is an euryhaline and eurytherm teleost that presents the ability to live under different environmental conditions of salinity and temperature (Azaza *et al*., 2008; Cardona, 2006; Rabeh *et al*., 2013). It is known that temperature and salinity are the key factors in teleost fish that cause fluctuations in the fluidity of cell membranes (Soengas *et al*., 2007) which largely depends on their lipid components. In this context, changes in membrane lipid composition is a key molecular mechanism of adaptation that is commonly called homeoviscous adaptation (Hazel,1995).

In the current study, lipid class composition and the fatty acid profiles of the two fish groups analyzed were shown to respond selectively and significantly to environmental conditions. Our data revealed that the lipids of the examined fractions from the seawater fishes were characterized by higher values for PUFA n-3, particularly in the PI fraction. However, a high content of PUFA n-6 was recorded in the PI fraction of the geothermal group. In addition, a substantial amount of SFA was observed in the TAG for the two groups. Our findings corroborate with those recorded by some authors who have reported that long-chain fatty acids were probably important for PI to successfully conduct particular cellular functions, including the role in cell growth, signal transduction processes and the membrane anchoring

of proteins in plants (Riekhof and Benning, 2009). Consequently, we hypothesize that an increase in PUFA n-6 in the PE of geothermal water fish might compensate for the effects of warm and lower salinity water, which tends to increase lipid rigidity.

The detailed investigation of *C. labrosus* groups considered in this study indicated that among the major fatty acids of the different lipid classes are C16:0, C18:1, C22:5n3, C18:2n6, C20:4n6, EPA, and DHA. First, with respect to saturates, the highest levels are usally contained in PE and TAG in the two *C. labrosus* groups. Among SFA, the major components were C16:0, followed by C18:0. Previous studies have reported that SFA, mainly palmitic acid, which is the main fatty acid synthesized *de novo* in fish, is classically associated with HUFAs in the diacyl phospholipids of fish (Steffens, 1997; Li *et al*., 2011). In fact, the important levels of SFA may result from a lipogenic activity (Dias *et al.,* 1998) that can be biosynthesized by fish through a conventional pathway catalyzed by cytosolic fatty acid synthetase (Sargent *et al.,* 2002). In addition, the TAG fraction in fish can also contain high levels of monoenoic C16-C18 fatty acids that are intensively synthesized in so-called "fatty" fish species to provide energy reserves. Likewise, the findings of Arts *et al.* (2009) revealed that triacylglycerol synthesis notably begins with a polar lipid that mostly has one of five common FA (e.g., myristic, C14:0; palmitic, C16:0; palmitoleic, C16:1n-7; stearic, C18:0; or oleic, C18:1n-9) in the *sn-1* position.

The highest MUFA detected in the lipid class of both fish groups was C18:1 with markedly impotant levels in the PC of the two studied groups. This is presumably due to its dominance in the commercial feed used in this survey. Another aspect to be taken into account is that C18:1 is a typical MUFA in fish which is most often considered from the standpoint of its energy importance (Sargent *et al.,*1989). It is also interesting to point out that PC in fish tissues is commonly rich in C18:1 and appears to be more easily influenced by dietary fatty acids than other phosphoglycerides (Tocher, 2003) and it should be the precise reason for the preponderance of these fatty acids.

It is remarkable that the phospholipids of the geothermal groups were found to contain significantly higher contents of C18:2n-6 compared to the seawater groups. The same conclusion was reached in a experiment in which *M. cephalus* fry were acclimatized to freshwater (El Cafsi *et al.,* 2003). The drop in salinity in the geothermal habit may have led to an increase in the percentages of PUFAs and particularly the n-6 series in the PE fraction. On the other hand, it is also possible that the mechanism involved in the catabolism of C18:2n-6 was more active in the fresh water fish than those from seawater (Sargent *et al.,* 1989; Kheriji *et al.,* 2003). It is also noteworthy that C18:2n-6, which is a well-known key dietary compoment constitutes a good energy source from fish (Castell *et al.,* 1972; Glencross, 2014). Herein, the dominant FA in the food is C18:2n-6 (29%), which allow us to explain the high levels of PUFA n-6 in the fillet of *C. labrosus*. Indeed, dietary lipids are a source of fatty acidswhich are required for the synthesis of new cellular lipids and for the turnover of existing lipids.

In the present study, an increased proportion of physiologically significant FAs (C22:6n-3 and C20:5n-3) in the phospholipid class was observed for both groups. It has been well established that fish phospholipids are usually considered as a physiologically crucial lipid classes since they are rich in PUFA, predominately DHA and EPA (Sargent *et al.,* 1993). Typically, EPA occurs in lower proportions than DHA due to the availability of these FA as dietary sources. Interestingly, C22:6n-3 is abundant in PI and PS particularly in geothermal specimens. The importance of DHA in PS was previously attributed to the ability of *C. labrosus* to assimilate this FA. The abundance of DHA observed in the polar lipids started with recognition that this FA has a unique conformation dictacted by helical or an angle iron shape with an overall length similar to that of C16:0 (Applegate *et al.,* 1986). This structure favors the formation of the hexagonal phase in phosphoglycerides, above all in C22:6n-3 phosphoglycerides containing small head groups such as phosphatidylserine and this will facilate very fast conformational changes in membranes (Brown,1994). In a general way, such essential FA are, for example, involved in the modulation of the properties of the lipid phase of the cell membrane, membrane bound enzymes as well as the precursor of functionally important lipooxygenase products (Koven *et al.,* 2011). Previous investigations have shown that variations in polar lipid contents and their individual fractions particularly in muscle are key compensatory mechanisms in organisms that guarantee optimum performance of several membrane-bound enzymes under different environmental conditions (Los, 2001; Cengiz *et al*., 2012).

5. CONCLUSIONS

This study provides initial insight into the lipid class composition of *C. labrosus* reared under different conditions. From the obtained results we can conclude that variations in the salinity and temperature chiefly affected the PUFA group. Indeed, we noticed that the juvenile *C. labrosus* reared in seawater conditions were characterized by the predominance of the n-3 series in phospholipid fractions. As for specimens from the geothermal system, high levels of n-3 PUFA were recorded in the PS and PI fraction, while PC and PE were dominated by the n-6 series. This suggests the aibility of *C. labrosus* to remodel its lipid composition to adapt to extreme environmental conditions. The obtained results can provide useful basic information that can help in the management of inland aquaculture practices. However, futher investigations are needed to better understand the impact of abiotic parameters on the lipid metabolism of of this promising species.

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Effect of tunisian pomegranate peel extract on the oxidative stability of corn oil under heating conditions

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SUMMARY: The effect of pomegranate peel extract (PPE) on the oxidative stability of corn oil during heating was studied. Oxidation was followed by determining peroxide value (PV), *p*-anisidine value (*p*-AV), free fatty acid value (FFA), conjugated dienes (CD), conjugated trienes hydroperoxides (CT) and the calculated total oxidation value (TOTOX). Polyphenol (TPC) and *ortho*-diphenol (TOPC) contents as well as the antioxidant activity of each oil sample were evaluated before and after heating. PPE showed a significant inhibitory effect on lipid oxidation. Heating samples for 8 hours supplemented by PPE to a level of 1000 ppm resulted in the highest significant decreases in investigated indices compared to the control and BHT values. It was concluded that the antioxidant activity of PPE delayed oxidation and can be used in the food industry to prevent and reduce lipid deterioration in oil.

KEYWORDS: *Corn oil; Heating; Lipid oxidation; Oxidative stability; Pomegranate peel extract; Quality indexes.*

RESUMEN: ***Efecto del extracto de cáscara de granada tunecina sobre la estabilidad oxidativa del aceite de maíz en condiciones de calentamiento***. Se estudió el efecto del extracto de cáscara de granada (ECG) sobre la estabilidad oxidativa del aceite de maíz durante condiciones de calentamiento. La oxidación se siguió mediante la determinación del índice de peróxido (IP), el índice de *p*-anisidina (*p*-AV), el índice de acidez (IA), los dienos conjugados (DC), los hidroperóxidos de trienos conjugados (TC) y el valor calculado de la oxidación total (TOTOX). Se evaluó el contenido de polifenoles totales (PT) y de *orto*-difenoles (*o*-DF), así como la actividad antioxidante de cada muestra de aceite, antes y después del calentamiento. El ECG mostró un efecto inhibidor significativo sobre la oxidación de lípidos. El calentamiento de las muestras, durante 8 horas suplementadas con ECG a un nivel de 1000 ppm, dio como resultado una significativa disminución de los índices investigados en relación con los valores de control y con BHT. Se concluyó que la actividad antioxidante de los ECG retrasó la oxidación y que se puede utilizar en la industria alimentaria para prevenir y reducir el deterioro de los lípidos del aceite.

PALABRAS CLAVE: *Aceite de maíz; Calentamiento; Estabilidad oxidativa; Extracto de piel de granada; Índices de calidad; Oxidación de lípidos.*

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1. INTRODUCTION

Fried foods are very popular because of their desirable flavors, colors and textures. It is well known that frying oils used as a cooking medium at high temperatures in the presence of oxygen and water from the food to be fried are subjected to very complex reactions of oxidation, polymerization and hydrolysis (Aladedunye *et al.,* 2017; Jiménez *et al.,* 2017). These reactions produce undesirable constituents, such as polar compounds (Seo Yeong Gim *et al.,* 2017), *trans* fatty acids (Bhardwaj *et al.,* 2016) and diacylglycerols (Aniołowska and Kita, 2016). These oxidized lipids are responsible for undesirable aromas and decrease the nutritional quality of the fried product (Bhardwaj *et al.,* 2016; Esposto *et al.,* 2015) and their excessive ingestion induces a detrimental effect on health. This is why research on lipid oxidation, especially on PUFA oxidation, has been the subject of numerous studies. The addition of antioxidants is a practical strategy to control the rate of lipid oxidation in foods during processing, storage, and exhibition (Seo Yeong Gim *et al.,* 2017). Synthetic antioxidants such as butyl-hydroxy-anisole (BHA), butyl-hydroxy-toluene (BHT) and tertiary-butyl-hydro-quinone (TBHQ) are widely used in the food industry as potential inhibitors of the oxidation of lipids (Aladedunye *et al.,* 2017; Maskan and Horuz, 2017). The addition of these antioxidants to improve the oxidative stability of edible oils is discouraged because of their toxicity and carcinogenicity. As a result, there is a trend towards the use of plant-based natural antioxidants to replace these synthetic antioxidants (Aladedunye *et al.,* 2017; Esposto *et al.,* 2015; Kmiecik *et al.,* 2015).

In recent years, attention has been focused on industrial waste. Vegetable co-products are interesting because they contain important molecules such as phenolic compounds that include simple phenols, flavonoids and anthocyanins. Many co-products have been studied as a source of antioxidants and their use is encouraged regarding their high biological activities. Pomegranate skin (*Punica granatum* L.) is a co-product with high nutritional value. It has numerous preventive and curative effects against several diseases due to its richness in polyphenols (Amri *et al.,* 2017c) which show various biological activities such as the elimination of free radicals, inhibition of oxidation and decreased risk of cardiovascular diseases and certain cancers (Amri *et al.,* 2017a). The effectiveness of fruit extracts and/or fruit peels in preventing the oxidation of vegetable oils during heat treatment has been reported (Iqbal *et al.,* 2008).

Among vegetable oils, sunflower oil has been used as a model to investigate the ability of various plant extracts in preventing its peroxidation (Iqbal *et al.,* 2008; Poiana, 2012). However, no study has ever been conducted on the efficacy of pomegranate peel in preventing the lipid peroxidation of corn oil under frying conditions. Hence this work aims to study the evolution of the quality of corn oil during heat treatment in the presence of natural antioxidants from pomegranate skin. Oxidative changes were monitored by the peroxide value (PV), *p*-anisidine value (*p*-AV), free fatty acid value (FFA), conjugated dienes (CD), conjugated trienes hydroperoxides (CT) as well as the calculated total oxidation value (TOTOX). Subsequently, polyphenol (TPC) and *ortho*-diphenol (TOPC) content as well as the analysis of oil's antioxidant power during heating were characterized.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All chemical standards including phenolic acids and reagents were purchased from Sigma-Aldrich Co. Ltd (St. Louis, MO. USA). Refined corn oil was recovered from the Agrimed group (Sfax, Tunisia).

2.2. Plant sample preparation

Fruits were harvested from Gabsi pomegranate trees from the Mahdia region in Tunisia. Fruits were washed and hand-peeled. Fruit peel was sundried and ground using a laboratory mill. The resulting powder was freeze-dried and protected from light under vacuum pack. 0.5 g of sample was extracted with 10 mL of methanol in the dark for 24 hours using an orbital shaker. Extractions were made in triplicate. The extracts were then filtered through a Whatman No.4 paper filter and concentrated under vacuum at 50 °C with a rotary evaporator. The crude extracts obtained where then freeze-dried, vacuum packed and stored under refrigeration until further analyses.

2.3. Phytochemical screening

The methanolic extracts of peels were evaluated for the content of: TPC, TOPC, anthocyanins (TAC), flavonoids (TFC), and total condensed tannins (TTC). All absorbance measurements were carried out using a UV-1601 Shimadzu spectrophotometer.

The TPC and TOPC of the methanolic fractions were determined according to the method of Montedoro *et al*. (1992) and expressed on dry weight (DW) basis as mg gallic acid equivalents (GAE)/g and mg hydroxytyrosol (HT)/g of sample. For TPC, PPE and diluted Folin–Ciocalteu reagent were mixed. Na_2CO_3 was added after 1 min and the mixture was incubated for 1 h. The wavelength used was 765 nm. For TOPC, 1 mL of HCl (0.5 N), 1 mL of a mixture of $NaNO_2$ and $NaMoO_4 \cdot 2H_2O$ in 100 mL·H_2O, and 1 mL of NaOH (1 N) were added to PPE. The absorbance was measured at 500 nm after 30 min.

TFC, expressed on a dry weight (DW) basis as mg catechin equivalents (CEQ)/g of sample, were evaluated according to the colorimetric assay developed by Zhishen *et al.* (1999). Diluted PPE was mixed with distilled water. At time zero, 5 min and 6 min, 0.3 mL of $NaNO_2$ (5% w/v), 0.3 mL of $AlCl_3$ (10% w/v) and 2 mL NaOH (1 M) were added, respectively. To made up the volume to 10 mL, 2.4 mL of distilled water were added. The absorbance was measured at 510 nm.

TAC were measured according to the method of Padmavati *et al*. (1997) and modified by Chung *et al.* (2005). They were expressed as mg cyanidin 3-glucoside equivalents per g of dry weight (mg CyE/g DW). PPE was mixed with acidified methanol (1% HCl/methanol) at room temperature, in the dark, for 2 h. After centrifugation for 15 min, at 1000g, the anthocyanin concentration in the supernatant was measured at 530 and 657 nm, respectively.

TTC was evaluated according to the procedure reported by Julkunen-Tiitto (1985) and expressed as mg tannic acid/g of dry weight (mg TA/g DW). PPE was mixed with 1.5 mL of vanillin (4%). Concentrated HCl was then added. The mixed solution was incubated for 20 min in the dark at ambient temperature. The absorbance was measured at 500 nm.

To evaluate the antioxidant activity of PPE, the DPPH test was used according to the method described by Braca *et al*. (2001). Different dilutions of the phenolic extract were prepared for each variety and then added to 1 mL DPPH (0.1 mM, in methanol). The reaction mixture was kept at room temperature. The optical density (OD) of the solution was measured at 517 nm. The optical densities of the samples in the absence of DPPH were subtracted from the corresponding OD with DPPH. The percentage reduction values were determined and compared to appropriate standards. The inhibition of the free radical DPPH, in percent (I_{DPPH} %) was calculated using the following equation:

$$I_{DPPH}\% = ((A\ blank - A\ sample)/A\ blank) \times 100$$

Where A blank is the absorbance of the control reaction (containing all reagents except the tested compound), and A sample is the absorbance of the tested compound.

2.4. Application of PPE to corn oil

Refined corn oil, free of synthetic antioxidants, was divided into five portions. Three of them were supplemented with 200, 500 and 1000 ppm PPE. The fourth one was mixed with synthetic antioxidant BHT at the permitted legal limit of 0.02% and was prepared for comparative purposes. The last portion, without additive, served as control. Before supplementation, PPE and BHT were mixed separately with a minimum amount of methanol to ensure dispersion in oil in an ultrasonic water bath. Then they were added to corn oil in brown glass bottles, and mixed for 30 min at 50 °C to get a diffusion of compounds from the PPE to the corn oil (Sultana *et al.,* 2007). All experiments were performed twice.

2.5. Heating processes

Samples with PPE, BHT and control samples were induced to oxidation by heating under simulated frying conditions using an incubator maintained at 200 °C. Samples were separately heated for 2, 4, 6, and 8 hours with temperature control using a calibrated chromel-alumel thermocouple (HI 935009, Hanna Instruments). The samples were taken out of the incubator and cooled after each heating time and stored at -20 °C until analysis.

2.6. Oxidative stability evaluation

The progress of lipid oxidation was evaluated by measuring standard chemical indices: FFA, PV, *p*-AV, CD, CT and TOTOX.

The FFA was determined by the titration of a solution of oil dissolved in ethanol/ether (1:1, vol/vol) with an ethanolic solution of potassium hydroxide (0.1M). The result was expressed as % of oleic acid.

The PV was determined using the standard titration method by the American Oil Chemists' Society (AOCS) (AOCS, 1994). This method determines all components, peroxides or other similar products of fatty acid oxidation. The results were expressed in milliequivalents of peroxide per kg of oil that oxidizes potassium iodide under test conditions ($mEqO_2/kg$).

The *p*-AV measures the carbonyl content in the oils or fats and was determined according to the AOCS official methods. In the presence of acetic acid, *p*-anisidine reacts with the aldehydiccarbonyl bond in oils, and leads to the formation of yellowish reaction products. The absorbance of the colored solution was measured at 350 nm (AOCS, 1994).

The CD and CT formed were determined using the standard method of AOCS (1994). The results were expressed as the specific extinction values K232 and K270.

TOTOX was used to estimate the oxidative deterioration of lipids. The TOTOX value was defined as the sum of both values (PV and *p*-AV) to total oxidation and was calculated according to the formula (Poulli *et al*., 2009):

$$\text{TOTOX value} = 2*\text{PV} + p\text{-AV}$$

2.7. Statistical analysis

All assays were run in triplicate. The results are reported as mean values of three analyses and standard deviation. Data was subjected to statistical analysis using the SPSS program, release 11.0 for Windows (SPSS, Chicago, IL, USA). The one-way analysis of variance (ANOVA) followed by Duncan's multiple range test were employed to study the differences between individual means and seemed to be significant at $p < 0.05$. Principal component analysis (PCA) was carried out using XLSTAT (2014) for Windows (Addinsoft, New York, USA).

3. RESULTS AND DISCUSSION

3.1. Phytochemical composition of PPE

The total analysis of the compound group defined by the total polyphenol (TPC), total flavonoid (TFC), total *ortho*-diphenol (TOPC), total tannin (TTC) and total anthocyanin (TAC) contents of PPE, were estimated (Figure 1). The results show that PPE is very rich in phenolic compounds which are known for their antioxidant power. In fact, the TPC of PPE reached a value of 171.57 mg GAE/g DW, lower than those reported by Amri *et al*. (2017c) (382 mg GAE/g DW) and by Harzallah *et al.* (2016) (215.54 mg GAE/g DW). The TOPC of PPE was 73.5 mg HT/g DW, which is much higher than those found by Mekni *et al*. (2013) where TOPC levels did not exceed 3 mg HT/g DW. The TFC of PPE was 95.12 mg QCE/g DW, similar to those found by Amri *et al*. (2017c). It is reported that tannins were the main phenolic compounds present in pomegranate. The PPE contained 2.28 mg TA/g DW of TTC. Concerning TAC, PPE contained moderate amounts (29.11 mg CyE/g DW) compared to those found by Amri *et al.* (2017c).

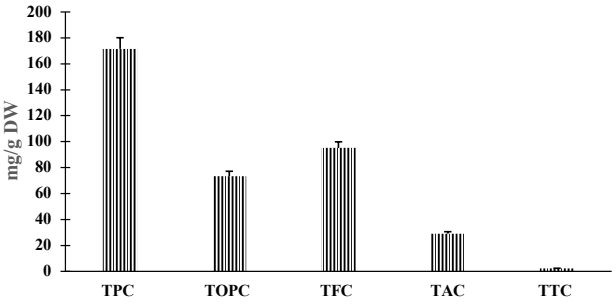

FIGURE 1. Phytochemical Composition of pomegranate peel extract. Results are expressed as means ± standard deviation (n = 3). TPC: total plolyphenols (mg GAE/g DW); TOPC: total ortho-diphenol (mg HTE/g D); TFC: total flavonoids (mg QCE/g DW); TTC: total tannins (mg TA/g DW); TAC: total anthocyanins (mg CyE/g DW).

3.2. Physicochemical properties of the frying/heating oils

3.2.1. Change in free fatty acid (FFA) contents

Hydrolysis is one of the most common reactions that causes frying oil degradation and therefore increased free fatty acid content. Therefore, free acidity is an important factor for oil quality (Amri, *et al.,* 2017b). The formation of free fatty acids might be an important measure of the rancidity of foods (Iqbal and Bhanger, 2007). The FFA value for fresh corn oil was 0.25%. This acidity is lower than that found in some edible oil such as olive oil, indicating that corn oil is suitable for edible

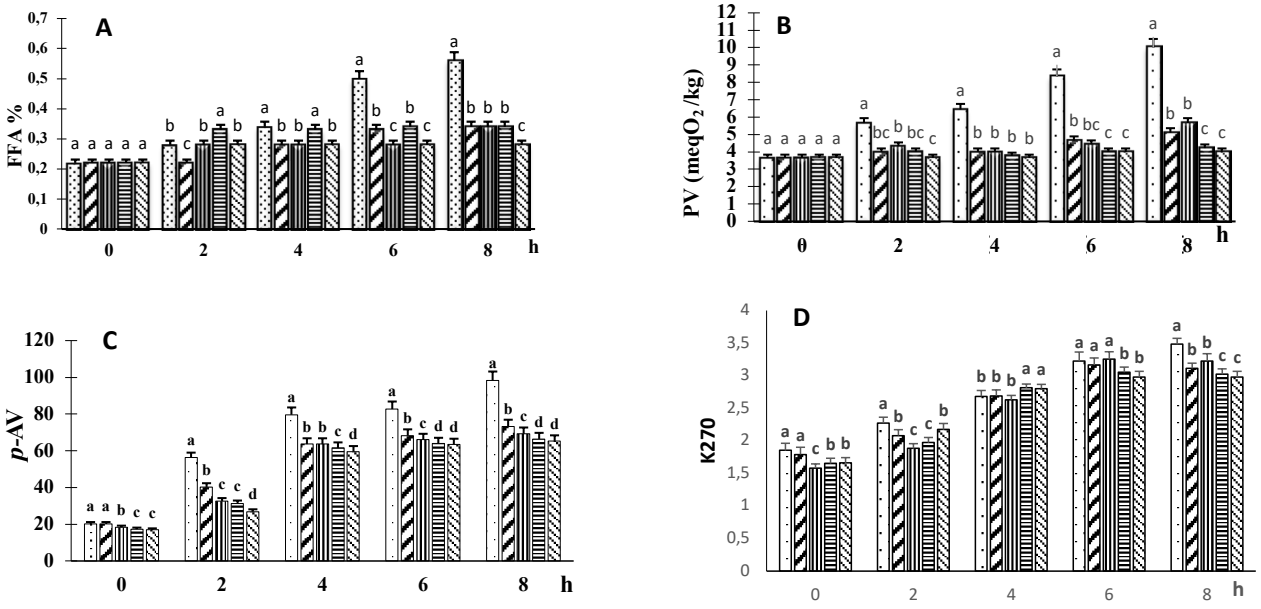

Figure 2: A: Changes in free fatty acid (FFA) contents in oils during heating. B: Changes in peroxide values (PV) in oils during heating. C: Changes in *p*-Anisidine values (*p*-AV) in oils during heating. D: Changes in conjugated triene values (CT) in oils during heating. Results are expressed as means ± standard deviation (n = 3). Bars (mean value ± SD) with different letters after each heating period are significantly different ($p < 0.05$) according to the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

purposes. As shown in Figure 2.A, FFA performed remarkably well with the increase in the oil heating period for all the samples. Initially, there was no significant difference in FFA content between the different samples. After 4 hours, significant changes were detected between the control oil (CO) and oils with different supplements (by BHT (CO-BHT) or various doses of PPE: CO-200, CO-500 and CO-1000). The most relevant change in the free fatty acid content was observed in the CO sample. In fact, after 8 hours of heating, CO exhibited the highest FFA, while CO-1000 exhibited the lowest value (Figure 2.A). The FFA equal to BHT for CO-200 and CO-500. In addition, from 0 to 8 hours, the FFA of CO increased by 56% compared to 28% for CO-BHT, CO-200 and CO-500 and 16% for CO-1000. From the present results, it may be concluded that over long heating periods, PPE at 200 and 500 ppm had the same preventive effect as BHT but at 1000 ppm it was more effective. Thus, the increase in FFA content during heating would indicate oil degradation. This result agrees well with the previous investigations carried out by various researchers on corn oil (Sultana *et al.,* 2007). It was reported that FFAs were formed due to the hydrolysis of fats, especially polyunsaturated fatty acids, diglycerides and monoglycerides produced by thermal treatments (Iqbal and Bhanger, 2007; Maskan and Horuz, 2017).

Supplementation with PPE and BHT markedly reduced the FFA increase and inhibited the development of rancidity. BHT was often used traditionally as an antioxidant in oil products to retard oxidation. However, it is allowed for use within legal limits in the food industry due to its toxic and carcinogenic effects (Tan *et al.,* 2015). In addition, it is very effective during the storage and transport of oils and fats, but is less effective at frying temperatures due to its volatility (Nor *et al.,* 2008). In this case, natural extracts such as PPE are a viable source of natural antioxidants for the prevention of frying oil oxidation at high processing temperatures. The preventive effect of PPE against lipid oxidation was reported previously by Iqbal et *al.,* (2008) who demonstrated that the methanolic extracts of PP were more resistant than the conventional synthetic antioxidant, i.e. BHA. In fact, PPE was heated for different intervals at 180 °C and subjected to antioxidant activity evaluation in a linoleic acid system. After 80 min heating, PPE exhibited 66.23% antioxidant activity, i.e. inhibition of lipid peroxidation which is better than BHA.

3.2.2. Change in peroxide value

Peroxide value is an indicator of the extent of initial oxidation in oil and fats (Farhoosh and Moosavi, 2009). It was used to measure the primary oxidation products, the hydroperoxides which can be degraded to a complex range of secondary products (Poiana, 2012). A continuous and tremendous increase in PV with the increase in heating period was observed for CO (Figure 2.B). After 8 hours of heating, the PV of CO increased by 3 times, from 3 to 10. Such observations have been confirmed by Sultana *et al.* (2007) after microwave heating of corn oil for 21 min and by Karoui *et al.* (2011), who reported that PV increased from 0.46 to 9.37 meq O_2/kg after the heating of corn oil from 25 to 200 °C.

At any heating time, the PVs measured in oil samples containing PPE (natural antioxidant) and BHT (synthetic antioxidant) were significantly lower than that of the control. In addition, the PVs for these samples increased but this increase was very slow. Initially, PPE at various doses and BHT at 200 ppm were comparable up to 6 hours of heating, when the PVs for PPE at 500 ppm and 1000 ppm were significantly lower (4 and 4.5, respectively) than that of BHT (Figure 2.B). After 8 hours, the effectiveness compared to the control was 60, 55 and 50%, respectively, for PPE at 1000 ppm, PPE at 500 ppm and BHT at 200 ppm. This suggests that PPE was more stable and effective than BHT. The PVs found in corn oil stabilized by PPE are far lower than those of corn oil stabilized with corncob extract at 500 and 1000 ppm (Sultana *et al.,* 2007), sunflower oil stabilized by grape seed extract (Poiana, 2012) and palm oil by Za'atar essential oils (Maskan and Horuz, 2017) and similar to those of corn oil stabilized with thyme (Karoui *et al.,* 2011). The antioxidant efficacy of PPE has been also confirmed previously in the stabilization of sunflower oil (Iqbal *et al.,* 2008).

3.2.3. Change in p-anisidine value

p-AV is a reliable measurement of the amount of secondary oxidation products which were formed by the decomposition of primary oxidation products (hydrogen peroxides). Alcohols, carboxylic acids, aldehydes and ketones are the major secondary oxidation products (Nor *et al.,* 2008) and are generally responsible for the off-flavors and off-odors of edible oils (Poiana, 2012). The data on *p*-AV with time of heating and the effect of PPE and BHT antioxidants were presented in Figure 2.C. As can be seen, the *p*-AV of all the oils significantly increased as a function of heating time ($p < 0.05$). After heating, the *p*-AV of CO increased from 20 to 98 with the highest increment level. In addition, at any heating time, statistical analyses revealed that the *p*-AVs of CO were significantly higher than those of the supplemented oils. This rapid increase in *p*-AV indicates the lipid deterioration of corn oil by oven heating at 180 °C. The same effect was observed for corn oil after microwave heating for 21 min (Sultana *et al.,* 2007) and for other edible oils such as palm olein (Maskan and Horuz, 2017), sunflower oil (Poiana, 2012), soybean oil (Saoudi *et al.,* 2016). On the other hand, the addition of BHT and various levels of PPE resulted in a significant decrease in *p*-AV ($p < 0.05$) compared to the control sample. In fact, *p*-AV in CO-BHT, CO-200, CO-500 and CO-1000 increased marginally during heating compared to CO and the lowest level was ever recorded for CO-1000. The inhibition of *p*-AV rise by PPE was dose-dependent but at the end of heating, CO-500 and CO-1000 had statistically the same effect. They inhibited the *p*-AV rise by 33% compared to 28 and 23 for CO-200 and CO-BHT, respectively. PPE at 500 and 1000 ppm seemed to be more stable than the BHT. This data was in agreement with those reported by Poiana (2012), who found that grape seed extract was more stable than BHT against the *p*-AV rise in sunflower oil subjected to convection and microwave heating up to 240 min under simulated frying conditions. Other natural extracts such as rosemary and curcumin extracts (Ravi Kiran *et al.,* 2015) inhibited the secondary oxidation of oil by more than 30% compared to BHT. The protective effect of PPE against the secondary oxidation of oil can be attributed to its richness in polyphenolic compounds. In a recent study, gallic acid (GA), a potent antioxidant phenolic compound was grafted in chitosan (GA-g-CS) and its effect on the oxidative stability in bulk oil was tested at 60 and 140 °C. Results showed that GA-g-CS and GA acted as antioxidants at 140 °C by the inhibition of the conjugated dienoic acid value and *p*-AV increase (Seo Yeong Gim *et al.,* 2017).

3.2.4. Change in conjugated diene and triene contents

The measurements of CD and CT are indicators of the oxidative deterioration of oils. CD and CT

are generated by the oxidation of polyunsaturated fatty acids (PUFA). In fact, during heating PUFA are oxidized with the formation of hydroperoxides and their double bonds suffer a rearrangement and generate CD. CT are formed through the conjugation to include three or more double bonds. The resulting CD exhibit intense absorption at 232 nm and quantified by K232; similarly, CT absorb at 272 nm and quantified by K272. Thus, the higher the proportion of PUFA in the oil, the higher the levels of CD and CT formed during frying (Karoui *et al.,* 2011) the greater the levels of CD and CT the lower the oxidative stability of the oil will be.

Figure 2.D shows the relative increase in CT contents of corn oil under oven heating at 180 °C, as a function of heating time. Initially, the CD and CT values for fresh corn oil were 3.75 and 1.75, respectively, which were relatively higher than that found in another plant oils such as soybean oil (2.78 and 0.73) (Ravi Kiran *et al.,* 2015), and olive oil (2.52 and 0.2) (Saoudi *et al.,* 2016). This result confirms that corn oil is much more oxidized than these oils. There were no significant changes in CD values for all the tested oils (data not shown). Thus, it was clear that the thermal treatment of corn oil at 180 °C had no impact on CD content. These results are inconsistent with those reported by Karoui *et al.* (2011), who reported that corn oil supplemented by thyme extract heated from 25 to 200 °C showed an increase in absorption at 232 from 1.8 to 4.89. Furthermore, Yu *et al.* (2018) observed a linear increase in CD with frying time for some edible oils such as coconut oil, soybean oil and pure olive oil.

The CT contents increased with the increase in heating time at a greater rate for the control. After up to 6 hours of heating, CO-1000 showed the lowest level of CT followed by CO-500 followed by BHT. Based on these results, the antioxidant activity of pomegranate peel extract at 500 and 1000 ppm was better than BHT at its legal amounts. These findings are in accordance with those reported by Iqbal *et al.* (2008) sunflower oil supplemented by 1000 ppm of PPE appreciably resisted the increase in CT. The inhibitory effect of PPE against lipid oxidation can be explained by its richness in tannins, powerful antioxidant compounds (Amri *et al.,* 2017a; Amri *et al.*, 2017c). In a recent study, tannic acid (TA), tannyl stearate (TS), molecules synthesized from TA, BHA and BHT synthetic antioxidants were investigated for their power to retard and inhibit linoleic acid oxidation. The results revealed that TA exhibited the best antioxidant activity and its derivative (TS) was more stable than synthetic antioxidants against lipid oxidation as established by the Rancimat method and the addition of TS to frying oils offered good protection against oxidation (Soliman *et al.,* 2017).

3.2.5. Change in TOTOX value

TOTOX is useful for quantifying oxygen-directed oil degradation. The results in Table 1 show that the TOTOX value for oil samples subjected to thermal treatments at 180 °C markedly increased with increasing heating time. As was observed in the case of *p*-AV, PV and FFA, oils supplemented with antioxidants PPE and BHT were more stable to oxidative rancidity than the CO. In fact, after each heating period, the greatest TOTOX value was registered for CO, while the lowest value was recorded for CO-1000. At the end, after 8 hours of heating, the TOTOX value for CO increased by 4 times with a 76% increase compared to 68% for CO-200, 67% for

Table 1. Effect of PPE and BHT on TOTOX values during corn oil heating at 180 °C

h	CO	CO-BHT	CO-200	CO-500	CO-1000
0	27.51±0.13^{a}	27.51±0.38^{a}	25.65±0.24^{b}	24.63±0.29^{b}	24.24±0.32^{b}
2	67.73±0.21^{a}	48.33±0.70^{b}	41.28±0.62^{c}	39.35±0.008^{c}	34.12±0.04^{d}
4	92.5±0.49^{a}	71.63±0.39^{b}	71.63±0.20^{b}	69.04±1.02^{bc}	66.91±0.71^{c}
6	99.35±0.17^{a}	77.58±0.21^{b}	74.83±0.83^{c}	71.88±0.16^{d}	71.40±0.37^{d}
8	118.25±0.58^{a}	83.48±0.29 ^{b}	80.54±0.24^{c}	74.89±0.17^{d}	73.16±0.23^{e}

Results are expressed as means ± standard deviation (n = 3). For each condition, values in the same row with different letters are significantly different at ($p < 0.05$) according to the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. PPE: pomegranate peel extract; TOTOX: total oxidation value; CO: Corn oil; CO-BHT: oil supplemented with BHT; CO-200: oil supplemented with 200 ppm PPE; CO-500: oil supplemented with 500 ppm PPE; CO-1000: oil supplemented with 1000 ppm PPE.

CO-BHT and CO-500 and 66% for CO-1000. Thus, the addition of PPE to oil inhibited the TOTOX value increase by more than 10% compared to the control. The best inhibitory effect was attributed to the 1000 ppm of PPE. PPE at a dose of 500 ppm and BHT inhibited lipid oxidation in a similar manner. Poiana (2012) found the same results for the sunflower oil added with grape seed extract and they explained that natural antioxidants of grape seed prevented the lipid oxidation developed in the heating time by surrounding the interface of the lipid system.

3.2.6. Change in phenolic composition

Phenolic content is a primary parameter for vegetable quality evaluation and directly involved in the prevention of oxidation and oil preservation (Amri *et al.,* 2017b). Figure 3 provides the evolution of TPC (Figure 3.A) and TOPC (Figure 3.B) in the different oils during thermal oxidation. The initial phenol contents in the oils supplemented with various doses of PPE were significantly higher than that in the CO and CO-BHT, which had the same contents (27.75 mg GAE/kg oil). In fact, before heating, the TPC provided by CO-1000 (87.43 mg GAE/kg oil) was 3.5 times more than that found in CO and CO-BHT. This marked difference might be attributed to the diffusion of phenols from PPE into the oil, supplementing those already present (Karoui *et al.,* 2011). On exposure to 180 °C, the TPC of all oils decreased significantly as a function of heating time. After each thermal treatment, a significant difference was observed among the different oils, which are classified according to their phenolic content as follows: CO-1000 > CO-500 > CO-200 > CO-BHT > CO. The CO and CO-BHT had the lowest phenol contents and statistically no significant differences were detected between them. CO seemed to be resistant to phenolic degradation after 8 hours of thermal oxidation at 180 °C. However, Karoui *et al.* (2011) demonstrated that refined corn oil (76.74 mg GAE/ kg oil) exposed to 150 °C for just 30 min, lost its TPC. The difference in the remaining contents in phenols did not reflect resistance to phenol degradation. In fact, a decrease of 47, 39, 25, 48 and 49% compared to the initial concentration was verified at the end of the treatment for CO, CO-BHT, CO-200, CO-500 and CO-1000, respectively. So, CO-200 seemed to be the most stable system to TPC degradation followed by CO- BHT; whereas CO and CO-1000 had the same resistance degree. These results were not in agreement with those found by Esposto *et al.* (2015) who evaluated the TPCs evolution of refined oil supplemented with four doses of olive phenolic extract (OPE) after 30 minutes, 1, 2, 4, 6, 8, 10 and 12 hours of frying and compared them to EVOO (extra virgin olive oil, 1237.6 mg/kg TPC). The results demonstrated that oils mixed initially with high doses of OPE were more stable and resistant to phenolic degradation and at a concentration of at least 400 mg/kg of polyphenols. OPE was able to reduce oxidation. So, the thermal degradation of phenolic compounds, and consequently the formation of their oxidation products, was proportional to the initial concentration of OPE added to the oil.

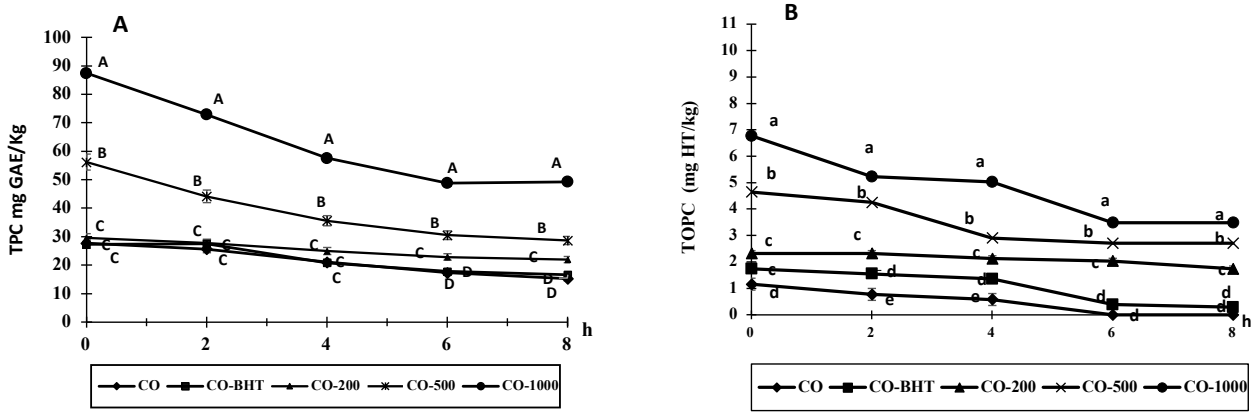

FIGURE 3. Heating impact on phenolic composition of corn oil supplemented with PPE and BHT. A: Total phenol contents (TPC), B: Total orthodiphenol contents (TOPC). Results are expressed as means ± standard deviation (n = 3). Data with different letters for the same heating time are significantly different ($p < 0.05$) according to the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

The changes in the TOPC in the different oils during heat treatment are shown in Figure 3.B.

A significant difference ($p < 0.05$) between the TOPC of different oils was observed before and after each treatment period. The initial amount of TOPC in the CO (1.16 mg HT/kg oil) was significantly ($p < 0.05$) lower than that in the PPE supplemented oil. Thus, in CO-1000, it was six times more than the initial amount. TOPC declined as a function of heat treatment in all oils. After 6 h of heating, the TOPC disappeared completely in CO and CO-BHT and decreased by 50% in CO-1000 (3.48 mg HT/kg) and CO-500 (2.71 mg HT/kg) compared to initial values. These contents remained stable until 8 hours of heating. As observed in TPC, the thermal degradation of TOPC was proportional to the initial concentration of PPE added to the oil. CO-200 seemed to be the most stable system to TOPC degradation followed by CO-BHT. In CO-1000 and CO-500, the percent of TOPC loss was similar to that of CO. It can be concluded that, at a level of 200 ppm of PPE, the remaining phenols (TPC or TOPC) would be responsible for protecting the oil but higher than that, the pro-oxidant effect would be observed.

3.2.7. DPPH radical scavenging activities of heated oils

The DPPH radical scavenging activities of CO and supplemented oils significantly decreased as a function of the heating time (Figure 4). Initially, oils supplemented by PPE have high radical scavenging activities. Thus, PPE provided the oil with greater radical scavenging capacity in a dose-dependent manner. DPPH loss in CO decreased from 62 to 54%. Yu *et al.* (2018) reported that DPPH radical scavenging activities of refined coconut oil, refined soybean oil, pure olive oil and vegetable shortening significantly decreased after 80 cycles of repeated frying. It has been suggested that radical scavenging compounds such as a-tocopherol or phenols in edible oil are degraded during thermal oxidation in accordance with the decrease in the DPPH radical scavenging activity of the oil. As oxidation time increased to 8 h, DPPH loss in CO-BHT, CO-200, CO-500 and CO-1000 decreased respectively by 13, 16, 12 and 3% after 8 hours' treatment. This implies that the decrease in the DPPH radical scavenging activity observed in CO was inhibited by the added antioxidants. PPE was more stable than BHT at doses of 500 and 1000.

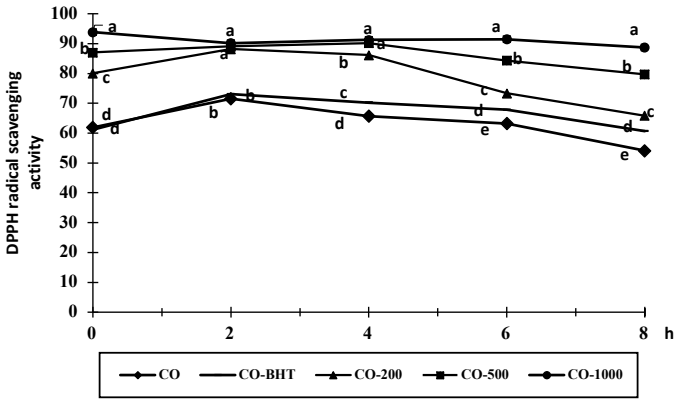

FIGURE 4. Heating impact on DPPH radical scavenging activity of corn oil supplemented with PPE and BHT. Data with different letters for the same heating time are significantly different ($p < 0.05$). Results are expressed as means ± standard deviation (n = 3) according to the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

3.2.8. Principal components analysis

A multivariate statistical analysis of the data was performed using PCA to analyze the oxidative stability of different oil samples (CO, CO-BHT, CO-200, CO-500 and CO-1000) before and after 8 hours heating. Figure 5 was plotted according to the correlation among oxidative parameters (FFA, PV, CD, CT, *p*-AV, and TOTOX), phenolic compounds (TPC and TOPC) and DPPH antioxidant activities. PC1 accounted for 75.95% of the total variance (87.75%), and PC2 accounted for 11.80%. The position of each variable in the loading plot describes its relationship with the others. Variables that are close to each other had high correlations. Variables on the same side of the origin (0.0) were positively correlated and those on the opposite side of the origin were negatively correlated. Different corn oil samples could be discriminated on the PCA plane.

PC1 was positively related to TPC, TOPC and DPPH antioxidant activities. PC2 was related more closely to FFA, PV, *p*-AV, TC, DC and TOTOX. CO-1000, CO-500 and CO-200 were located on the positive side of PC1; whereas CO and CO-HBHT were located on the negative side.

This data revealed that the lowest levels for TPC and TOPC were observed in CO and CO-BHT, with the greatest extent of the oxidative deterioration, expressed by PV, *p*-AV, FFA, CT, CD and TOTOX. However, high levels were

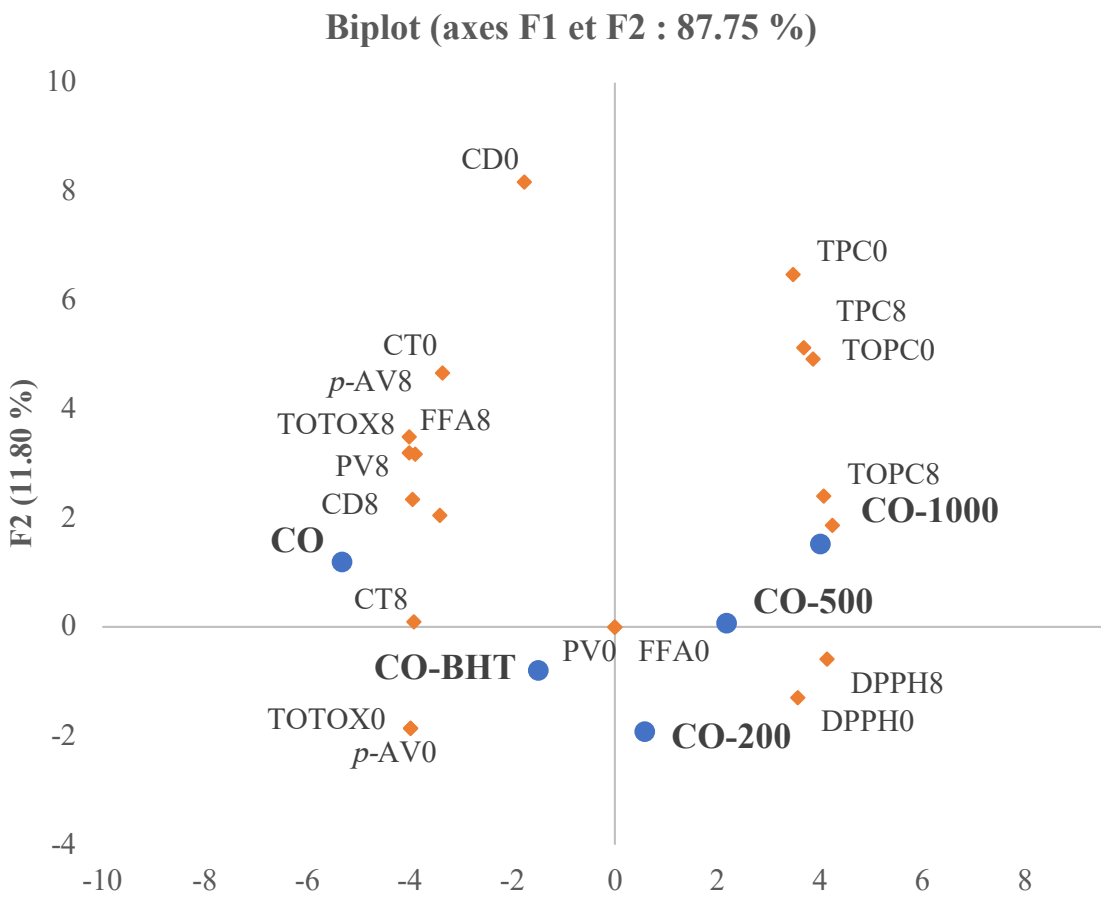

FIGURE 5. Principal component analysis (scores and loading plots, Biplot) applied to the data set for oxidative parameters, phytochemical contents (polyphenols and orthodiphenols) and DPPH antioxidant activities of different corn oil samples before and after 8 hours' heating. PV: peroxide value, *p*-AV: *p*-anisidine value, FFA: free fatty acid value, CD: conjugated dienes, CT: conjugated trienes, TOTOX: total oxidation value, TPC: polyphenol content, TOPC: ortho-diphenols.

registered for PPE-supplemented oils (200, 500 and 1000 ppm) with the lowest TOTOX values. This could be attributed to the protective action of TPC against thermo-oxidative degradation, explaining the negative correlation observed between TOTOX values and these phenolic compounds before and after 8 hours' heating. On the other hand, significant proportions of the primary and secondary oxidation products (PV, CD, CT and *p*-AV) were observed for CO, with negative correlations with CO-BHT and PPE-enriched oils (CO-200, CO-500 and CO-1000) respectively, according to PC2 and PC1.

The PCA results confirmed the deterioration effect on corn oil quality due to heating and the effectiveness of various PPE extracts used (200, 500 and 1000ppm) compared to BHT against the formation of primary and secondary oxidation products.

4. CONCLUSION

According to the results, it can be concluded that PPE exhibited significant potential to stabilize corn oil under heating conditions. They decreased the thermal deterioration of oil by enhancing its hydrolytic stability, inhibiting double bond conjugation and reducing the loss in polyunsaturated fatty acids. PPE at concentrations of 500 and 1000 ppm have potential stabilization efficiency compared to synthetic antioxidant (BHT). Therefore, PPE, a potential antioxidant source, can be recommended to extend the shelf-life of unsaturated vegetable oils.

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Investigation on chemical composition, antioxidant activity and SARS-CoV-2 nucleocapsid protein of endemic *Ferula longipedunculata* Peşmen

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SUMMARY: The essential and fatty oils were investigated and a quantitative analysis of the root, green and stem parts of *F. Longipedunculata* was performed by GC-MS and HPLC-TOF/MS and their antioxidant (DPPH method) activities and potential binding of phytochemicals against SARS-CoV-2 nucleocapsid were determined using Molegro Virtual Docker software. In the root part of the plant, the prominent components of oil were *β*-phellandrene (53.46%), ocimene (6.79%), 4-terpineol (5.94%) and santalol (5.03%). According to the quantitative results, vanillic acid (141.35 mg/kg), ferulic acid (126.19 mg/kg) and 4-hydroxybenzoic acid (119.92 mg/kg) were found in the roots; quercetin-3-*β*-*O*-glycoside (1737.70 mg/kg), quercetin (531.35 mg/kg) and ferulic acid (246.22 mg/kg) were found in the in the green part; and fumaric acid (2100.21 mg/kg), quercetin-3-*β*-*O*-glycoside (163.24 mg/kg), vanillic acid (57.59 mg/kg) were detected in the stem part. The antioxidant activity of all parts of the plant was higher than the control with BHT. Silibinin, rutin, and neohesperidin exhibited a stronger affinity than nucleotides. In the silico analysis, many of the phytochemicals were attached with strong hydrogen-bonds and electrostatic effects to the amino acids to which nucleotides are bound. The results indicated that the plant showed antioxidant effects and can be effective against SARS-CoV-2 thanks to the different phytochemical compounds it contains.

KEYWORDS: *Antioxidants; Chemical composition;* Ferula longipedunculata *Peşmen; COVID 19; SARS-CoV-2*

RESUMEN: ***Investigación sobre la composición química, la actividad antioxidante y la proteína nucleocápsida del SARS-CoV-2 de la endémica* Ferula longipedunculata *Peşmen.*** Se analizó el aceite esencial y la grasa de la raíz, la parte verde y el tallo de F. *Longipedunculata* mediante GC-MS y HPLC-TOF/MS y sus actividades antioxidantes (método DPPH) y posible unión de fitoquímicos contra el SARS-CoV-2 nucleocápside utilizando el software Molegro Virtual Docker. En la parte de la raíz de la planta, los componentes prominentes del aceite fueron *β*-felandreno (53,46%), ocimeno (6,79%), 4-terpineol (5,94%) y santalol (5,03%). Los resultados cuantitativos mostraron los siguientes valores: ácido vainílico (141,35 mg/kg), ácido ferúlico (126,19 mg/kg) y ácido 4-hidroxibenzoico (119,92 mg/kg) en la raíz, quercetina-3-*β*-*O*-glucósido (1737,70 mg/kg), quercetina (531,35 mg/kg) y ácido ferúlico (246,22 mg/kg) en la parte verde y ácido fumárico (2100,21 mg/kg), quercetina-3-*β*-*O*-glucósido (163,24 mg/kg) y ácido vainílico (57,59 mg/kg) en la parte del tallo, respectivamente. La actividad antioxidante de todas las partes de la planta fue mayor que el control de BHT. La silibinina, la rutina y la neohesperidina exhibieron una afinidad más fuerte que los nucleótidos. En el análisis silico, muchos de los fitoquímicos se pueden unir con fuertes enlaces de hidrógeno y con efectos electrostáticos a los aminoácidos a los que se unen los nucleótidos. Los resultados indicaron que la planta tiene un efecto antioxidante y puede ser eficaz contra el SARS-CoV-2 gracias a los diferentes compuestos fitoquímicos que contiene.

PALABRAS CLAVE: *Antioxidante; Composición química; COVID-19;* Ferula Longipedunculata *Peşmen; SARS-CoV-2*

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1. INTRODUCTION

The Apiaceae family consists of flowering and aromatic plants which are best known for their characteristic flowers, fruits (Heywood, 2007) and volatile substances (Widodo *et al*., 2014). *Ferula longipedunculata* Peşmen, Apiaceae, is a wild plant which is indigenous to Turkey. It grows in the central Anatolia region of the country. This plant has been used in Turkish folk medicine for stomach pain and as a wound healing remedy. Also, the roots and leaves of the *Ferula* plant are consumed as tea in Antolia in order to increase the aphrodisiac effect and sperm count. It has also been reported to be used to increase milk yield and fertility in goats and sheep (Pakdemirli, 2020). The *Ferula* species has been the subject of many studies on the chemicals often used in the characterization of compounds identified in the world as well as in the medical field. In the biochemical analysis, coumarins, methanolic, benzoic acid, antibacterial sesquiterpenes, ferulenol, terpenoids, steroidal esters, methanol, ethanol, sulfides, sinkiangenorin C have been found in many compounds and have been reported to be used in medicine (Duran *et al*., 2020; Li *et al*., 2015; Yang *et al*., 2006).

Antioxidants are gaining importance in the human health and food industry worldwide. Antioxidants are substances that prevent the easy degradation of the structure even in small quantities and the deterioration of the structure of oxidized substances (Brewer, 2011). Antioxidants are the main defense mechanism in the body and act as free-radical scavengers. They are manufactured inside the body and involve catalase, dismutase and peroxidase enzymes. BHT is the most widely used antioxidant and is a lipophilic organic compound, chemically a derivative of phenol, which is beneficial for antioxidant activity. Its aims to decelerate the effect of free-radical deterioration in several areas, especially the food, biomedical, rubber, plastic, oil, and petroleum industries (Yehye *et al*., 2015)

SARS coronavirus-2 (SARS-CoV-2) is a pathogen which is easily transferred from human to human. It is the main cause of the worldwide pandemic with serious diseases and death rates (Raoult *et al*., 2020). The coronavirus nucleocapsids (N) play a delicate role in improving the activity of virus transcription and assembly. Therefore, they were suggested as targets for drugs to combat CoVs (McBride *et al*., 2014). Plants are rich sources of natural compounds with antiviral effects (Sytar *et al.,* 2021). The therapeutic potential of many phytochemicals has been reported with *in silico* techniques to combat coronavirus (Adem *et al*., 2020; Galanakis *et al*., 2020). Molecular docking studies are actively used to describe biologically active compounds with the potential to bind the SARS-CoV-2 Nucleocapsid protein. However, no biotechnologically detailed studies on *Ferula longipedunculata* Peşmen plant have been found.

The aim of this study was to investigate the affinities of the phytochemicals found in the Endemic *Ferula longipedunculata* Peşmen towards SARS-CoV-2 nucleocapsid in *silico*. The constituents of the root, stem and green parts of the plant were investigated as the main reason for the chemical composition, antioxidant activities and SARS-CoV-2 nucleocapsid of *Ferula longipedunculata* Peşmen.

2. MATERIALS AND METHODS

2.1. Plant Material

Parts of *Ferula longipedunculata* Peşmen were collected from the Berit mountain province, (Figure 1), central Anatolia, Turkey during the flowering stage (June 15, 2015). After identification of the plant by Prof. Dr. Ömer Saya, a voucher (No. 1416) was deposited in the KOSAF herbarium of Turkey. The collected plant materials were air-dried in the shade.

2.2. Extraction Procedure

122 g (root), 82 g (stem) and 75 g (green-aerial) parts of the plant were dried at room temperature and cut into small pieces before being macerated three times (24h each time) with methanol/H_2O (80%). After filtration and evaporation, the obtained extract was partitioned with solvents in increasing polarity: chloroform, ethyl acetate and *n*-butanol. Each extract was evaporated under reduced pressure. The obtained extract contained (6.1 g root) $CHCl_3$, (0.9 g stem) EtOAc and (1.3 g green part) *n*-BuOH. Antioxidant activity analyses were performed with 10 grams of each plant material set on a balloon flask and 100 ml methanol and acetone solvents were added to each one. Extraction was then carried out for two hours, using conventional extraction methods (Khan *et al*., 1988).

FIGURE 1. *Ferula Longipedunculata* growing collection location Berit mount, Kahramanmaraş, Turkey. longitude: 37° 30' 93' 70'' E; latitude: 42° 031' 22'' N; altitude: 2100-2409 m above sea level

2.3. Isolation of the essential oils

The air-dried root of *F. longipedunculata* was subjected to methanol-distillation for 2 hours, using a Clevenger-type apparatus, according to the method recommended by the (European Pharmacopia procedure, 1983) to produce oils. The obtained essential oil was dried and after filtration, and stored at 4 °C until analysis.

2.4. Gas Chromatography (GC)

Fatty acids were analyzed by GC-MS (Agilent Technologies 7890A model GC system, 5975C inert MSD with Triple-Axis Detector/USA) using a BPX-20 capillary column (30 m x 0.25 mm, 0.25 µm film thickness; 5% phenyl polysilphenyl IN-siloxane), 70 eV ionization voltage, and FID detector. The oven temperature was between 50 and 120 ºC at 5 ºC/min and 120-240 ºC at 10 ºC/min and held for 5 minutes. 1.0 µL of diluted extracts 300:1 was injected in the split mode. The injector and detector temperatures were adjusted to 220 ºC and 290 ºC, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. The samples were determined with 1/1000 dilutions (Demirtas and Sahin, 2013).

2.5. Gas Chromatography/Mass spectrometry (GC/MS)

GC/MS analysis was performed by gas chromatography-mass spectrometer using a BPX20 column with autosampler and column (30 m x 0.25 mm x 0.25 µm film). A GC/MS detection system was used for electron ionization (ionization energy 70 eV). Helium was used as carrier gas at a a flow rate of 1.3 mL/min and diluted to 1/1000 (Demirtas and Sahin, 2013).

2.6. Molecular Docking Study

The docking studies used Molegro Virtual Docker software. The Crystal Structure of the N-terminal RNA binding domain of the SARS-CoV-2 nucleocapsid protein (PDB ID:6M3M) was downloaded from the online PDB database (www.pdb.org), and prepared for molecular docking using Molegro Virtual Docker Tools. The score function used was the MolDock score with the coordinates of the position X: 8.50 Y: -34.91 and Z:-28.06 at 16 Å3 radius, and 0.30 grid resolution. The docking region of the protein was selected according to previously reported studies (Dinesh *et al*., 2020; Kang *et al*., 2020). The 3D structure of the phytochemicals was downloaded from the website https://www.ncbi.nlm.nih.gov/pccompound, and geometrically optimized utilizing MarvinSketch 19.27 software.

2.7. Quantitative analysis by HPLC-TOF/MS

A HPLC analysis was performed with an Agilent Technology 1260 Infinity HPLC System equipped with 6210 Times of flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 x100 mm, 3.5 µm) column. Mobile phases A and B were ultra-pure water

with 0.1% formic acid and acetonitrile, respectively. The flow rate was 0.6 mL/min and column temperature was 35 ºC. Injection volume was 10 µL. The solvent program was as follow: 0-1 min 10% B; 1-20 min 50% B; 20-23 min 80% B; 23-30 min 10% B. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogen gas at 325 ºC, nitrogen gas flow of 10.0 L/min, nebulizer of 40 psi, a capillary voltage of 4000 V and finally, fragmentor voltage of 175 V. For sample analysis, dried crude extracts (200 ppm) were dissolved in methanol at room temperature. Samples were filtered through a PTFE (0.45µm) filter with an injector to remove particulates (Demirtas and Sahin, 2013; Abay G *et al*., 2015).

2.8. DPPH radical-scavenging activity

Different methods can be used to evaluate antioxidant activity but a rapid, simple and inexpensive method to measure the antioxidant capacity of food is DPPH, which is widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate antioxidant activity (Kedare SB *et al*., 2011).

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the investigation of the free-radical scavenging activity of the extracts (Nabavi *et al*., 2008). Different concentrations of extract were added to the same volume of a methanol and acetone solution of DPPH (100 mM). Absorbance was recorded at 517 nm after 30 min in the dark at room temperature for reaction to take place. All tests were carried out three times. BHT was used for standard controls. The inhibition of free-radical DPPH in percent (I%) was calculated as follows:

$$I\% = [(A_{blank}-A_{sample})/A_{blank}]x100,$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

3. RESULTS and DISCUSSION

3.1. Chemical composition of the fatty acids

The analysis of fatty acid compositions of root, green and stem parts of *F. longipedunculata* plant was performed using gas chromatography (GC-MS). The results obtained from the GC and GC–MS analysis of the fatty acids of the plant are presented in Table 1. 16, 6 and 4 components of the root, green and stem parts, respectively, were identified as fatty acids representing 100%. For all parts (root, green and stem) the major compound was linoleic acid at 70.37, 35.38 and 53.58%, respectively. Our research showed that the stem part had more fatty acid than the root and green parts. A literature search showed that *Ferula* oils are rich in fatty acids (El-feraly and Khan, 2001; Garg and Agarwal, 1988; Nagatsu *et al*., 2002).

3.2. Chemical composition of the essential oil

The GC-MS analysis of the essential oil of the *F. longipedunculata* root part is presented in Table 2. Eighteen compounds, representing 99.9% of the essential oil, were identified and characterized. Monoterpene *β*-phellandrene (53.46%) was the major compound in this plant. Other major monoterpene compounds included ocimene (6.79%), 4-terpineol (5.94%) and sesquiterpene santalol (5.03%).

After comparing the chemical composition of *Ferula longipedunculata* essential oil with other species of the *ferula* genus some differences and similarities were found. The main key components of the essential oil of *Ferula persica* were dillapiole (57.3%) and elemicine (5.6%) (Javidnia *et al*., 2005). Guaiol (58.76%), (E)-nerolidol (10.16%) and α-eudesmol (3.05%) were found to be the major (key) compounds of the oil of *Ferula ferulaoides* (Shatar, 2005). These components were not present in *Ferula longipedunculata* essential oil.

In the essential oil analysis of *Ferula elaeochytris* with GC-MS, nonane (27.1%), *α*-pinene (12.7%) and germacrene B (10.3%) were obtained as the main compounds (Başer *et al.*, 2000). In a study conducted in Iran, the essential compounds of the *Ferula szowitsiana* plant were obtained as *α*-pinene (12.6%), germacrene D(12.5%) and *β*-pinene (10.1%) (Rustaiyan *et al*., 2006). As expected, compounds such as *α*-pinene and *β*-pinene were not obtained as the main compounds for the *F. longipedunculata* plant. In addition, *α*-pinene was identified in the *Ferula longipedunculata.*

Moreover, the major components in the oil of *F. gummosa* were found to be *β*-pinene (50.1%), *α*-pinene (18.3%), 3-carene (6.7%), *α*-thujene (3.3%) and sabinene (3.1%) (Eftekhar *et al*., 2004).

Table 1. The fatty acid composition of the root, green and stem parts of *Ferula longipedunculata*

No	Compounds[a]	RT	% in oil		
			Root	Green	Stem
1	γ-cadinene	17.742	0.35	-	-
2	Acoradien	19.762	0.54	-	-
3	Bisabolene	20.203	0.70	-	-
4	Bicyclo[3.3.1]nonane-2,6-diol	20.947	0.46	-	-
5	Sesquisabinene hydrate	22.475	1.04	-	-
6	p-Mentha-2,8-diene, 1-hydroperoxide	22.961	0.70	-	-
7	2,3-Dimethylhydroquinone	23.562	0.93	-	-
8	trans-8-Hydroxy-bicyclo(4,3,0)non-3-ene	23.682	0.41	-	-
9	3-Hydroxy-2-(2-methylcyclohex-1-enyl)propionaldehyde	24.380	1.03	-	-
10	Pentadecanoic acid, methyl ester	24.912	1.44	-	-
11	6-[1-(Hydroxymethyl)vinyl]-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-2(1H)-naphthalenone	25.301	2.85	-	-
12	Palmitic acid methyl ester	27.579	8.62	22.16	24.76
13	Cyclododecane methanol	31.092	0.53	-	-
14	Linoleic acid, methyl ester	32.173	70.37	35.38	53.58
15	9- octadecanoic acid, methyl ester	32.248	9.30	-	-
16	Linolenic acid, methyl ester	32.276	-	25.04	12.47
17	Octadecanoic acid, methyl ester	32.562	0.74	4.26	-
18	3-Heptadecen-5-yne	26.926	-	6.01	-
19	Phytol	32.431	-	7.15	-
20	Stearic acid, methyl ester	32.563	-	-	9.19

[a]Compounds are listed in order of their elution from the BPX-20 capillary column, RT-retention time. min.

Table 2. Chemical composition of the essential oil of *F. longipedunculata* root parts

No	Compounds[a]	RT	% Composition
1	*α*-Thujene	12.411	0.8±0.43
2	*α*-Pinen	12.711	1.41±0.05
3	*β*-Phellandrene	14.003	53.46±0.64
4	beta-Myrcene	14.328	0.91±0.05
5	*α*-Terpinen	15.387	1.46±0.06
6	*β*-Cymene	15.657	4.12±0.08
7	*α* -Pinen	15.887	1.89±0.13
8	Ocimene	16.295	6.79±0.01
9	γ-Terpinen	16.822	3.98±0.12
10	2,3-Heptadien-5-yne, 2,4-dimethyl-	17.556	1.99±0.11
11	2,3,4,5-Tetramethylcyclopent-2-en-1-ol	20.681	1.17±0.05
12	4-Terpineol	21.163	5.94±0.01
13	Santalol	30.721	5.03±0.05
14	Epiglobulol	32.844	2.18±0.02
15	alpha-Caryophyllene	33245	2.47±0.08
16	6-[1-(Hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-2(1H)- naphthalenone	34.330	0.94±0.03
17	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2one	34.579	2.32±0.04
18	alpha-Bisabolol	37.069	3.13±0.01
Total			99.99

[a]Compounds are listed in order of their elution from the BPX-20 capillary column, RT-retention time. min.

The genera *Ferula* is rich in its essential content, which is also named Ferula oil. Of the genera, *F. assafoetida, F. gummosa* and *F. badrakema* contain essential oils. Those essential oils give a strong aromatic smell to the plant species. Furthermore, these oils have been documented to possess antifungal and antibacterial activities (Sahebkar and Iranshahi, 2011). Among the components of the essential oil, alpha-pinene and beta-pinene are of the major compounds (Benevides *et al*., 2001; Kim *et al*., 2006).

3.3. Identification and quantification of phenolic acids by HPLC-TOF/MS

The *n*-BuOH extract was obtained from the root, green and stem parts of *Ferula longipedunculata* and analyzed by HPLC-TOF/MS. The identification was performed based on their retention times and mass spectrometry by comparison with those of different standards. The results show the presence of 43 compounds including 17 organic and phenolic acids (Table 3), 26 flavonoids and phenolics (Table 4). Some phenolics were detected in a very small amount and barely reached detection limits (trace) because their concentration had not been seen. The main compounds of *F. longipedunculata* were fumaric acid, quercetin-3-*β*-D-glucoside, quercetin, ferulic acid, vanillic acid, and 4-hydroxybenzoic acid. The highest amounts were determined as vanillic acid in the root part, quercetin-3-*β*-D-glucoside in the green part and fumaric acid in the stem part. The green part of the plant contains more flavonoids than other parts of the plant. In terms of the phenolic acid richness of the plant parts, it was determined as stem, green and root part, respectively. As a result, *F. longipedunculata* is rich in flavonoids and phenolic compounds.

3.4. DPPH radical-scavenging Activity

The antioxidant activity may be due to different mechanisms, such as the decomposition of peroxides, prevention of chain initiation, reducing capacity, prevention of continued hydrogen abstraction, free-radical scavenging and binding of transition metal ion catalysts (Mao *et al*., 2006). The radical scavenging activity of organic extracts was determined from the reduction in the optical absorbance at 517 nm due to the scavenging of stable DPPH free radicals. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen contribution ability. DPPH is a stable free radical and accepts an elec-

Table 3. Quantitative results of organic and phenolic acids in plant extracts (mg phenolic/kg plant)

Organic and phenolic acids	RT	Root	Green	Stem
Gallic acid	2.4	Trace	Trace	Trace
Fumaric acid	3.2	75.49±2.14[a]	6.63±0.51[a]	2100.21±5.15[a]
Gentisic acid	4.5	24.73±1.04	120.21±2.89	16.51±1.53
Chlorogenic acid	5.5	5.13±0.01	72.35±1.95	16.19±1.06
4-Hydroxybenzoic acid	7.0	119.92±0.29	183.42±1.24	34.20±0.84
Protocatechuic acid	7.1	22.05±0.39	74.22±1.79	23.43±0.85
Caffeic acid	7.6	6.72±0.27	82.00±1.25	6.42±0.47
Vanillic acid	7.9	141.35±0.68	239.88±1.34	57.59±0.96
Syringic acid	8.1	116.57±0.52	214.24±1.14	50.87±0.38
4-Hydroxybenzaldehyde	9.4	13.25±0.57	89.11±1.01	Trace
Ellagic acid	9.7	Trace	127.58±2.08	Trace
Sinapic Acid	10.5	Trace	2.04±0.28	Trace
Ferulic Acid	10.6	126.19±0.72	246.22±1.7	nd
p-Coumaric acid	12.1	Trace	Trace±	Trace
Protocatechuic acid ethyl ester	12.8	Trace	Trace	Trace
Salicylic acid	13.1	51.14±1.88	185.69±1.3	11.13±1.23
Cinnamic acid	15.2	9.62±0.04	10.77±1.26	nd

RT-retention time. min, [a]Values expressed are means ± S.D. of three parallel measurements
nd: not detected

Table 4. Quantitative results of flavonoids and phenolics in plant extracts (mg phenolic/kg plant)

Flavonoids and phenolics	RT	Root	Green	Stem
Catechin	5.8	10.36±0.66	11.02±1.01	nd
Rutin	9.2	Trace	10.08±0.51	Trace
Polydatine	9.6	Trace	Trace	Trace
Scutellarin	9.7	Trace	16.45±1.80	Trace
Quercetin-3-*β*-D-Glucoside	9.8	7.39±0.53	1737.70±36.5	163.24±2.97
Naringin	10.5	Trace	213.29±3.67	19.02±2.04
Diosmin	10.6	38.70±0.82	46.38±1.63	47.57±1.28
Taxifolin	10.6	Trace	Trace	Trace
Hesperidin	10.8	Trace	278.38±1.07	Trace
Apigetrin	10.9	Trace	Trace	Trace
Neohesperidin	11.1	Trace	2.55±0.16	Trace
Myricetine	11.9	Trace	Trace	Nd
Baicalin	12.0	Trace	Trace	Trace
Fisetin	12.1	Trace	Trace	Trace
Morin	13.0	12.95±1.97	17.20±0.31	14.51±0.97
Resveratrol	13.0	Trace	Trace	Trace
Quercetin	14.0	10.54±0.67	531.35±2.45	3.93±0.6
Silibinin	15.1	Trace	Trace	nd
Apigenin	15.6	Trace	Trace	Trace
Naringenin	15.7	Trace	Trace	Trace
Kaempferol	15.7	Tr	60.51±2,51	tr
Diosmetin	16.1	Trace	Trace	Trace
Neochanin	17.7	Trace	Trace	Trace
Eupatorin	18.9	Trace	Trace	Trace
Wogonin	19.8	Trace	Trace	Trace
Biochanin A	20.5	Trace	Trace	nd

RT-retention time. min. [a]Values expressed are means ± S.D. of three parallel measurements
nd: not detected

tron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997).

The DPPH radical-scavenging activity of *F. Longipedunculata* root oil and its methanol and acetone extract are shown in Table 5. The methanol root extract at 0.1 mL concentration had the highest antioxidant value (98.5%). In the acetone solvent, it was found that the parts of green and stem at 0.3 mL concentration had the highest antioxidant value (86.8%). Among the solvent extracts from different parts of *F. longipedunculata,* the lowest concentration of methanol extract had the best antioxidant activity, whereas the stem part of the acetone extract showed the lowest activity. Interestingly, the results of the DPPH free-radical scavenging assay showed that the extracts had higher activities than the positive control (BHT) in all concentrations and higher activities in lower concentrations in methanol extracts as seen in Table 5. The reason for the high antioxidant activity is due to the phenolic compounds it possesses. The extract of *F. assafoetid*a exhibited a good antioxidant activity in all models studied. The extracts had good Fe^{2+} chelating ability, DPPH radical and nitric oxide scavenging activity (Dehpour *et al*., 2009). *Ferula-assafoetida* leaves are free-radical scavengers and may act as primary antioxidants, which react with free radicals by donating hydrogen (Nabavi *et al*., 2011). Research shows that the *ferula-assa-foetida* leaves have different kind of flavonoides, phenolic compounds (Dehpour *et al*., 2009). All these compounds probably contribute to the main reason for its significant radical-scavenging activity. Research-

TABLE 5. DPPH free radical scavenging activity of *F. longipedunculata* root, green and stem parts (methanol and acetone extract)

		DPPH free radical scavenging (%)					
		***F. longipedunculata* extract**			**BHT**		
		Concentration (ml)			Concentration (ml)		
Parts of Plant	Solvents	0.1	0.2	0.3	0.1	0.2	0.3
Root part	Methanol	98.5	98	98.3	90.7	92.1	97.9
Green part	Methanol	97.2	97	96.6	90.7	92.1	97.9
Stem part	Methanol	98.1	97.9	97.7	90.7	92.1	97.9
Root part	Acetone	79.5	80.6	82.8	77.6	75.1	74.8
Green part	Acetone	80.6	82	86.8	77.6	75.1	74.8
Stem part	Acetone	79.1	85.4	86.8	77.6	75.1	74.8

BHT (Butylated hydroxytoluene): as control, DPPH: 1,1-diphenyl-2-picryl hydrazyl

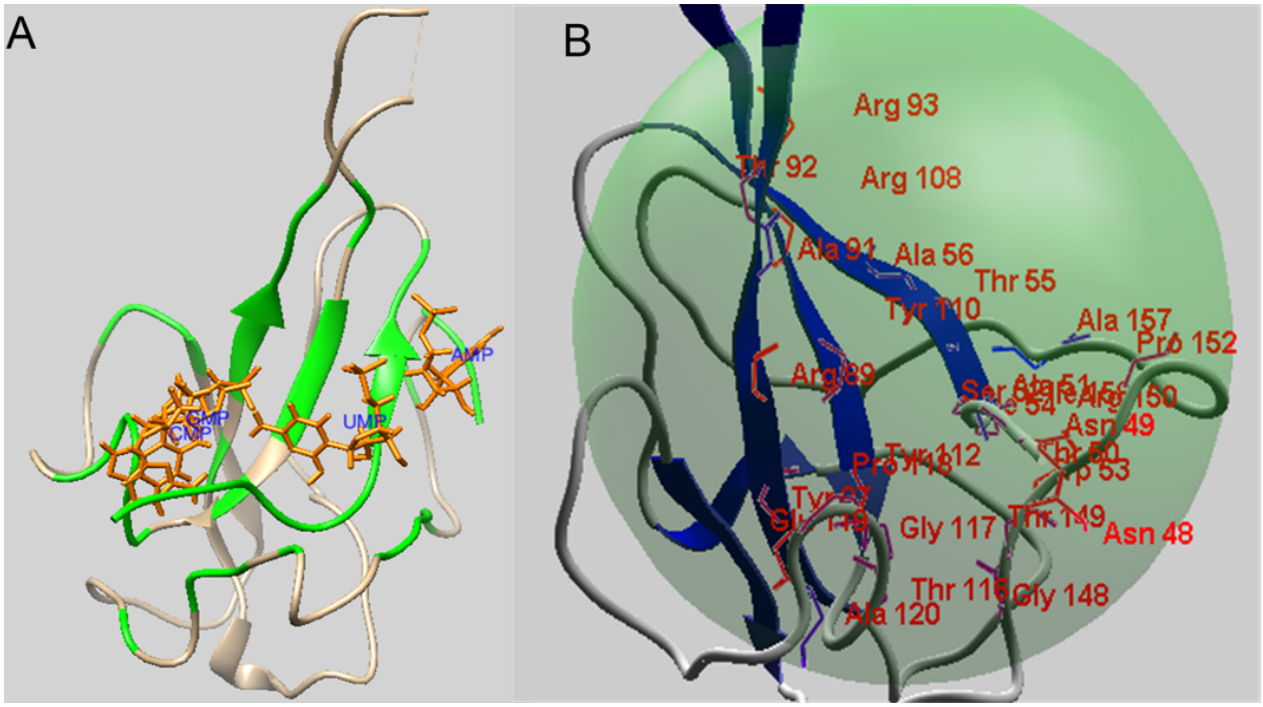
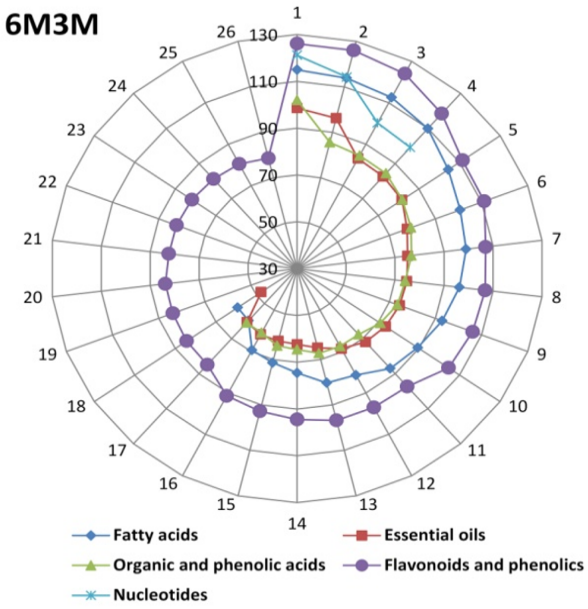


FIGURE 2. The docking region of SARS-CoV-2 nucleocapsid protein. (A: Active site (green) and binding of nucleotides (orange); B: Amino acid residues at docking cavity)

ers recently obtained better results regarding natural antioxidant compounds like gallic acid, coenzyme Q10, rosmarinic acid, tannins and flavonoids from medicinal herbs rather than artificial antioxidants (Tavafi and Ahmadvand, 2011). Natural antioxidants compared to artificial antioxidants are much safer and more beneficial and also have fewer side effects (Craft *et al*., 2010).

3.5. Docking Results

The SARS-CoV-2 nucleocapsid is a vital protein in the RNA genomic packing, viral transcription, and assembly in an infectious cell (Raoult *et al*., 2020). Therefore, it is considered an excellent target to battle against SARS-CoV-2. The possible interaction areas with nucleotides and RNA of the SARS-CoV-2 N protein N-terminal domain were previously determined (Dinesh *et al*., 2020; Kang *et al*., 2020). The site selected for docking, the binding sites of nucleotides and some amino acids are shown in Figure 2. Uridine 5'-monophosphate (UMP), adenosine 5'-monophosphate (AMP), cytidine 5'-monophosphate (CMP), and guanosine 5'-monophosphate (GMP) were used to compare the binding domain and affinity scores of



No	Fatty acids	Essential oils	Organic and phenolic acids	Flavonoids and phenolics	
1	Linolenic acid	6-[1 (Hydroxymethyl)vinyl]-4,8a-dimethyl-	Chlorogenic acid	Silibinin	19.Myricetine
2	9-octadecanoic acid	6-[1-(Hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-2(1H) naphthalenone	Sinapic acid	Rutin	20.Kaempferol
3	Linoleic acid	Epiglobulol	Caffeic acid	Neohesperidin	21. Quercetin
4	Phytol	alpha-Bisabolol	Ferulic acid	Naringin	22. Biochanin A
5	Palmitic acid	alpha-Caryophyllene	Ellagic acid	Diosmin	23. Apigenin
6	Pentadecanoic acid	Santalol	Syringic acid	Hesperidin	24. Morin
7	3-Heptadecen-5-yne	3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	p-Coumaric acid	Scutellarin	25. Taxifolin
8	Bisabolene	Ocimene	Cinnamic acid	Apigetrin	26. Neochanin
9	6[1(Hydroxymethyl) vinyl]4,8adimethyl-4a,5,6,7,8,8a hexahydro-2(1H) naphthalenone	2,3-Heptadien-5-yne, 2,4-dimethyl-	Protocatechuic acid ethyl ester	Polydatine	**Nucleotides**
10	Sesquisabinene hydrate	2,3,4,5Tetramethylcyclopent-2-en-1-ol	Vanillic acid	Baicalin	**1) AMP**
11	Cyclododecane methanol	beta-Myrcene	Gallic acid	Quercetin -3-β-D Glucoside	**2) GMP**
12	Acoradien	α-Thujene	4-Hydroxybenzoic acid	Wogonin	**3) UMP**
13	γ-cadinene	β-Phellandrene	Gentisic acid	Fisetin	**4) CMP**
14	3-Hydroxy-2(2methylcyclohex-1 enyl)propionaldehye	γ-Terpinen	Fumaric acid	Resveratrol	
15	p-Mentha-2,8-diene, 1hydroperoxide	β-Cymene	Protocatechuic acid	Naringenin	
16	trans-8-Hydroxy-bicyclo(4,3,0)non-3-ene	α-Terpinen	4Hydroxybenzaldehyde	Catechin	
17	Bicyclo[3.3.1]nonane-2,6-diol	4-Terpineol	Salicylic acid	Diosmetin	
18	2,3Dimethylhydroquinone	α-Pinen		Eupatorin	

FIGURE 3. Radar graphic representation of molecular docking results of phytochemicals and list of molecules

phytochemicals. Our study shows that several phytochemicals present in the endemic *Ferula longipedunculata* Peşmen presented significant predicted binding activity towards the SARS-CoV-2 nucleocapsid protein. Figure 3 shows the binding affinity information of our phytochemicals, and details of their estimated binding scores were demonstrated in Table 6. Also, many of the phenolics present in endemic plant have

Table 6. Details of docking results of some phytochemicals

Name	MolDock Score	Hydrogen Bond	Amino acids involved in hydrogen bonding	Electrostatic	Nucleotides bound to the same region
Silibinin	-126.107	-10.155	Arg150, Tyr 112, Asn 49, Asn 48, Gly117, Thr 149		UMP, CMP, GMP
Rutin	-126.039	-16.549	Arg108, Tyr110, Thr58, Tyr 173, Gln 161, Leu 160, Aln 161, Ala174		AMP, UMP, CMP, GMP
Neohesperidin	-124.043	-13.436	Arg 89, Tyr112, Ser52, Tyr110, Thr 92, Thr 149		UMP, CMP, GMP
Naringin	-118.464	-12.895	Tyr 112, Thr 50, Thr 149, Asn 48, Asn 49		UMP, CMP, GMP
Diosmin	-111.507	-9.955	Arg 150, Tyr 110, Tyr 112, Ser 52, Ala 56		UMP, CMP, GMP
Hesperidin	-111.133	-17.762	Asn 49, Ser 52, Phe 54, Arg 150, Arg 89, Tyr 112, Arg103, Tyr 110		UMP, CMP, GMP
Scutellarin	-107.010	-9.490	Tyr 112, Thr 149, Asn 48, Thr 50, Asn 49, Arg89		UMP, CMP, GMP
Apigetrin	-106.803	-18.390	Tyr 112, Ser 52, Phe54, Arg150, Tyr110, Arg89, Thr 149, Thr 50		UMP, CMP, GMP
Polydatine	-106.120	-14.276	Leu 160, Ala 174, Gln 161, Thr 58, His 60, Tyr 173, Gln 161		AMP
Chlorogenic acid	-102.058	-14.141	Ser 52, Thr 50, Gly117, Thr149, Arg 89, Tyr 112	Arg 150	UMP, CMP, GMP
Sinapic Acid	-85.529	-12.355	Arg 89, Arg 90, Asp 129	Arg 89	CMP, GMP
Linolenic acid	-114.959	-1.023	Arg 89, Tyr 112	Arg 89	GMP, CMP
9- octadecanoic acid	-113.834	-2.468	Tyr 110, Arg 108	Arg 108, Arg 93	AMP, UMP
6-(1-Hydroxymet hylvinyl)-4,8a-dimethyl	-98.624	-5.959	Gly 117, Thr 149, Thr 50		GMP, CMP
6[1(Hydroxymethyl) vinyl]4,8a-dimethyl-4a,5,6,7,8,8ahexahy-dro-2(1H)-naphthalenone	-96.182	-7.907	Tyr 173, Thr 58, Gln 161, Lue 160		AMP
AMP	-121.197	-13.876	Arg93, Arg108, Ala 56, Thr 58, Tyr 173, His 60	Arg 108, Arg 93	
GMP	-114.272	-3.803	Arg89, Tyr112, Thr50, Thr 149	Arg 89	
UMP	-100.274	-7.321	Tyr 110, Arg 150, Tyr 112, Ser 52, Phe 54	Arg 93, Arg 108	
CMP	-99.163	-14.516	Tyr 112, Asn49, Ala51, Thr 149	Arg 89	

significant binding affinity with this target. Some of the flavonoids and phenolics are silibinin, rutin, neohesperidin, naringin, diosmin, hesperidin, scutellarin, apigetrin, and polydatine. Table 6 presents the binding score and amino acid residues that make their hydrogen bond. Figure 4 demonstrates the possible binding modes of some phytochemicals. Silibinin exhibited the highest binding energy at the active site of SARS-CoV-2 nucleocapsid protein. It formed hydrogen bond interactions Arg 150, Tyr 112, Asn 49, Asn 48, Gly 117, Thr 149. Active site residues Gln 192, Thr 190, Arg 188, His 164, Gln 189, Glu 166, Gly 143, Ser 144, and Cys 145 participated in hydrogen bond interactions with rutin. Chlorogenic acid and sinapic acid with -106.120 and -85.529 MolDock scores exhibited the most effective phenolic acids against the target as *in silico*. The computer analysis results suggest that two phenolic acids had electrostatic potential in the interaction. The results of the prepared study shown that Ser 52, Thr 50, Gly 117, Thr 149, Arg 89, and Tyr 112 were critical residues in the hydrogen bonding of chlorogenic acid with protein. It also interacts electrostatically with Arg 150. The docking results in Table 6 demonstrate that chlorogenic acid interacted with the region where UMP was connected. Arg 89, Arg 90, Asp 129 amino acids were responsible for sinapic acid-binding in the SARS-CoV-2 nucleocapsid protein. It acted electrostatically with the Arg 89 ami-

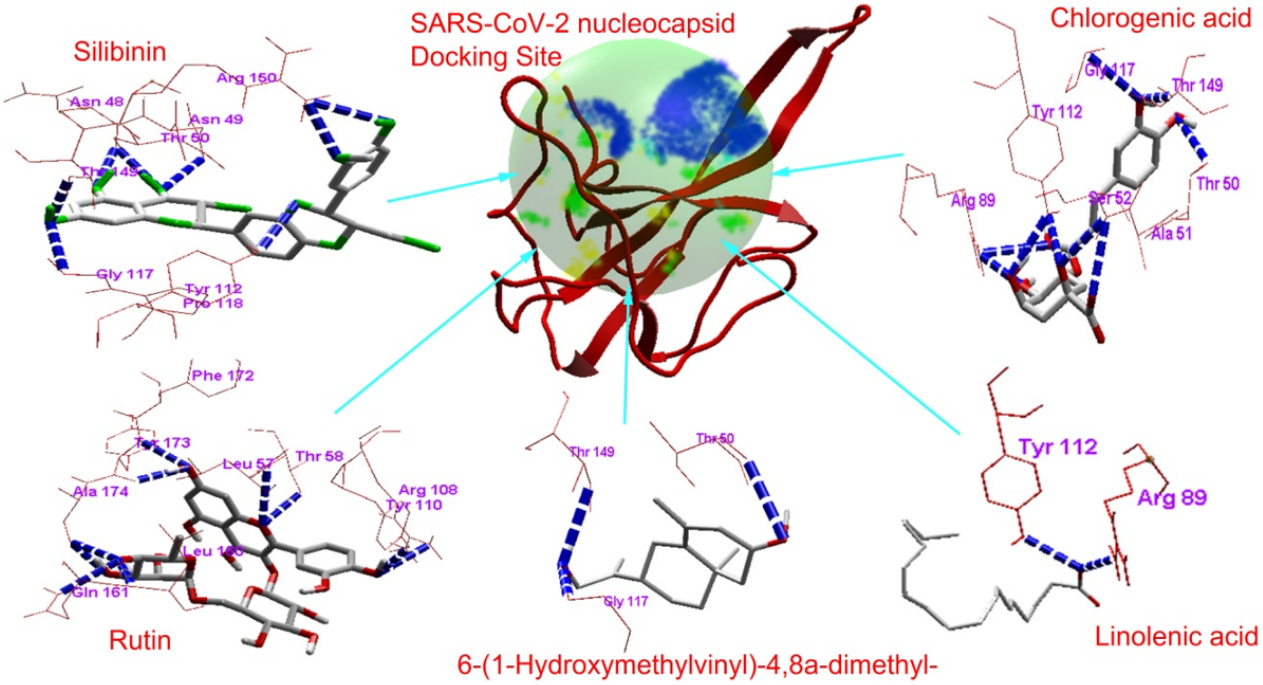


FIGURE 4. Protein binding site and 3D animation of possible binding of some phytochemicals (The dotted blue line shows hydrogen bonds)

no acid, in which GMP and GMP interacted as electrostatic. Two compounds, linolenic acid and 9-octadecanoic acid, showed the highest docking scores (-114.959 and -113.834, respectively) among all the fatty acids. Linolenic acid formed hydrogen bonds with Arg 89 and Tyr 112, and made an electrostatic interaction with Arg 89. This phytochemical was found to share the same region with CMP and GMP in the target protein. 9-octadecenoic acid showed a hydrogen bond with Tyr 110 and Arg 108, and was found to have electrostatic interaction with Arg 108 and Arg 93. It interacted with the same amino acids as AMP and UMP nucleotides.

6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-, the most active compound in the essential oils, formed hydrogen bonds with Gly 117, Thr 149, and Thr 50. The hydrogen bond interaction of 6-[1-(Hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-2(1H)- naphthalenone was formed with Tyr 173, Thr 58, Gln 161 and Lue 160 residues of protein. Both compounds made hydrogen bonds with similar amino acids to nucleotides GMP, UMP, and CMP.

4. CONCLUSIONS

F. longipedunculata flowers were investigated for their chemical composition. The extracts from the different plant parts exhibited well. The results of the present work indicate that the antioxidant activity of the methanol and acetone extracts of *Ferula longipedunculata* is higher than the control, such as BHT. The methanol and acetone extracts of the plant might be an alternative additive in foods, medicine and cosmetics, instead of toxic artificial antioxidants. The different results achieved in this study may be caused by factors such as the use of different parts of the plant, environmental and genetic differences and species diversity. These results interestingly encourage to continue the work to isolate the active molecules responsible for the antioxidant and assessment of biological activity of each compound individually and the need for in-depth studies on the plant extract.

The study also provided important insights into the first step of the COVID-19 infection, viral entry into cells, and defined potential phytochemicals for antiviral intervention. Although confirmation with an infectious virus is pending, our results indicate that natural compound responses raised against SARS-S could offer some protection against COVID-19 infection, which may have implications for outbreak control.

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Ultrasound-assisted extraction of red mombin seed oil (*Spondias purpurea* L.): phenolic profile, fatty acid profile and chemical characterization of the cake, residue from the oil extraction

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SUMMARY: The ultrasound-assisted method was used to extract oil from the red mombin seed, mainly aiming to analyze yield. A multivariate analysis served to define optimized parameters (6.46 minutes and S/S ratio of 1:23.10 mass:volume) for ultrasound-assisted extraction (UAE) with the objective of maximizing yield, using the response surface methodology (RSM) and desirability graph with central variables and axial points determined by the central composite rotatable design (CCRD). In addition to the optimization of oil extraction, oil was chemically characterized in terms of antioxidant capacity and nutritional aspects to test the quality and chemical characteristics of red mombin seed oil extraction residue (cake). Analyses showed 32% unsaturated fatty acids, such as palmitoleic acid, linolelaidic acid, and α-linolenic acid, and the presence of phenolic compounds, especially catechin. High dietary fiber content and the presence of phenolic compounds, such as chlorogenic acid, vanillin, and gallic acid, were found in the cake, which allows the possibility of incorporating this material into food products.

KEYWORDS: *Bioactive compounds; Emerging technologies; Fruit processing waste; Nutraceuticals; PUFAs; Red mombin cake.*

RESUMEN: Extracción asistida por ultrasonidos del aceite de semillas de ciruelas rojas (*Spondias purpurea *L.): perfil fenólico, ácidos grasos y caracterización química de la torta, residuo de la extracción del aceite. Se utilizó el método asistido por ultrasonido para extraer el aceite de semillas de ciruelas rojas, principalmente con el objetivo de analizar el rendimiento. Un análisis multivariante permitió la elección de los parámetros óptimos (6.46 minutos y una relación S/S de 1:23.10 masa:volumen) para extracción asistida por ultrasonido (EAU) con el objetivo de maximizar el rendimiento, utilizando la metodología de superficie de respuesta (RSM) y un gráfico de optimización, con variables centrales y puntos axiales determinados por el diseño giratorio de compuesto central (CCRD). Además de la optimización de la extracción del aceite, éste se caracterizó químicamente con respecto a la capacidad antioxidante y los aspectos nutricionales, para probar la calidad y las características químicas la torta, residuo de la extracción del aceite de semilla de ciruela roja. Los análisis mostraron un 32% de ácidos grasos insaturados, como el palmitoleico, linolelaídico y ácido α-linolénico, y la presencia de compuestos fenólicos, especialmente catequina. En la torta se encontró un alto contenido de fibra dietética y la presencia de compuestos fenólicos, como ácido clorogénico, vainillina y ácido gálico, lo que permite la posibilidad de incorporar este material en los productos alimenticios.

PALABRAS CLAVE: *Compuestos bioactivos; Nutracéuticos; PUFA; Residuos del procesamiento de frutas; Tecnologías emergentes; Torta de ciruela roja*

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1. INTRODUCTION

Fruit production has increased in Brazil since the 1990s, with cultivation conditions and novel technologies being applied in the market. Moreover, industry expansion regarding the processing of plant material is also developing, such that fruit processing may originate agricultural and industrial waste, adding up to 0.5 billion tons of waste worldwide, thus generating environmental, social and economic impacts (Banerjee *et al.,* 2017; Papargyropoulou *et al.,* 2014; Girotto *et al.,* 2015; Parfitt *et al.,* 2010).

The red mombin (*Spondias purpurea* L.) is a tropical fruit belonging to the Anacardiaceae family and is native to Central America, and is distributed on other continents, such as Asia and Africa, due to its ease of adaptation to varying climates (Engels *et al.,* 2012; Omena *et al.,* 2012; Maldonado-Astudillo *et al.,* 2017). The management, processing, and commercial activity of the seriguela are linked to regional development because its cultivation is still based on informal agricultural practices. Despite this, it is consumed and marketed in different ways, such as juices, nectars, sweets, and sorbets (Pereira and Santos 2016). The processing of the fruit is based on its composition so that in its ripe stage the seriguela, from northeastern Brazil, for example, has an average of 70% pulp, 14% peel and 16% seed (Maldonado-Astudillo *et al.,* 2014; Alia-Tejacal *et al.,* 2012). Therefore, the processing of seriguela generates on average 540 tons of seed residue (Pereira and Santos, 2016).

Red mombin seeds are a type of waste originated from juice and nectar industries and have been the subject of studies assessing nutritional quality of interest to industries, especially for foods and pharmaceuticals, due to compounds such as vitamin C, bioactive compounds, carotenoids, and oil (Omena *et al.,* 2012; Maldonado-Astudillo *et al.,* 2017). Within this context, red mombin seeds have been shown to present compounds with interesting biological activities, such as anticholinesterasic activity, due to high concentrations of chlorogenic acid, and antioxidant capacity, which has been attributed to compounds such as rutin, quercetin, rhamnetin, and kaempferol, which also present anti-inflammatory and antitumor activities (Engels *et al.,* 2012; Omena *et al.,* 2012; da Silva and Jorge 2014a; Lesjak *et al.,* 2018; Maldonado-Astudillo *et al.,* 2017; Silva e Lima and Meleiro 2012).

Conventionally, oil extraction from seeds is carried out by pressing followed by the use of solvents, such as n-hexane. However, this type of extraction is applied to oilseeds that present oil contents above 30%. Novel extraction methods are necessary in order to increase the yield and quality of seeds with lower oil concentrations. Ultrasound-assisted extraction (UAE) has been used as an alternative to reduce problems related to conventional extraction, such as high temperatures, long periods of extraction, and to reduce environmental concern regarding the use of high concentrations of solvents (Zhang *et al.,* 2008; Sicaire *et al.,* 2016).

Thus, UAE presents advantages compared to conventional extractions, since this method increases the transfer from mass into liquid using cavitation forces because the bubbles formed during this process disturb cell walls, therefore increasing the release of components of interest into the medium (Chielle *et al.,* 2016). This prevents damages to the structures and properties of compounds in matrices because the procedure can be conducted at low or even room temperatures. Therefore, the amount of n-hexane used in oil extraction decreases, less time is needed, and maintaining performance becomes desirable, which is an essential issue for industries due to economic and environmental reasons (Zhang *et al.,* 2008; Sicaire *et al.,* 2016).

Thus, the objective of the present study was to optimize the ultrasound-assisted extraction of red mombin seed oil, evaluating yield as the main variable, using the response surface methodology (RSM) under various extraction conditions, such as time and solid:solvent ratio. In addition, the study also aimed to characterize the oil obtained regarding its antioxidant capacity and nutritional aspects, to test its quality and evaluate the characteristics of red mombin seed oil extraction residue, herein called "cake", for possible applications of this material.

2. MATERIAL AND METHODS

2.1. Standards and chemical reagents

The chemical reagents ABTS ((2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), β-carotene, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), DPPH (2,2-di-

phenyl-1-picrylhydrazyl), Tween 40, and phenolic compound standards (gallic acid, catechin, chlorogenic acid, caffeic acid, ferulic acid, *trans*-cinnamic acid, vanillin, quercetin, *m*-coumaric acid, *p*-coumaric acid, *o*-coumaric acid) were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Methanol (HPLC grade) was obtained from J-TBacker (Alfragide, Lisbon, Portugal). In turn, the Folin-Ciocalteu reagent was obtained from Dinâmica (Indaiatuba, São Paulo, Brazil). Acetone, acetic acid, gallic acid, linoleic acid, methanol, and chloroform were obtained from Vetec (Saint Louis, Missouri, USA), while sulfuric acid was obtained from Merck (Darmstadt, Hessen, Germany). Ethanol, was obtained from Synth (Diadema, São Paulo, Brazil), while n-hexane and potassium persulfate were obtained from Neon (São Paulo, São Paulo, Brazil). All reagents were analytical grade, and stock and buffer solutions were prepared using distilled water.

2.2. Red mombin seed preparation

Red mombin seeds were donated by the company Frutos do Brasil, located in the municipality of Goiânia, state of Goiás, Brazil. Fruits were originally collected from the municipality of Santa Maria da Vitória, state of Bahia (13° 23' 41" S, 44° 11' 19" W, 436 m), Brazil, during the 2016/2017 harvest. Seeds were washed and sanitized using a 200 ppm sodium hypochlorite (NaOCl) solution for 30 minutes to eliminate all vegetative forms of microorganisms, thus avoiding seed deterioration during the storage period. The drying process was carried out by placing a portion of red mombin seeds in a forced-air convection oven at 50 °C (TE 394/4, Tecnal, Piracicaba, Brazil) until they reached constant weight, stored in low-density polyethylene bags, and kept in a freezer at -18 °C until analyses. Red mombin seeds were defrosted at room temperature and dried using a forced-air convection oven (TE 394/4, Tecnal, Piracicaba, Brazil) at 50 °C until they reached constant weight. Seeds were pulverized using a knife mill for subsequent oil extraction.

2.3. Ultrasound-assisted extraction (UAE) and experiment design

Ultrasound-assisted extraction (UAE) was used to extract red mombin seed oil. An ultrasonic bath (USC2800A, Logen Scientific, São Paulo, Brazil; frequency 40 Khz; internal dimension: 293 x 235 x 150 mm) was used for this procedure. Extraction was carried out under various experimental conditions which were defined in pre-tests and presented two independent factors: time (x1, 5 - 15 min), and solid:solvent ratio (x2, 1:5 - 1:20) (mass:volume).

Table 1. Ultrasonic-assisted red mombin seed oil extraction matrix for the construction of the central rotational compound design (DCCR) and the experimental result of the yield

Variables	Levels				
	Axial (-)	-1	0	+1	Axial (+)
Time (x1,min)	3	5	10	15	17
S/S ratio (x2,m:v)	1.90	5	12.5	20	23.1
No	Blockes	Time (x1, min)	S/S ratio (x2, m:v)	Oil Yield (%)	
				Experimental	Predicted
1	1	-1 (5)	-1(1:5)	4.99	8.73
2	1	-1(5)	1(1:20)	58.93	58.99
3	1	0(15)	-1(1:5)	7.99	15.19
4	1	0(15)	1(1:20)	54.90	58.41
5[c]	1	0(10)	0(1:12.5)	44.97	45.71
6 [c]	1	0(10)	0(1:12.5)	47.98	45.71
7[*]	2	-1.41(3)	0(1:12.5)	42.96	45.92
8[*]	2	+1.41(17)	0(1:12.5)	52.00	45.92
9[*]	2	0(10)	-1.41(1:1.90)	0.00	-6.23
10[*]	2	0(10)	+1.41(1:23.10)	60.89	59.86
11[c]	2	0(10)	0(1:12.5)	44.92	45.71
12[c]	2	0(10)	0(1:12.5)	44.97	45.71

[C] Central point; [*] Axial point.

N-hexane was used as the solvent and the temperature was held at 30 °C during extraction. The extract obtained was filtered using a qualitative filter paper into previously dried and zeroed 50 mL beakers to determine lipid content (P_0). Next, the material was placed in an oven at 105 °C and dried until reaching constant weight. Lipid weight was then determined. Relationships between response and variables were established according to a central composite rotatable design (CCRD) (Table 1).

The experiment design consisted of 12 trials, including two central points, determined to assure data repeatability (runs 5, 6, 11, and 12), and two axial points per block. Extraction yield was selected to combine independent variables. Thus, maximum yield shown by the design was chosen for multiple extractions, from which the solvent was removed using a rotary evaporator at 50 °C and traces of the solvent were removed using a vacuum oven (MA 120/TH, Marconi Equipamentos Para Laboratórios Ltda, Piracicaba, São Paulo, Brazil) for 24 hours at 30 °C. The oil extracted was stored in amber bottles and covered with aluminum foil until the characterization and oxidative profile analyses.

A full description of the experimental design requires a cubic model. Lipid yield (Y) is related to independent variables coded as x_i and x_j, according to the second-degree polynomial, such as in equation 1, where β_0 is the interaction coefficient, β_i is the linear term, β_{ii} is the quadratic term, and β_{ij} is the interaction term.

$$Y = \beta_0 + \sum \beta_i x_i - \sum \beta_i x_i^2 + \sum \beta_{ii} xj - \sum \beta_{ii} x_j^2 - \sum \beta_{ij} xixj \quad (1)$$

2.4. Determination of extraction yield

After extraction, the determination of red mombin seed oil yield followed the methodology described by Chanioti and Tzia (2017):

$$Yield\ (\%) = \frac{P_0}{P_a} * 100 \quad (2)$$

Where P_0 and P_a refer to lipid content in the sample and sample weight (g), respectively.

2.5. Characterization of red mombin seed cake and oil

The objective of characterizing the red mombin seed extraction residue, or cake, was to test its possible technological applications. Analyses were conducted in triplicate and presented proximal composition, such as moisture, ashes, proteins, and total carbohydrates (TC) by difference (TC = 100 - (moisture + ashes + proteins + lipids)), as well as hydrogenionic potential, total titratable acidity, expressed in g·100 g^{-1} of organic acids, soluble solids (AOAC 2016), and lipids using the Bligh-Dyer method (Bligh and Dyer, 1959). Results were expressed in g·100 g^{-1}. Caloric value was calculated according to Atwater and Woods (Atwater and Woods, 1896).

With the aim of characterizing red mombin seed oil, the following tests were conducted: hydrogenionic potential; acidity index; refraction (AOCS 2013); and peroxides, using a semi-quantitative Quantofix® Peroxide kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany).

2.6. Cake extracts

The preparation of extracts to evaluate the antioxidant capacity of the cake followed a modified version (Zieliński and Kozłowska 2000). In sum, five different extracts were obtained: ether extract (ETE); alcoholic extract (ALE); aqueous extract (AQE); methanol:acetone:water extract (50% methanol:70% acetone:distilled water) (MAWE), at a proportion of 2:2:1; and hydroalcoholic extract (70% ethanol (v/v)) (HAE). In order to obtain ETE, 2.5 g of the sample were homogenized under agitation and sheltered from light using a 1:20 (m/v) ethyl extracting solution for one hour at room temperature. The extract was then filtered, transferred to a volumetric flask, and the final volume was adjusted according to the extracting solution volume initially used. The residue recovered was used to obtain other extracts following the same procedure, although with ethanol to obtain ALE and distilled water to obtain AQE.

Regarding the preparation of MAWE, 20 mL of 50% methanol were added to the sample and left to rest for 1 hour. The extract was then filtered and transferred to a 100-mL volumetric flask. Soon after, 70% acetone was added and the same procedure was again conducted, such that the filtrate was added to the same flask that had been used previously. At the

end, the volume was completed using distilled water. In turn, HAE was prepared by adding 50 mL of 70% ethanol and macerated for one hour, followed by filtration. At the end of the extractions, all extracts were placed in amber bottles and stored in a freezer at -18 °C until the determination of antioxidant capacity (Rufino *et al.,* 2010).

2.7. Extracts of the red mombin seed oil

A methanolic extract was used to evaluate reducing capacity (Wang *et al.,* 2017). In sum, 1 g of oil was weighed and placed into a centrifuge tube with 2.5 mL of n-hexane and 3 mL of a methanol:water solution (60:40, v/v). The mixture was agitated for 3 minutes in a vortex equipment, and the tube was then placed in a refrigerated centrifuge (5403, Eppendorf AG, São Paulo, Brazil) at 3,500 g for 10 minutes at 4 °C. Thus, the lower methanolic layer was separated and this operation was repeated three times. The methanolic extract was evaporated at 35 °C using a forced-air convection oven until completely dry. Next, the extract was resuspended in 1 mL of the methanol:water solution (60:40, v/v). The extract was stored in an amber bottle and refrigerated at 4 °C until analyses.

In order to determine antioxidant capacity using the DPPH and ABTS methods, the red mombin seed oil and isopropyl alcohol (1:10 p/v) extract was prepared based on its solubilization (Martins *et al.,* 2013).

2.8. Antioxidant capacity

Red mombin seed cake extracts – ETE, ALE, AQE, MAWE, HAE – and oil methanolic extract were used to determine reducing capacity using the Folin-Ciocalteu method, expressed in mg of gallic acid equivalents per 100 g of sample. The cake extracts and the isopropyl alcohol extract were used to determinate DPPH (2,2-diphenyl-1-picrylhydrazyl), expressed in IC_{50} ($mg \cdot mL^{-1}$) and ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), expressed in µmol of Trolox equivalents per gram of sample. In addition, the determination of antioxidant capacity using the ferric reducing antioxidant power (FRAP),expressed in µmol of $FeSO_4$ per gram of sample, and β-carotene/linoleic acid method, expressed in percentage of protection, was only made for the cake extracts (Rufino *et al.,* 2010; Zieliński and Kozłowska 2000).

2.9. Phenolic compound profiles in red mombin seed cake and oil

The extract was used to identify phenolic compounds in the cake through the chromatographic method (Ramaiya *et al.,* 2013a). For this extraction, 2.5 g of red mombin seed cake was homogenized in 20 mL of 70% HPLC grade methanol (v/v) for one hour in an ultrasonic bath at room temperature. The extract obtained was centrifuged at 14,000 rpm for 15 min at 4 °C and filtered using a qualitative filter paper. In order to inject samples, extracts were filtered again using 0.45-µm porous membrane filters.

In turn, the extract used to identify phenolic compounds in red mombin seed oil using the chromatographic method was prepared following Wang *et al.* (2017). The extract was refrigerated at 4 °C until analysis.

2.10. Phenolic compound profiles

The quantification and identification of phenolic compounds in red mombin seed oil and cake were carried out using high-performance liquid chromatography (HPLC-DAD/UV-Vis) (Gonçalves *et al.,* 2017), with a Shimadzu model (Shimadzu Corporation, Kyoto, Japan) equipped with four high-pressure pumps (model LC-20AT), photodiode array detector (model SPD-M20A), degassing unit (model DGU-20A5), CBM-20A interface, CTO-20AC column oven, and an autosampler (model SIL-20A). Separation was done by means of a Shimadzu Shimpack ODS GVP-C18 column (4.6 x 250 mm, 5 mm) connected to a pre-column (Shimadzu-pack ODS GVP-C18, 4.6 x 10 mm, 5 µm). The mobile phase consisted of 2% (v/v) acetic acid in deionized water (mobile phase A) and a 70:28:2 (v/v) ratio of methanol:water:acetic acid (mobile phase B) at a flow rate of 1.0 $mL \cdot min^{-1}$ with a gradient elution program: 20% B (0-5 min), 45% B (25-43 min), 80% (50 min), 0% B (55-65 min), and execution time of 65 minutes. Injection volume was 20 µL and analyses were conducted at 15 °C. Phenolic compounds were detected at 280 nm. Standard solutions were diluted in methanol (HLPC grade) and calibration curves were obtained by injecting ten different concentrations in duplicate. Phenolic compounds were identified by comparing retention times and standards (gallic acid, catechin, chlorogenic acid, caffeic acid, ferulic acid, *trans*-cinnamic acid, vanillin, quercetin, *m*-coumaric

acid, *p*-coumaric acid, *o*-coumaric acid). Results were expressed in mg of phenolic compound·100 g^{-1} of the sample.

2.11. Fatty acids profile

Methylation of the oil extracted (AOCS, 2013) was carried out with the objective of producing fatty acid methyl esters (FAME). The esters which resulted from this esterification step underwent a gas chromatography analysis using GC 2010 SHIMADZU equipment with flame ionization detector (FID) and capillary column (100 m x 0.25 mm x 0.2 μm). The chromatographic conditions were: a) Injector: 1-μL of the sample was injected with execution time of 60 min operating in the split mode, using helium drag gas at a flow rate of 1.0 mL·min^{-1}; b) Column: initial temperature of 140 °C, held for 5 minutes, and raised at a rate of 4 °C·min^{-1} to 240 °C. The stationary-phase column consisted of biscyanopropyl polysiloxane. Fatty acids were identified and quantified by comparing the retention time of esters in the Supelco 37 standard of the components in the FAME mixture (CRM47885 - CAS 75-09-2) with the samples.

2.12. Statistical analysis

The design and coefficient of the predictive models were obtained using Statistic 12 software (Statsoft, Tulsa, USA). The analysis of variance (ANOVA) was used to analyze data from red mombin seed oil extraction tests and indicate significant differences ($p < 0.05$). The variable presenting the lowest p value (or highest F value) indicated the most significant effect ($p < 0.05$) of responses. The response surface methodology (RSM), combined with the desirability function, was used to evaluate process optimization. The results from the red mombin seed cake and oil characterization analyses were expressed in mean ± standard deviation and were conducted in triplicate. The analyses of residue antioxidant capacity were submitted to a 5%-probability Tukey's test for extract variables.

3. RESULTS AND DISCUSSIONS

3.1. Effects of ultrasound-assisted extraction on red mombin seed oil yield

The experimental value and the predicted yield of red mombin seed oil extraction carried out through various experiments, under various conditions, are shown in Table 1.

The highest oil extraction yield was obtained in experiment 10, in which extraction conditions were 10 min and solvent amount was 23.10 mL of solvent per gram of solid. In light of the yield obtained, Table 2 demonstrates the effect of independent variables on the dependent variable. The independent variable solid:solvent (mass:volume ratio) presented a positive effect ($p < 0.05$) on the linear and quadratic term, while "time" and "interaction among factors" did not have any effect on red mombin seed oil extraction ($p > 0.05$).

3.2. RSM analysis

Model adequacy was evaluated using the analysis of variance (ANOVA), shown in Table 3. The regression model adjusted for the experiment results presented a coefficient of determination (R^2) of 0.9682, which indicates that 96.82% of total variability of responses was attributed to the experimental variables studied. In turn, the adjusted R value of 0.9417 showed a direct relationship between experiment values and predicted yield values. The F value of the model was 36.5808 and was lower than the p value (0.05), which along with the p value for lack of ad-

TABLE 2. **Analysis of the effect of the conditions (Time and S/S ratio) on the yield of red mombin seed oil extraction.**

Factors	Efects	SD	t (3)	p
Mean	45.7115	0.7565	60.4212	0.0000
S/S ratio (g·mL^{-1}) (L)	46.7398	1.0699	43.6854	0.0000
S/S ratio (g·mL^{-1}) (Q)	-18.8956	1.1962	-15.7962	0.0000
Time (min) (L)	2,9404	1.0699	2.7482	0.0708
Time (min) (Q)	-1.8622	1.1962	-1.5568	0.2173
S/S ratio x Time (L)	-3.5146	1.5130	-2.3228	0.1028

L: linear; Q: quadratic. ($p < 0.05$).

TABLE 3. **Analysis of variance (ANOVA) of the model adjusted for yield.**

Source	Sum of squares	DF	Mean square	Fvalue	Ftab	R^2	Radj
Model	4976.259	5	995.2518	36.5808	4.39		
Residual	163.241	6	27.2069	-	-		
Lack of fit	156.373	3	52.124	22.7670	9.28		
Pure error	6.868	3	2.289	-	-		
Correlation Total	5139.500	11	-	-	-	0.9682	0.9417

justment indicated that the model precisely adjusted to the experimental data.

The mathematical model codified for red mombin seed oil extraction yield is a second-order polynomial and is represented by equation 3.

$$Y = 45.71 + 46.74x_1 - 18.90x_1^2 \quad (3)$$

A tridimensional (3D) response surface graph was constructed and is shown in Figure 1. These types of graphs are useful to determine maximum and minimum points. In turn, contour graphs are useful to determine how variables influence the desired responses (Li *et al.,* 2012). Thus, the effects of solid:solvent ratio, time, and response analyzed were assessed and the yield value increased until it reached a level > 60%, with a solid:solvent ratio of 1:23.10. In addition, taking into account the industrial extraction process, in order to reduce energy and time expenditure, experiment 2 showed a yield close to the maximum, at around 59%, but with half the extraction time applied in experiment ten. In order to reach maximum yield value, the minimum time required is approximately 2 min.

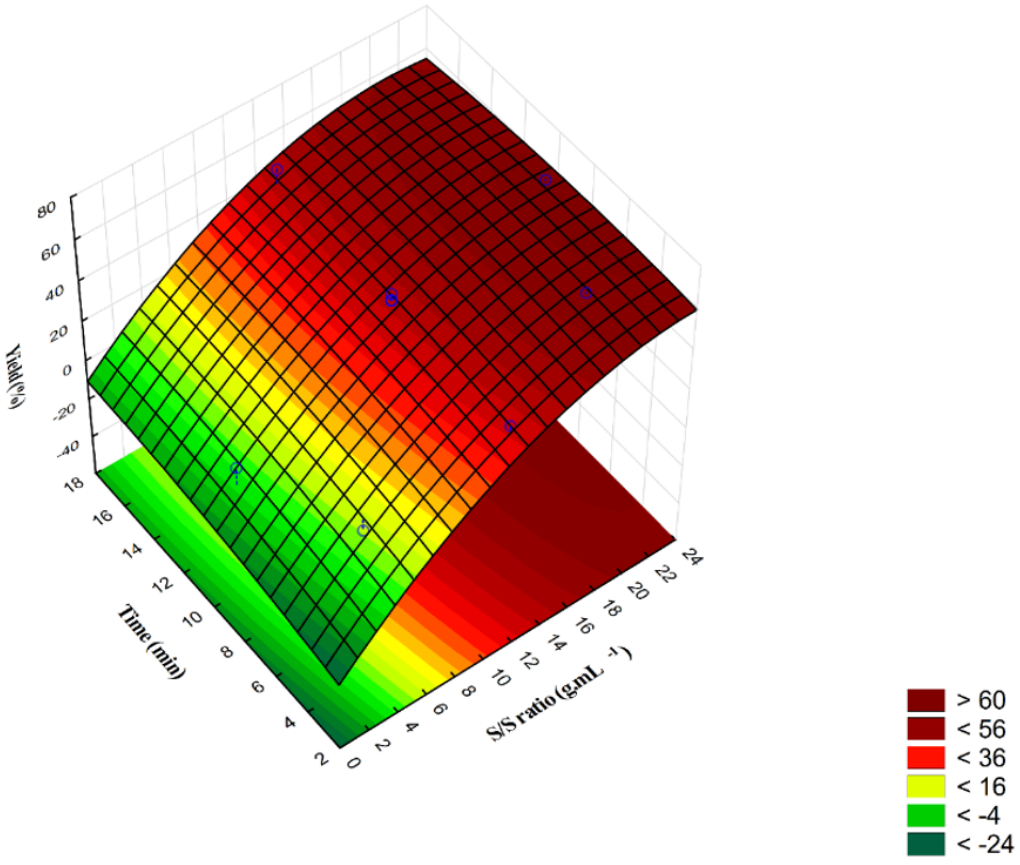

FIGURE 1. Response surface of the yield from red mombin seed oil extraction.

3.3. Optimization of the conditions for red mombin seed oil extraction

The desirability function was applied to optimize the ultrasound-assisted extraction of red mombin seed oil. The optimized values are shown in Figure 2.

As observed in Figure 2, the optimized point to obtain a yield value above 60% would be after 6.46 min, with a solid:solvent ratio of 1:23.10. Under these optimized conditions, the predicted value for extraction yield was 60.89%, while the experimental value observed was 62.85 % (n = 3), thus validating the model. The strong correlation observed between real and predicted values confirms that the model was adequate to reflect the optimization expected. Only the optimized values of extracted red mombin seed oil and its cake were characterized.

A satisfactory adjustment of the quadratic model was observed for the ANOVA and parametric analyses, such as R^2. The results showed that predicted and experimental values were significantly different. However, the present study showed that the combination of emerging ultrasound extraction technology and natural raw material, such as the residue from red mombin seeds, is an economic alternative for traditional extraction methods, considering industry demands and sustainable development. However, more advanced studies should be conducted, using less harmful solvents and other extraction conditions.

3.4. Phenolic compound and fatty acid profiles

The identification of phenolic compounds in red mombin seed cake and oil is shown in Table 4. A few classes of phenolic compounds could be identified in red mombin seed cake, such as simple phenols, cinnamic acids, and flavonoids. This shows that after extracting the oil, the residue still presented phenolic compounds with biological activities of interest,

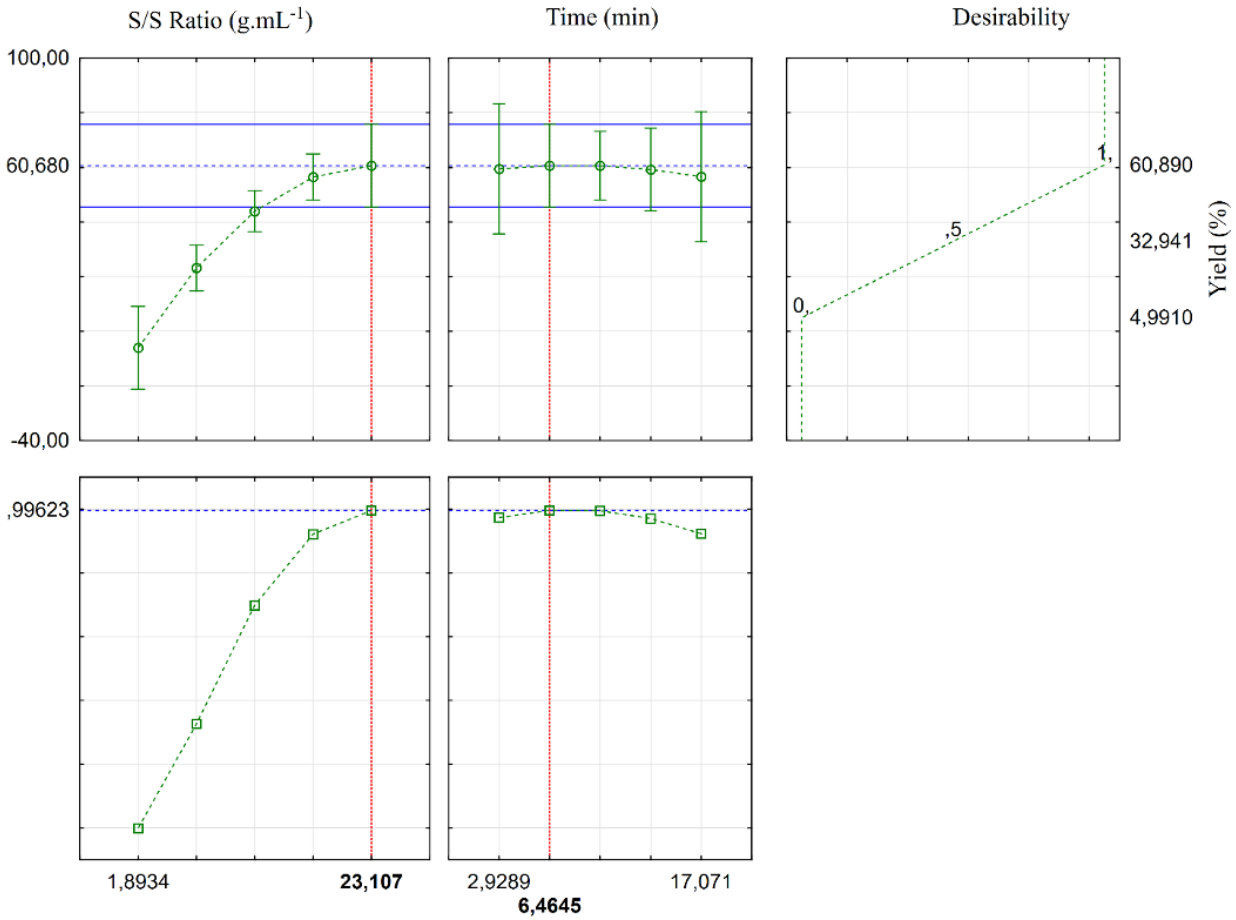

FIGURE 2. Desirability graph of the yield from the red mombin seed oil extraction.

TABLE 4. **Profile of phenolic compounds of the cake and oil and fatty acids of red mombin seed oil obtained by USA.**

Phenolic compounds	**Retention time (min)**	**Phenolic profile (mg·100g^{-1})**	
		Red mombin seed cake	**Red mombin seed oil**
Acid galic	6.82	2.71±0.24	0.56±0.02
catechin	10.80	30.70±4.79	N.D.
Chlorogenic acid	12.56	N.D.	1.08±0.04
Caffeic acid	15.05	N.D.	N.D.
Vanillin	17.65	N.D.	0.52±0.06
M-coumaric acid	24.42	N.D.	N.D.
Ferulic acid	27.79	N.D.	N.D.
Quercetin	37.59	N.D.	N.D.
Trans-cinnamic acid	51.66	0.36±0.02	N.D.
Fatty Acid		**Red mombin seed oil (g·100g^{-1})**	
Saturated			
C8:0		0.50±0.00	
C12:0		0.57±0.24	
C13:0		1.04± 0.57	
C14:0		1.30±0.42	
C15:0		0.48±0.17	
C16:0		16.32±0.87	
C18:0		8.69±0.15	
C20:0		40.77±2.89	
C22:0		0.22±0.02	
C24:0		0.29±0.02	
Monounsaturated			
C16:1		0.47±0.05	
Polyunsaturated			
C18:2 *trans* n-6		30.89±2.14	
γ C18:3 n-6		0.43±0.03	
C18:3 n-3		1.56± 0.19	
SFA		70.18	
MUFA		0.47	
PUFA		32.68	
PUFA/SFA		0.47	
n-6/n-3		20.07	

Mean±SD (n=3). N.D.: Not detected; SFA: Saturated Fatty Acid; MUFA: Monounsaturated Fatty Acid PUFA: Polyunsaturated Fatty Acid.

such as antioxidant, antimicrobial, and anti-inflammatory activities attributed to gallic acid, catechin, and *trans*-cinnamic acid (Chen *et al.,* 2011; Kang *et al.,* 2018; Samavat *et al.,* 2016).

Regarding the oil extracted from red mombin seeds, there were also only a few classes of phenolic compounds present, such as benzoic acids, gallic acid, vanillin, and cinnamic acids, such as chlorogenic acid. These compounds may contribute to antioxidant capacity (Omena *et al.,* 2012). In comparison with the oil extracted from other seeds, all compounds identified in the present study were also found in camellia seed oil (*Camellia* L.) (Wang *et al.,* 2017; Fang *et al.,* 2015), while only vanillin

was reported in oils extracted from soybean, grape seed, pumpkin seed, and rice bran (Siger *et al.,* 2008). Moreover, gallic acid has also been identified in olive oil (Rosenblat *et al.,* 2008). Despite the presence of these compounds in oils extracted from other seeds, quantitative differences were observed among them.

The composition of fatty acids in red mombin seed oil, identified using gas chromatography with a flame ionization detector (GC-FID), is described in Table 4. This analysis yielded a total of 14 fatty acids. The most abundant were eicosanoic acid, followed by linolelaidic acid (a *trans* isomer of linoleic acid), palmitic acid, and finally, stearic acid. In total, the oil comprised 67.92% saturated fatty acids (SFA), 31.63% polyunsaturated fatty acids (PUFA), and only 0.45% monounsaturated fatty acids (MUFA).

Considering this percentage, data from the literature indicate that PUFA reduces total cholesterol and regulates levels of HDL and LDL cholesterol in the blood (Berto *et al.,* 2015). Other authors stated that n-3 fatty acids have immunosuppressor effects, and that n-6 fatty acids help regulate immune responses when activated (Perini *et al.,* 2010). Therefore, high n-6:n-3 ratio values may interfere in mechanisms related to inflammation, especially when there is an increase in n-6 PUFA due to their pro-inflammatory effect, and because enzymes involved in PUFA metabolism are mapped in a region that is often associated with cancer (Azrad *et al.,* 2013). The n-6:n-3 ratio values were close to those found in a study on *umbu* seed oil (a fruit from the Anacardiaceae family), for which values for this ratio were between 13.4 and 18.0 (Dias *et al.,* 2019).

In addition to the n-6:n-3 ratio, the PUFA:SFA ratio also provides important information. According to the Department of Social Health and Security (Department of Health and Social Security 1994), diets should present PUFA:SFA ratios ≥ 0.45 to be considered beneficial to human health. However, when this ratio is below the stipulated value it can lead to an increase in cholesterol levels in the blood (Berto *et al.,* 2015). Thus, despite the ratio between n-6 and n-3 PUFA, this relationship can be considered beneficial in case of consumption of red mombin seed oil.

3.5. Physicochemical characterization of red mombin seed cake and oil

The physicochemical characterization of red mombin seed oil is presented in Table 5. The refraction index represents the content of unsaturated fatty acids and indicates if this seed's oil presents edible oil qualities, given that the digestion of edible plant oils is determined by PUFA composition (da Silva and Jorge 2014; Rezig *et al.,* 2018). The refraction index value obtained (1.475) indicated high levels of unsaturated fatty acids, as in soybean oil (1.477), grape seed oil (1.472), and *cajá-manga* seed oil (1.4598) (Nehdi *et al.,* 2012; FAO/WHO 1995). The acidity index was similar to the *cajá-manga* seed oil (*S. mombin* L.), which belongs to the same family (Anacardiaceae) as the red mombin. However, *cajá-manga* seeds present lower values than those recommended by FAO/WHO, indicating possible oxidative degradation of the material evaluated. The presence of peroxides was not observed, indicating that lipid oxidation did not occur in this oil (Böger *et al.,* 2018). Therefore, the process used to obtain the oil preserved its final quality (Eromosele and Paschal, 2003).

The reducing capacity of red mombin seed oil was partially attributed to the phenolic compounds pres-

Table 5. **Characterization of red mombin oil and antioxidant profile.**

Parameters	**Red mombin seed oil**	**CODEX ALIMENTARIUS (2003)**	**BHT**
Refractive index	1.475±0.00	-	-
Peroxide index	N.D.	15 meq·kg^{-1}	-
Acidity level (%oleic acid)	1.33±0.05	4.0 mg KOH·g^{-1}	-
CR (mg AGE·100g^{-1})	38.43±1.80	-	-
DPPH (IC_{50}) (mg·L^{-1})	186.64±1.17	-	85.2±0.7
ABTS (μmol de Trolox·g^{-1})	206.20±1.72	-	-

N.D.: Not detected

ent in the methanolic fraction extracted, since three phenolic compounds were identified (gallic acid, chlorogenic acid, and vanillin). However, based on the total reducing capacity (38.43 mg GAE·100 g^{-1}) found in the present study, and given the interfering substances in this analysis, it is known that this assessment is not specific to phenolic compounds but also for compounds from the -OH functional group. Therefore, since the oil studied was a raw extract, other compounds such as vitamin C, sugars, amino acids, amines, and sulfur-containing compounds may interfere with the final results (Chen *et al.,* 2015). In addition, only 6.84% (2.63 mg·100 g^{-1}) of the total reducing capacity was related to phenolic compounds. The same behavior was observed in the methanolic fraction of grape seed oil (165.5 mg GAE·100 g^{-1}/6.14 mg·100 g^{-1}), passion fruit seed oil (262.3 mg GAE·100 g^{-1}/9.28 mg·100 g^{-1}), and pumpkin seed oil (268.6 mg GAE·100 g^{-1}/1.21 mg·100 g^{-1}) (da Silva and Jorge, 2014).

In turn, the antioxidant capacity of red mombin seed oil was completely determined using an extract prepared with isopropyl alcohol because other compounds with different polarities were considered. Thus, the DPPH method showed that 186 mg·mL^{-1} of antioxidants would be necessary to reduce the initial concentration of oxidation reactive species by 50%. Antioxidant capacity through ABTS showed a higher value than what was observed by Da Silva and Jorge (2014a) for grape seed oil (138.8 μmol of Trolox.g^{-1}).

The chemical characterization of red mombin seed cake, after oil extraction, is shown in Table 6. Moisture values were shown to guarantee cake preservation and stability, since water content was below the value (15%) recommended in Codex alimentarius (FAO/WHO) (1999). With this moisture content it is possible that the cake would help with mixture fluidity and maintaining ingredient portions when used in a food product (Zago *et al.,* 2015). Considering that red mombin seeds before oil extraction presented approximately 1.87 g·100 g^{-1} of lipid content, 43% of the initial content was recovered.

Since the cake is considered a raw material with a high content in dietary fibers, especially insoluble fibers (ANVISA 2012), it can be considered a functional co-product. Fibers present biological functions of interest, such as assisting intestinal transit and influencing the modulation of intestinal microbiota, generating various metabolic products and functions depending on the microorganisms present (Danneskiold-Samsøe *et al.,* 2019). According to the Institute of Medicine of the National Academy of Sciences (FNB/IOM 2009), mean fiber intake should be 14 g for every 1,000 kcal. Thus, by consuming 100 g of red mombin seed cake, a total of 83.73 g of dietary fiber is absorbed, which represents six times the recommended intake value.

Table 6. Proximal and chemical composition of red mombin seed cake.

Parameters	Red mombin seed cake
Moisture (g·100g^{-1})	4.31 ± 0.21
Ash (g·100g^{-1})	2.42 ± 0.05
Protein (g·100g^{-1})	4.81 ± 0.21
Lipids (g·100g^{-1})	0.82 ± 0.02
Total carbohydrates (g·100g^{-1})	87.74 ± 0.20
Insoluble fiber (g·100g^{-1})	80.45 ± 0.50
Soluble fiber (g·100g^{-1})	3.28 ± 0.50
Dietary fiber (g·100g^{-1})	83.73 ± 0.50
Caloric value (Kcal·100g^{-1})	377.64 ± 0.57
pH	3.78 ± 0.03
TTA (g·100g^{-1})	0.36 ± 0.01
SS (°Brix)	3.00 ± 0.58

Some authors state that, in fact, waste produced by agricultural and industrial activities are potential sources of nutrients and bioactive compounds (Banerjee *et al.,* 2017; Girotto *et al.,* 2015), especially for pharmaceutical industries (Mirabella *et al.,* 2014). In this case, the presence of bioactive compounds was observed by determining antioxidant capacity using various methods (Table 7) and solvents. Part of the values obtained in these trials could be attributed to the phenolic compounds identified using HPLC-DAD/UV-Vis. Statistical differences ($p < 0.05$) were observed among the solvents evaluated in all antioxidant capacity methods investigated. MAWE was the solvent that extracted the most compounds present in the cake because the mixture of solvents involved (50% methanol:70% acetone:distilled water (2:2:1) (v:v:v)) can act directly on cell walls and membranes, favoring the process of compound extraction and consequently causing their lixiviation (Lapornik *et al.,* 2005).

Taking into account the existing literature on the nutritional compounds and antioxidant capacity of

Table 7. **Antioxidant profile of the red mombin seed cake.**

Extract	RC (mg AGE.100g^{-1})	DPPH (IC_{50})	ABTS (µmol de Trolox.g^{-1})	FRAP (µmol de FeSO4.g^{-1})	β-Carotene bleaching (%Protection)
ETE	6.94±0.13^{e}	85.16±5.97^{e}	305.67±2.85^{d}	80.35±0.69^{e}	41.34±5.45^{c}
ALE	45.05±1.00^{d}	13.62±1.26^{d}	166.01±3.72^{e}	70.07±0.70^{d}	34.32±3.97^{d}
AQE	106.33±4.16^{c}	6.96±0.87^{c}	356.83±3.50^{c}	256.74±5.90^{c}	62.83±1.32^{a}
MAWE	493.87±6.27^{a}	1.90±0.21^{a}	2734.73±2.16^{a}	984.30±2.81^{a}	54.39±1.62^{b}
HAE	454.55±2.87^{b}	2.33±0.03^{b}	1274.98±5.21^{b}	811.45±4.24^{b}	44.63±3.67^{c}

Results correspond to means ± standard deviation of three replicates. Lowercase letters on the same line and uppercase letters in the same column do not differ statistically from the Tukey test at 5% ($p < 0.05$). RC: reducing capacity; ETE: ether extract; ALE: alcoholic extract (95% alcohol); AQE: aqueous extract; MAWE: methanol + acetone + distilled water extract; HAE: hydroalcoholic extract (70% alcohol).

red mombin seed cake, the present study represents the first contribution to the subject. Red mombin seed cake presented interesting nutritional values from a preservation and fiber content point of view. Conveniently, the cake that originates from the extraction of red mombin seed oil may eventually be used as an ingredient for new types of food, with the objective of providing nutritional enrichment.

4. CONCLUSIONS

The red mombin seed is considered a waste in the processing of pulps by the food industry, causing numerous environmental problems, so this study collaborates to reduce this organic waste. Thus, it was possible to obtain a maximum yield in the extraction of the oil in experiment ten under the conditions of 23.10 mL of solvent per gram of seed and extraction time of 6.46 minutes, obtaining a 60% yield, contributing to the discovery of new sources of antioxidant compounds, mainly from agro-industrial waste, aiming for the development and application of new active products by the pharmaceutical medicinal industries and nutraceutical companies due to the presence the unsaturated fatty acids and phenolic compounds.

Futhermore, in this study it was also possible to obtain other conditions for the extraction of oil from the red mombin seeds that benefit the industry in terms of energy and process savings, maintaining a yield close to the maximum and with reduced time.

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DATA AVAILABILITY

The data referring to this article are part of the development of the master's thesis and are not disclosed in any repository.

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Assessment of obtaining sunflower oil from enzymatic aqueous extraction using protease enzymes

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SUMMARY: The aim of this work was to maximize the enzymatic aqueous extraction (EAE) of sunflower seed oil using protease enzymes from the evaluation of various temperatures, pH and enzyme concentrations, using a Box-Behnken experimental design. The effect of a thermal pre-treatment of sunflower seeds on free oil yield (FOY) and oil quality was also determined. In the experimental range adopted, a lower temperature (40 °C) provided higher FOY values, as well as the intermediate pH (8.00) and maximum enzyme concentration (9% v/v). Thermal pre-treatment provided an increase in FOY in the initial extraction times (60 to 180 min) and decreased of the extraction time of 4 to 3 h to obtain the highest FOY value (~16%). The fatty acid composition of the oils obtained showed a predominance of oleic (~47.5%) and linoleic acids (~39.5%). The total phytosterol content in the samples was hardly affected by the heat pre-treatment of the seeds, while the fatty acid profile, tocopherol content and oxidative stability were not altered.

KEYWORDS: *Alcalase; Enzymatic extraction; Free oil yield;* Helianthus annuus *L.*

RESUMEN: ***Evaluación de la obtención de aceite de girasol a partir de extracción acuosa enzimática usando enzimas proteasa.*** El objetivo de este trabajo fue maximizar la extracción acuosa enzimática (EAE) de aceite de semillas de girasol utilizando la enzima proteasa a partir de la evaluación de las variables temperatura, pH y concentración de la enzima, utilizando un diseño experimental de Box-Behnken. Además, se determinó el efecto del pretratamiento térmico de las semillas de girasol sobre el rendimiento (RA) y la calidad del aceite. En el rango experimental adoptado, las temperaturas más bajas (40 °C) proporcionaron valores de RA más altos, así como el pH intermedio (8,00) y la concentración máxima de enzima (9% v/v). El pretratamiento térmico proporcionó un aumento del RA en los tiempos de extracción iniciales (60 a 180 min) y una disminución del tiempo de extracción de 4 a 3 h para obtener el valor de RA más alto (~ 16%). La composición en ácidos grasos de los aceites obtenidos mostró predominio de los ácidos oleico (~47,5%) y linoleico (~39,5%). El contenido total de fitosteroles en las muestras se vio poco afectado por el pretratamiento térmico de las semillas, mientras que el perfil de ácidos grasos, el contenido de tocoferoles y la estabilidad oxidativa no se vieron afectados.

PALABRAS CLAVE: *Alcalasa; Extracción enzimática;* Helianthus annuus *L.; Rendimiento de aceite.*

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1. INTRODUCTION

The sunflower (*Helianthus annuus* L.) is cultivated in all continents, and the plant is a dicotyledon, originating from the North American continent (Saydut *et al*., 2016). This crop is an oilseed which contains around 38 to 50% high quality oil and has a great capacity of adaptability in different soil and climate conditions (Castro and Leite, 2018). In 2018, the worldwide production was 50 million tons, and Ukraine is the largest producer in the world, follow by Russia and Argentina (FAO, 2020). Sunflower oil is mainly used for human consumption, such as edible oil, margarine and salad sauce (Sánchez-Muniz *et al*., 2016). Furthermore this oil is used in pharmaceutical, cosmetics and biodiesel production (Saydut *et al*., 2016).

The oil quality is associated with fatty acid profile. Sunflower oil stands out for being rich in these compounds, with a predominance of unsaturated fatty acids, mainly linoleic acid (60 to 70%), followed by oleic acid (20 to 30%) (Aquino *et al*., 2019). The consumption of vegetable oil with high quantities of linoleic and oleic acids can help to decrease low-density lipoproteins (LDL cholesterol), and consequently reduce the risk of heart disease (Sánchez-Muniz *et al*., 2016). The oil extracted from sunflower seeds is also composed of natural antioxidants, such as a-tocopherols, phytosterols, vitamins A, D and E, which aid in oxidative stability (Aquino *et al*., 2019; Chen *et al*., 2020), These components are in minor quantity and provide additional nutritional value.

In order to achieve less harmful processing to the environment, without the use of toxic and flammable solvents, vegetable oil extraction with petroleum-based solvent can be replaced by enzymatic aqueous extraction (EAE), a sustainable method which is considered a green process (Cheng *et al*., 2019). Enzymatic extraction is characterized by using water as a solvent and enzymes for hydrolysis of the cell wall, which is responsible for trapping oil in the oleaginous (Yusoff *et al*., 2015). Thus, it is necessary to break up the cell wall and membranes of the oilseed by enzymatic hydrolysis, to release of oil, which is in intracellular vacuoles (Liu *et al*., 2016). Enzymatic hydrolysis, in addition to breaking the wall cell, is effective in breaking down the molecular complex lipoprotein and lipopolishaccaride in simple molecules, releasing extra oil that would not be extracted by means of another method (Campbell *et al*., 2016; Yusoff *et al*., 2015).

In the primary plant cell wall, there is an insoluble micro-fibrillary phase consisting of cellulose and hemicellulose which provides support and constitutes its main structure where other components such as proteins and glycoproteins are incorporated and a non-cellulosic polymers phase, which consists of pectic polysaccharides (Broxterman and Schols, 2018). Due to the composition of the structure cell wall, the most commomly-used enzymes for the enzymatic aqueous extraction of vegetable oil are cellulose, hemicellulose, pectinase, protease and a-amylase (Liu *et al*., 2016). Extraction efficiency and oil quality depend on the application of one or a combination these enzymes (Yusoff *et al*., 2015).

Protease enzymes play a significant role in the cell's metabolism because these enzymes can digest long protein chains in shorter fragments through the hydrolysis of peptide bonds (Gong *et al*., 2017). Alcalase® is a bacterial alkaline protease produced by *Bacillus licheniformis* and has been considered by many researchers to be one of the best enzymes for protein hydrolysis (Memon *et al*., 2019). Proteases are one of the most important enzyme groups used commercially, constitute approximately 40% of world's enzyme market. They are widely used in the food, detergent, leather, pharmaceutical and biotechnology industries (Vijayaraghavan *et al*., 2014). In the extraction of vegetable oil, proteolytic enzymes hydrolyze oleosins, which are proteins that surround the body of oil in the oilseed, decreasing surface activity and promoting the release of the oil (Moura *et al*., 2008). Protease enzymes are used for oil extraction from sunflower (Ribeiro *et al*., 2016), *Camellia oleifera* (Meng *et al*., 2018), pecan nuts (Polmann *et al*., 2019) and pomegranate seeds (Goula *et al*., 2018).

The aim of this work was to maximize the enzymatic aqueous extraction of sunflower oil using protease enzymes and to evaluate the effects of the temperature (40 to 60 °C), pH (7.0 to 9.0) and enzyme concentration (1% to 9% (v/v)) on the free oil yield (FOY). In the condition of maximum FOY, the influence of thermal pre-treatment on the seeds and quality parameters of the oil were verified.

2. MATERIALS AND METHODS

2.1. Materials

Sunflower seeds were purchased at a local market in Umuarama (Paraná – Brasil). Alcalase® 2.4L FG (endo-protease that hydrolyzes most peptide bonds within a protein) with an activity of 2.4 $U \cdot mL^{-1}$ (Unit defined by the hydrolysis of casein to produce 1 mmole of tyrosine per minute at pH 7.5 and 37 °C), was provided by LNF Latino Americana. The reagents used to adjust the pH were sodium hydroxide (Nuclear, 95%) and Chloridric acid (Nuclear, 40%) and *n*-hexane was used to determine the non-lipid fraction in the free oil (Panreac, Castellar del Vallès, Barcelona). The solvents ethanol (95%, Anhydrol, Diadema, São Paulo, Brazil) and *n*-hexane (Panreac, Castellar del Vallès, Barcelona) were used in the oil recovery tests. The fatty acid profile was determined using methanol (≤ 99.9%, Panreac, Castellar del Vallès, Barcelona), sodium hydroxide (≤ 97%, Anidrol, Diadema, São Paulo, Brazil), a boron trifluoride-methanol solution (BF_3, B1252, Sigma-Aldrich, St. Louis, MO, USA) and heptane (Neon, Suzano, São Paulo, Brazil). The contents of phytosterols, tocopherols and free fatty acids were determined using *N,O*-Bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA, 15238, Sigma-Aldrich, St. Louis, MO, USA), heptane (Neon, Suzano, São Paulo, Brazil) and 5-α-Cholestane (≥ 97.0%, C8003, Sigma-Aldrich, St. Louis, MO, USA) and methyl heptadecanoate (≥ 99.0%, 51633, Sigma-Aldrich, St. Louis, MO, USA) as internal standards. Acylglycerols were quantified by external standardization using monoolein (purity ≥ 99%), 1,3-diolein (purity ≥ 99%) and glyceryl trioleate (purity ≥ 99%), purchased from Sigma-Aldrich (Saint Louis, Missouri, United States).

2.2. Sunflower seeds preparation

Sunflower seeds with moisture 4.3% ± 0.20 (determined in an oven at 105 °C) were crushed using a household blender and then the particles obtained were classified (set of Tyler-type sieves, Bertel) and a fraction with an average diameter of 0.725 mm was used in the extractions.

In the experiments which evaluated thermal pre-treatment effect of the seeds, before crushing and granulometric classification, the methodology described by Tian *et al*. (2019) was adopted. Thus, whole seeds were immersed in distilled water in the ratio of 1:3 (w/v). After 3 hours of immersion at room temperature, the excess water was removed and the seeds (150 g) were spread in a thin layer under a metal sieve. The sieve was put in an oven with air circulation (Marconi, Model MA035) at 120 °C for 60 minutes.

2.3. Enzymatic aqueous extraction

The maximization of enzymatic oil extraction from sunflower seeds with the enzyme Alcalase® 2.4L FG was carried out using the Box-Behnken experimental design with three factors, three levels and four central points. The values of the three variables evaluated were: temperature (A) 40, 50 and 60 °C; pH (B) 7.0, 8.0, and 9.0 and enzyme concentration (C) 1, 5 and 9% (v/v). The three values for the variables corresponded to the levels: -1 (low), 0 (central point) e +1 (high), respectively. The values adopted for the independent variables took into account the Novozyme (2019) indications, which report temperature and pH range for optimal activity of the Alcalase® 2.4L FG enzyme at 30 to 65 °C and pH 7.0 to 9.0, respectively. Regarding the enzyme concentration evaluated (in relation to the extraction medium volume), it was based on the enzymatic aqueous oil extractions performed by Jiang *et al*. (2010), Ribeiro *et al*. (2016) and Meng *et al*. (2018).

The enzymatic extraction was carried out in Erlenmeyer flasks (125 mL) the crushed sunflower seeds (10 g) and distilled water (40 mL), in the mass ratio of 1:5 (g of seeds/g of water), proportions used according to the study of Aquino *et al*. (2019). In sequence, the pH was adjusted according to the condition to be evaluated with NaOH solution (1 $mol \cdot L^{-1}$) and enzyme added in the concentration of the test. Flasks were put in an orbital shaker (Marconi, model MA 830/A) at 180 rpm, for 5 hours with controlled temperature according to the experimental design.

After extraction, the free oil was recovered using the steps and conditions described by Aquino *et al*. (2019). Therefore, the suspensions had pH adjusted to 5.0 and the flasks were incubated for 1 hour under shaking 180 rpm at 25 °C. Then, the samples were stored overnight in a refrigerator (Consul, 340) at 4 °C. After that, the samples were centrifuged twice at 2700 rpm for 15 minutes at room temperature and four phases were formed (solid, aqueous, emulsion and free oil). The free

oil in the upper phase was transferred to a petri dish and kept in an oven until reaching constant weight. To assess the free oil content in the upper phase, the determination of the non-lipid fraction in the sample was performed as described by Rodriguez *et al.* (2021), obtaining 4.7% ± 0.71 of non-lipid compounds from this phase. The free oil yield (FOY) was calculated by Equation 1:

$$\text{Free Oil Yield (\%)} = \frac{\text{Weight of recovered free oil (g)}}{\text{Weight of sunflower seed (g)}} \text{x100} \quad \text{(Eq. 1)}$$

The analysis of variance (ANOVA) was used to evaluate the effects of independent variables on the dependent variable (free oil yield). The experimental data were adjusted to a second-order polynomial mode, using the Statistica® 8.0 software. The generalized model used is expressed in Equation 2:

$$Y = \beta_0 + \Sigma_{i=1}^{3} \beta_i X_i + \Sigma_{i=1}^{3} \beta_{ii} X_i^2 + \Sigma_{i=1}^{2} \Sigma_{j=i+1}^{3} \beta_{ij} X_i X_j \quad \text{(Eq. 2)}$$

Where β_0, β_i, β_{ii} and β_{ij} are the regression coefficients (β_0 = constant term; β_i = linear term; β_{ii} = quadratic term; β_{ij} = linear interaction term) and Y is the response variable (free oil yield - FOY) observed in the experiments. X_i and X_j are independent variables: temperature, pH and enzyme concentration.

The experimental conditions provided the maximum FOY in the evaluated experimental range as determined by Equation 2. In this condition, verification experiments (triplicate) were carried out to evaluate the predictive capacity of Equation 2 and the experimental results were submitted to the Student's t-test (Excel®, 2016) to estimate differences between the experimental values and the predictions. In this same experimental condition, experiments were carried out in triplicate to verify the efficiency of the solvent recovery of the oil from the phases. 5 mL of solvent (ethanol or *n*-hexane) were added in the step where four phases were formed, and the flasks were shaken to ensure homogenization and in the sequence centrifuged at 2700 rpm for 15 minutes. This procedure was performed three times for each solvent. The oil with solvent was transferred to a petri dish, and kept in an oven until reaching constant weight.

The influence of the thermal pretreatment of sunflower seeds was evaluated in the experimental conditions which give maximum FOY, as obtained from Equation 2. The extractions (destructive) were performed in triplicate at the times 60, 120, 180, 240, 300 and 360 minutes for seeds with and without treatment.

2.4. Characterization of free oil

A gas chromatograph coupled to the mass spectrometer (GC-MS) (Shimadzu, model CG-2010 Plus, Tokyo, Japan) and equipped with an automatic injector (Shimadzu, model AOC-20i, Tokyo, Japan) was used to analyze the fatty acid profile and contents in phytosterols, tocopherols and free fatty acids.

For the fatty acid composition, the oil was previously prepared according to the procedure described by Stevanato and Silva (2019). 1.5 mL of a 0.5 mol·L^{-1} methanolic sodium hydroxide solution were placed in a test tube. The tube was shaken vigorously and heated in a thermostatic bath (Nova Ética, model 314/8, Piracicaba, São Paulo, Brazil) at 100 °C for 100 min. Subsequently, 2 mL of derivative agent BF_3 were added and the tube was subjected to heating again for 5 min. A 5-mL aliquot of heptane was added to the test tube and after phase separation the supernatant was collected and sent for analysis. The analytes were separated in a capillary column DB-Wax™ (Shimadzu, 30m × 0.25mm × 0.25 µm, Tokyo, Japan), using helium as carrier gas (1.0 mL·min^{-1}). The temperature of the injector, the ionic source and the interface were 250, 260 and 250 °C, respectively. The initial temperature of the column was 80 °C, which was elevated to 180 °C at a rate of 10 °C·min^{-1}, and then elevated again to 240 °C at 4 °C·min^{-1}, remaining constant for 2 min at this temperature. Fatty acid methyl esters (FAMEs) were identified using the NIST Spectrum Library Spectrum Library (version 2014). The FAME quantification was performed from the normative area of the chromatographic peaks, using the percentage of the relative area of each peak in relation to the sum of all peaks.

The contents of phytosterols, tocopherols and free fatty acids were determined using BSTFA/TMSC as derivatizing agent, following the method described by Stevanato and Silva (2019). A SH-Rtx-5MS™ capillary column (Shimadzu, 30m ×

0.25mm × 0.25 μm, Tokyo, Japan) was used to elute the compounds. The injection temperature was 280 °C. The oven was initially operated at 150 °C with an increase in temperature to 230 °C (10 °C·min^{-1}), then the temperature was increased again to 280 °C (15 °C·min^{-1}), and kept constant for 25 min. The identification was carried out as previously described and quantification was performed by internal standardization using 5-α-cholestane (5 mg·mL^{-1}) and methyl heptadecanoate (5 mg·mL^{-1}) as reference standards.

The acylglycerol composition was determined on a gas chromatograph (Shimadzu, GC-2010 Plus, Tokyo, Japan) equipped with flame ionization detector (FID), *on-column* injector (Shimadzu, Tokyo, Japan) and capillary column Zebron™ ZB-5HT inferno (Phenomenex, 10 m×0.32 mm×0.10 m, Torrance, CA, USA). The chromatographic conditions were previously described by Stevanato and Silva (2019). Chromatographic areas generated from the standard solutions of triolein (0.1 to 3.1 mg·mL^{-1}), diolein (0.03 to 2.5 mg·mL^{-1}), and monolein (0.05 to 2 mg·mL^{-1}) were plotted against the concentration to obtain the line equations ($R^2 > 0.99$).

The oxidative stability of the oil was determined using the Professional Rancimat Biodiesel equipment (Metrohm, model 823, Herisau, Switzerland). The samples (2.5 ± 0.003 g) were exposed to an air flow of 20 L·h^{-1} at a constant temperature of 130 °C. The secondary oxidation products were transferred to the measuring vessel containing 50 mL of distilled water. The induction period was automatically determined by the equipment, and measured from the increase in thermal conductivity of the distilled water.

2.5. Statistical analysis

To verify the influence of heat pre-treatment on sunflower seeds and oil quality, the results were evaluated by analysis of variance (ANOVA) and Tukey's test with a significance level of 5% (α=0.05), using the Statistica® 8.0 software. All treatments and analyses were performed at least in duplicate (n=4).

3. RESULTS AND DISCUSSION

3.1. Free oil yield (FOY)

The experimental condition and the free oil yield (FOY) obtained from each experimental condition, adopted in the experimental design, are presented in Table 1. Based on the results from this table, it can be verified that FOY varied from 7.02 to 15.59% and to identify the influence of each variable and its interactions on the response variable, the coded variable was adjusted to a second-order polynomial equation as expressed in Equation 3:

$$FOY\ (\%) = 7.82 - 1.39A + 0.36B + 1.70C + 3.24A^2 + 0.03B^2 + 0.32C^2 - 0.27AB - 0.93AC - 0.23BC \quad \text{(Eq. 3)}$$

Table 2 presents the ANOVA results which were used to validate the second-order polynomial model (Equation 3) adjusted to the experimental data, as well as to evaluate the influence of each variable on the response. The results showed that the model was significant ($p < 0.05$) only for the linear effects of the three variables and quadratics of the temperature variable (A). For the interactions among them, the model was significant only for the interaction of the varying temperature (A) and enzyme concentration (C). These results are shown by high values for F and low values for *p*.

Table 1. Experimental conditions applied and free oil yield (FOY) obtained in the experiment to assess the effects of the operating variables using a Box–Behnken design

Run	Variable[a]			FOY (%)
	A	B	C	
1	-1 (40)	-1 (7.0)	0 (5%)	11.61
2	1 (60)	-1 (7.0)	0 (5%)	9.64
3	-1 (40)	1 (9.0)	0 (5%)	13.07
4	1 (60)	1 (9.0)	0 (5%)	10.04
5	-1 (40)	0 (8.0)	-1 (1%)	10.21
6	1 (60)	0 (8.0)	-1 (1%)	9.03
7	-1 (40)	0 (8.0)	1 (9%)	15.59
8	1 (60)	0 (8.0)	1 (9%)	10.68
9	0 (50)	-1 (7.0)	-1 (1%)	6.04
10	0 (50)	-1 (7.0)	1 (9%)	9.76
11	0 (50)	1 (9.0)	-1 (1%)	7.02
12	0 (50)	1 (9.0)	1 (9%)	9.83
13	0 (50)	0 (8.0)	0 (5%)	8.06
14	0 (50)	0 (8.0)	0 (5%)	8.06
15	0 (50)	0 (8.0)	0 (5%)	7.61
16	0 (50)	0 (8.0)	0 (5%)	7.55

[a]A= Temperature; B= pH and C= Enzyme concentration.

TABLE 2. Analysis of variance (ANOVA) of the quadratic model for the maximization of free oil yield (FOY) from the enzymatic aqueous extraction of sunflower oil

Source	Sum of square	Degree of freedom	Medium square	F	p[a]
A (L)	15.374	1	15.374	198.624	0.0008
A (Q)	42.055	1	42.055	543.349	0.0002
B (L)	1.059	1	1.059	13.676	0.0343
B (Q)	0.003	1	0.003	0.039	0.8559
C (L)	22.984	1	22.984	296.954	0.0004
C (Q)	0.397	1	0.397	5.128	0.1085
A*B	0.281	1	0.281	3.629	0.1529
A*C	3.478	1	3.478	44.938	0.0068
B*C	0.207	1	0.207	2.675	0.2005
Lack of fit	0.262	3	0.087	1.127	0.4619
Pure error	0.232	3	0.077		
Total	86.332	15			

A= Temperature; B = pH; C = Enzyme concentration; L = Linear effect; Q = Quadratic effect; [a] Statistical significance ($p < 0.05$).

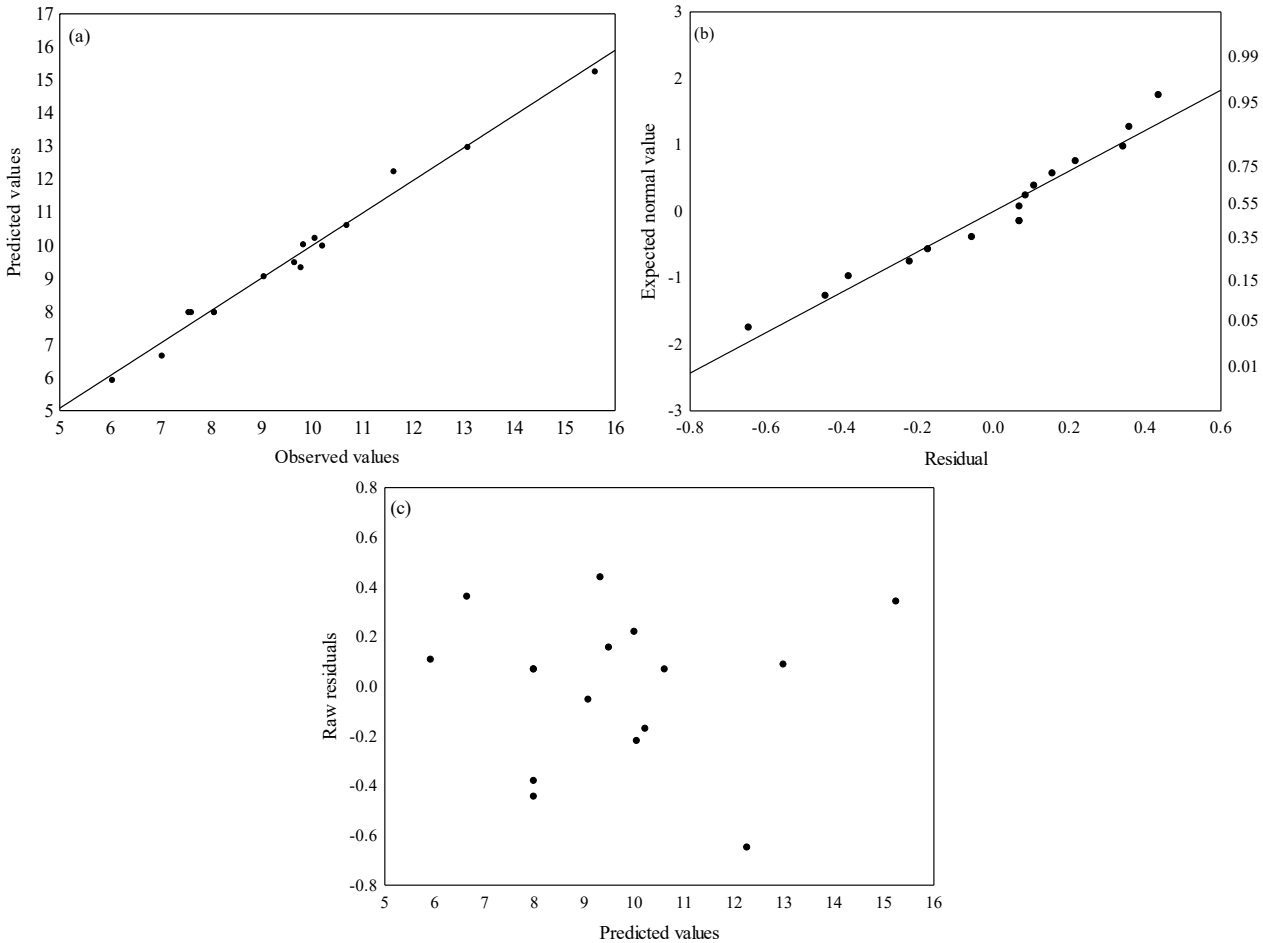

FIGURE 1. Diagnostic plots to verify the proposed model. (a) plot of predicted values versus observed values; (b) plot of normal probability of the residues; (c) raw residuals versus predicted values.

According to the ANOVA data, F_{calc} (123.28) was higher than F_{tab} (3.33); thus the polynomial model was valid in relation to the experimental data. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2Adj) of the model were 0.984 and 0.976, respectively, which indicates a high degree of correlation between the observed and predicted values.

In order to assess the adequacy of the predicted model, diagnostic graphs were generated, and are shown in Figure 1. Figure 1a shows good agreement between the experimental and predicted data, since the data are close to the straight line, which indicates a satisfactory fit. The graph of normal probability of the residues (Figure 1b) confirms the assumption of normality of the residues. This fact is characterized by the position of the points near the straight line, indicating that the errors are normally distributed. In the graph of raw residuals versus predicted values (Figure 1c), it can be seen that most of the residues were randomly distributed around zero, showing that there is no pattern of behavior between them and that the variance was constant. Thus, the information presented in Figure 1 confirms the adequacy of the model, as well as the validity of its predictions.

3.1.1. Effect of temperature

Temperature was the variable that had the greatest influence on the FOY and by maintaining the pH value and enzyme concentration parameters constant, the highest values for response variables were obtained with the lowest temperature (40 °C). This was evidenced by the results from runs 1, 3 and 7, which are shown in Table 1 as the highest FOY values. According Passos *et al*. (2009), enzymatic aqueous extraction is favored at 40 °C, a fact that allows energy saving and facilitates the preservation of enzymatic activity. In addition, the use of higher temperature increases energy costs (Cheng *et al*., 2019).

Enzymes normally have activities at temperatures between 35 and 60 °C and an increase in temperature results in protein denaturation (Yusoff *et al*., 2015). Consequently, this reduces the release of oil from oilseeds. However, it should be noted that within the optimal temperature range for enzymes, oleaginous matrix characteristics also have an influence at an appropriate temperature for the oil extraction process (Liu *et al*., 2016). The rate of enzymatic extraction is directly related to temperature, where high temperatures can increase extraction rate, but on the other hand they can darken the oil (Liu *et al*., 2016). The temperature influences oil quality, where a mild temperature does not deteriorate the oil due to the oxidation of polyunsaturated fatty acids and the development of rancidification (Ribeiro *et al*., 2016). Furthermore, high temperatures can also be the cause of the caramelization of the carbohydrates present in the extraction medium, which will reduce the quality and yield of the extracted oil (Yusoff *et al*., 2015).

In the enzymatic extraction of peanut oil using the Protizyme protease enzyme, Sharma *et al*. (2002) reported the highest oil yield at 40 °C. Li *et al*. (2011) evaluated five enzymes in the aqueous extraction process and the highest yield was obtained with the Alcalase® enzyme at 40 °C. According to Rui *et al*. (2009), the optimal temperature range for the hydrolysis of pectinase, cellulase and protease enzymes was between 40 to 55 °C.

3.1.2. Effect of pH

The increase in pH in the extraction medium favored the content of free oil. However, this increase was noted to be lower in magnitude than other parameters. Extraction efficiency can be maximized at the optimal pH; whereas each enzyme has an optimal specific value (Abdulkarim *et al*., 2005). The optimal pH should not be near the isoelectric point, which is 9.4 for Alcalase® 2.4L FG protease enzyme (Sigma-Aldrich, 2021), because in the specific isoelectric point the enzyme is insoluble and can make t oil extraction difficult (Kumar *et al*., 2017). The pH does not affect only enzyme activity, but also the separation of oil and protein. The enzymes can simultaneously solubilize and hydrolyze protein and break up polysaccharides, which facilitates oil extraction (Latif and Anwar, 2011).

When evaluating the enzymatic extraction of pine kernel oil with 2% (v/v) of Alcalase® at 50 °C, Li *et al*. (2011) obtained oil yield of 87 and 76% for extraction at pH 9.0 and 12.0, respectively. Meng *et al*. (2018) carried out the oil extraction of *Camellia oleifera* with Alcalase® enzyme and evaluated the effect of pH in the range of 8.5 to 9.5, obtaining the highest yield (93.5%) at pH 9.2. In obtaining pecan nut oil using aqueous extraction with the enzyme Alcalase®, Polmann et al. (2019) obtained an extraction yield of 65.3% at pH 8.0 and 52 °C.

3.1.3. Effect of enzyme concentration

The FOY was higher with the increase in enzyme concentration and this fact can be observed when comparing results achieved by varying the value for enzyme concentration and keeping temperature and pH values constant. For example, when comparing experimental runs 9 and 10, there was an increase of 62.6% in the FOY when enzyme concentration passed from 1 to 9% (v/v), respectively.

The enzyme amount is directly related to the substrate available for enzymatic hydrolysis and higher enzyme concentration increases the enzymatic interaction with substrate, which degrades the cell wall and releases the oil. Goula *et al*. (2018) reported that enzyme concentration affects hydrolysis and extraction yield. When extracting oil from pomegranate seeds, the greater the amount of enzyme used, the faster the extraction was and the higher the yield was. When higher enzyme concentrations of cellulase and protease (Peclyve V) enzymes, the oil yield was higher at 10%. However, there is a saturation point where the addition of more enzyme does not increase the yield, in addition to increasing processing costs and resulting in bitterness and darkening of the oil (Jiang *et al*., 2010; Latif and Anwar, 2011).

Siriwardhana *et al*. (2004) found that the extraction yield obtained from *Hizikia fusiformis* using Ultraflo® and Alcalase® 2.4 L FG protease enzymes increased when the enzyme concentration increased to 5%. In the enzymatic aqueous extraction of peanut oil using protease (Alcalase® 2.4 L FG) and cellulase (Cellulase AE80) enzymes, Jiang *et al*. (2010) reported that FOY increased with the increase in the amount of enzyme from 1 to 2%.

Although the enzyme represents a cost for the enzymatic aqueous extraction, Cheng *et al.* (2019), reported that the enzymatic extraction of soybean oil can be economically viable, because it requires less energy and the initial investment cost is lower when compared to the solvent extraction process. In addition, economic viability can be improved by recycling the enzyme and using the concept of biorefinery, where co-products, carbohydrates and proteins could be used as raw material for other processes (Sekhon *et al.*, 2018, Cheng *et al.*, 2019).

3.1.4. Interaction of variables

The contour graphics for the interaction between two independent variables were generated by keeping one variable at the central level (Figure 2). FOY increased with decreasing temperature and increasing pH value (Figure 2a) or enzyme concentration (Figure 2b). However, the effect of temperature was greater in combination with enzyme concentration than with pH, resulting in a more pronounced curve concavity (Figure 2b) and significant interaction. Figure 2c shows that the level curves of the variables pH and enzyme concentration did not show curvature. This linear behavior indicates that there were no considerable interactions between these independent variables and the FOY.

3.1.5. Maximization of FOY

To determine the set of variables that maximized of FOY from EAE, the desirability function was applied from Statistica® software, considering only the significant terms of Equation 3. The results showed that conditions were: temperature of 40 °C, pH 8.0 and enzyme concentration of 9% (v/v), which resulted in the theoretical FOY of 15.61%. To validate the efficiency of the predictive equation, the experiment was carried out under the conditions of maximum oil removal, in quintuplicate, and FOY obtained was 14.77% ±0.55. The efficiency of the model was verified by the Student´s *t*-test, which showed that there was no significant difference between real and predictive results.

Campbell *et al*. (2016) reported 39% sunflower oil at 50 °C, seed-to-water mass ratio of 1:10 and with the addition of 2% protease (Protex 7L) and 2% cellulase (Multifect CX 13L). The oil yield present in this work was determined with free oil and the article cited includes free and emulsified oil, which justifies the higher oil yield value than that presented in this work. Moradi and Rahimi (2019) extracted sunflower oil with a mixture of cellulase and pectinase enzymes and obtained free oil yield of 23.7% using 2% enzyme at 40 °C, pH 4.5 and seed-to-water mass ratio of 1:6. However, in order to separate the free oil it was washed with *n*-hexane, which may have contributed to obtaining higher oil mass. Thus, for comparative effects, experiments were performed in the condition of maximum FOY to verify the yield with the recovery of the emulsified

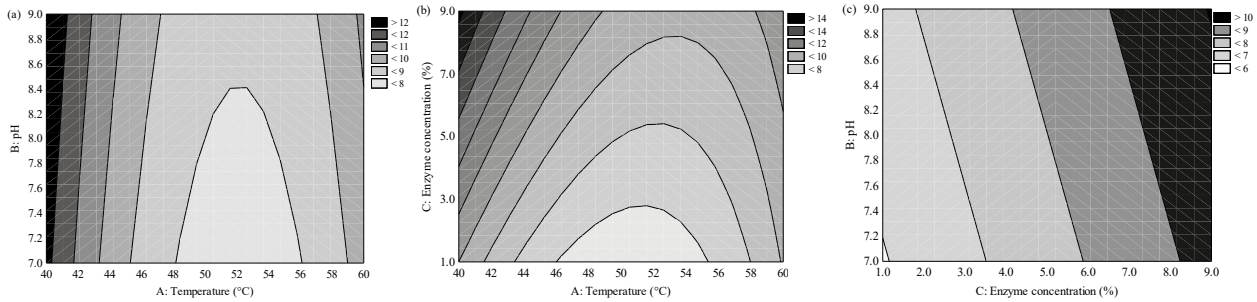

FIGURE 2. Contour plot of response surface showing the effects of binary interactions between independent variables on the free oil yield (FOY); (a) pH and temperature; (b) enzyme concentration and temperature; (c) pH and enzyme concentration.

oil, obtaining 19.04%±0.7 and 30.78%±0.79 of oil with the use of ethanol and *n*-hexane, respectively.

Ribeiro *et al*. (2016) obtained 36.6% of free oil in the enzymatic extraction with 10% of each enzyme, pectinase (Pectinex Ultra SPL), cellulase (Celluclast 1.5L) and protease (Alcalase® 2.4L FG), sunflower seed-to-water mass ratio of 1:6 at 55 °C and 8 hours of extraction. However, to obtain greater yield than that obtained in the present work, the concentration of the enzyme used by the authors was triple the amount in addition to the use of three different enzymes. Aquino *et al*. (2019) used Celluclast® 1.5 L enzyme and obtained 17.76% ± 0.46 of sunflower free oil in the enzymatic extraction. The authors used a temperature of 60 °C, seed-to-water mass ratio of 1:5 and 1% (v/v) enzyme. The value was close to that found in this study at 14.77% ± 0.55 using seeds of the same origin and lot. This difference can be justified by the action of the cellulase enzyme, which has different activity than the protease enzyme. The vegetable cell wall is composed of a higher amount of cellulose than protein (Szymanska-Chargot *et al*., 2015), so the action of cellulase enzyme in cellulose hydrolysis may have released a higher amount of oil. On the other hand, the protease enzyme can create protein hydrolysates which are better emulsifiers than native proteins. In this case the extracted oil is retained in the emulsion, decreasing FOY (Campbell *et al*., 2016).

3.1.6. Influence of thermal pre-treatment of sunflower seeds

Figure 3 presents the results for FOY obtained from extractions using seeds with and without thermal pre-treatment. Thermal pre-treatment influenced the FOY in the initial 180 minutes of extraction, thus the highest difference was observed after the first hour, with an increase of 71.34% in FOY. For

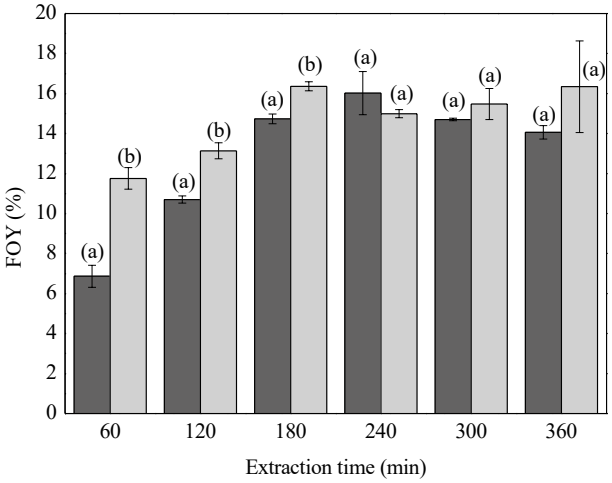

FIGURE 3. Free oil yield (FOY) from enzymatic aqueous extraction of oil from sunflower seeds after thermal treatment (light grey bars) and without thermal treatment (dark grey bar). Conditions: 40 °C, seed-to-water mass ratio of 1:5 (g/g), pH 8.0 and enzyme concentration of 9% (v/v). Data represent the means of duplicate analyses (n=3). Values with different superscript letters are significantly different ($p < 0.05$) for each extraction time. Differences were determined using the Tukey's test.

extractions of 2 and 3 hours, increases of 22.78% and 11.01% were obtained, respectively. After 240 minutes of extraction, no influence of heat treatment on FOY was observed. In this way, the process of enzymatic extraction with thermal pre-treated seeds reached equilibrium 1 hour before the process without thermal pre-treatment. This fact suggests that the oleosins around of a body oil and/or proteins are responsible for the emulsion stability in sunflower seeds and were de-naturated by the action of heat and humidity from the thermal treatment, which contributed to the increased FOY.

Li *et al*. (2016) treated thermally crude peanuts (150 °C for 20 minutes) and obtained an increase in the extraction oil yield and attributed the results to the

fact that temperature possibly affected the functional property of peanut proteins which are responsible for the stability of emulsion. Song *et al*. (2019) applied thermal treatment to peony seeds (110 °C and 0.48 MPa for 60 minutes) after immersed them in water at a seeds-water ratio of 1:5 (g/g) and obtained an enzymatic aqueous extraction of oil using pectinase enzyme. The treated seeds presented increased free oil yields of 77.13% to 89.45%. Furthermore, the microstructure of peony seeds with and without thermal treatment were analyzed through laser scanning microscopy, and it was possible to observe the rupture of oleic bodies and consequently the oil coalescence. Tian *et al*. (2019) applied a thermal pre-treatment (120 °C for 60 minutes) after immersion of colza seeds in water in a ratio of 1:3 (g/g) and related the increase in the yield to aqueous extraction. Thus, the authors concluded that the combination of humidity and higher temperature improved heat transfer and helped to irreversibly denature the layer of oleosin proteins that surround the oil bodies in the oleaginous seeds where oil coalescence occurs.

3.2. Oil characterization

The chemical composition of the oil obtained from sunflower seeds with and without heat pre-treatment obtained with 3 and 4 hours extraction time, respectively, is shown in Table 3. Oleic, linoleic, palmitic and stearic acids were the four main fatty acids present in the oil, with a predominance of oleic. According to Codex Alimentarius standards, sunflower oil obtained from EAE is classified as mid-oleic acid, whose content in this fatty acid is

Table 3. Effect of thermal pre-treatment on the chemical properties of sunflower oil obtained from enzymatic aqueous extraction

Property		Without thermal pre-treatment	With thermal pre-treatment
Fatty acids (%)	Capric	0.02±0.00[a]	0.02±0.00[a]
	Myristic	0.06±0.00[a]	0.07±0.00[a]
	Palmitic	5.72±0.07[a]	5.66±0.00[a]
	Palmitoleic	0.08±0.04[a]	0.08±0.00[a]
	Stearic	4.46±0.01[a]	4.41±0.02[a]
	Oleic	47.22±0.73[a]	47.70±0.18[a]
	Trans-Vaccenic	0.50±0.01[a]	0.49±0.01[a]
	Linoleic	39.67±0.73[a]	39.37±0.13[a]
	Arachidic	0.36±0.03[a]	0.36±0.01[a]
	Behenic	1.20±0.00[a]	1.18±0.02[a]
	Lignoceric	0.38±0.04[a]	0.40±0.01[a]
	Not identified	0.31±0.08[a]	0.25±0.01[a]
Phytosterols (mg per 100 g)	Campesterol	15.80±0.34[a]	15.99±0.14[a]
	Stigmasterol	21.46±0.60[a]	18.88±0.17[b]
	γ-Sitosterol	12.07±0.54[a]	8.72±0.01[b]
	β-Sitosterol	100.07±3.89[a]	90.08±1.13[b]
	Total phytosterols	149.41±5.33[a]	133.66±0.83[b]
Tocopherol (mg per 100 g)	α-Tocopherol	31.49±0.41[a]	34.55±1.32[a]
Free fatty acid (%)		0.57±0.08[a]	0.52±0.06[a]
Acylglycerols (%)	Triacylglycerols	86.86±0.65[a]	88.87±0.66[a]
	Diacylglycerols	3.36±0.03[a]	3.82±0.04[a]
	Monoacylglycerols	0.68±0.01[a]	0.64±0.01[a]
	Total acylglycerols	90.89±0.69[a]	93.33±0.71[a]
Induction period (h)		2.28±0.29[a]	1.95±0.04[a]

Means followed by the same lowercase letter (in each row) do not differ statistically ($p > 0.05$). Differences were determined using the Tukey's test (n=4).

in the range of 43.1-71.8% (Codex Alimentarius, 2019). The heat pre-treatment of the seeds did not influence the fatty acid profile of the oil, which showed high levels of monounsaturated fatty acids (~48%) and polyunsaturated fatty acids (~40%); while the saturated fatty acid content was low (~12%). The higher proportion of oleic acid than linoleic acid is an advantage to the quality of the oil. Smith *et al*. (2007) showed that sunflower oil with high oleic acid content has better oxidative and thermal stability than regular sunflower oil with high linoleic acid content (71.6%).

As shown in Table 3, four phytosterols (campesterol, stigmasterol, γ-sitosterol and β-sitosterol) and one tocopherol (α-tocopherol) were identified in sunflower oil. Among the phytosterols, β-sitosterol was the major component, representing on average 67.19% of the total composition. The high concentration of phytosterols in the oil can promote anti-lipid and hypolipidemic effects when ingested (Dai *et al*., 2013). In addition, phytosterols have antioxidant activity based on electron transfer and also have the ability to scavenge free radicals (Liu *et al*., 2019), attenuating lipid oxidation. The α-tocopherol present in the samples can also increase the oxidative stability of the oil due to its antioxidant capacity, which interrupts the chain reaction of free radicals (Liu *et al*., 2021). The heat pre-treatment slightly reduced (~10.54%) the total phytosterol content, due to the lower levels of stigmasterol, γ-sitosterol and β-sitosterol quantified in the oil. This effect can be attributed to the thermal degradation caused by heating during the pre-treatment. It is known that phytosterols undergo oxidation when subjected to heating, as reported by Chen *et al*. (2020) who studied the thermo-oxidative stability of soy germ phytosterols and reported that heating the oil to 120 °C for 60 min promoted a loss of ~8% in these phytosterols, which is in accordance with this study. However, α-tocopherol was not influenced by seed pre-treatment. The values obtained for phytosterol content were higher than those reported using the conventional method with n-hexane (Aquino *et al*., 2019) after 8 hours' extraction.

Sunflower oil had a low free fatty acid content (>0.6%), which indicates the absence of hydrolytic reactions responsible for causing rancidity and decomposing triglycerides (Goszkiewicz *et al*., 2020). Sunflower oil contains an average of ~92.11% total acylglycerols, which shows that aqueous enzymatic extraction showed high selectivity to this oil. The thermal pre-treatment did not modify the composition of acylglycerols, indicating that the heating time of the seeds was insufficient to hydrolyze the triacylglycerides into smaller components (MG and DAG).

The oil obtained in the present study had a longer period of induction compared to the studies by Ghosh *et al*. (2019) (0.56 h) and Ramos *et al*. (2020) (1.47 h), who evaluated the oxidative stability of sunflower oil at 130 °C. The high oxidative stability of sunflower oil can be attributed to the presence of active compounds, such as phytosterols and tocopherols. In addition, the mild conditions applied in the oil extraction of the oil can contribute to resistance to thermal oxidation. The oils obtained from seeds with and without heat treatment showed similar thermal stability. This result was expected, since susceptibility and oxidative resistance is mainly affected by the chemical composition of the oil and in this study the oil obtained from raw and pre-treated seeds showed similar compositions.

4. CONCLUSIONS

Temperature was the variable that had the greatest effect on the response variable, as the lower temperature favored the increase in free oil yield. In addition, the intermediate pH and the maximum level of enzyme concentration contributed to the increase in the response variable. Therefore, the conditions for maximum FOY (14.77% ± 0.55) from EAE using protease enzyme were 40 ºC, pH 8.0 and enzymatic concentration of 9% (v/v). The pre-treatment applied to sunflower seeds promoted an increase in FOY at the beginning of the extraction and decreased the extraction time by 1 hour when compared to enzymatic extraction with untreated seeds. Thus, FOY of 16.4%±0.8 was obtained in 3 hours of extraction. Therefore, the mild temperature, the short extraction time of 3 hours and the addition of just one enzyme, present the advantage of this extraction process, which provides a lower cost in relation to the enzyme and energy expenditure. The oil obtained by aqueous enzymatic extraction showed oleic and linoleic acid as major fatty acids. B-sitosterol was the main phytosterol present in the oil; while γ-tocopherol was the only tocopherol found. Still, the oils showed high resistance to thermo-oxidative degradation. The chemical composition of the oil was not affected by the pre-heat treatment of the seeds.

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