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Preparation and characterization of oleogels with tallow and partially hydrolyzed tallow as organogelators

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SUMMARY: The aim of this study was to evaluate the organogelation potential of tallow fat (TF) and partially hydrolyzed tallow fat (HTF) against saturated monoglyceride (MG) and a saturated monoglyceride + diglyceride mixture (MDG) as the organogelators. TF itself created oleogel at a 30% addition level, while HTF, MG and MDG oleogels were prepared at 10% addition levels. Fatty acid composition data showed that the oleogel of HTF (HTFO) was quite similar to those of MG and MDG oleogels. Solid fat content, free fatty acidity and peroxide values were found to be in acceptable ranges for HTFO. Thermal properties, crystal morphology and X-ray diffraction patterns were also evaluated. Rheological analyses indicated that all oleogels had higher storage modulus (G´) than loss modulus (G´´). The time-sweep test showed that after applying higher shear rates, the gels re-formed at rest. Further, all oleogels maintained their gelled consistency until around 54 °C. The results suggest that HTF could be a cheap, efficient, fast melting, safe and readily available organogelator.

KEYWORDS: *Microstructure; Organogelator; Partially Hydrolozed Tallow; Rheology; Tallow*

RESUMEN: ***Preparación y caracterización de oleogeles con sebo y sebo parcialmente hidrolizado como organogeladores.*** El objetivo de este estudio fue evaluar el potencial de la organogelación de grasa de sebo (GS) y grasa de sebo parcialmente hidrolizada (GSH) contra monoglicéridos saturados (MG) y mezcla de monoglicéridos + diglicéridos saturados (MDG) como organogelantes. Con la propia GS se creó un oleogel con un nivel de adición del 30%, mientras que los oleogeles de GSH, MG y MDG se prepararon con niveles de adición del 10%. Los datos de composiciones de ácidos grasos mostraron que el oleogel de GSH (OGSH) era bastante similar a los oleogeles de MG y MDG. El contenido de grasa sólida, la acidez grasa libre y los valores de peróxido se encuentran en rangos aceptables para OGSH. También se evaluaron las propiedades térmicas, la morfología del cristal y los patrones de difracción de rayos X. Los análisis reológicos indicaron que todos los oleogeles tenían un módulo de almacenamiento (G´) mayor que el módulo de pérdida (G´´). La prueba de barrido de tiempo mostró que después de aplicar velocidades de cizallamiento más altas, los geles se reformaron en reposo. Además, todos los oleogeles protegieron su consistencia gelificada hasta alrededor de 54 °C de temperatura. Los resultados han sugerido que GSH podría ser un organogelador barato, eficiente, de fusión aguda, seguro y fácilmente disponible.

PALABRAS CLAVE: *Microestructura; Organogelador; Reología; Sebo; Sebo parcialmente hidrolizado*

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1. INTRODUCTION

To fulfill the need for *trans*-free, low saturated and structured (plastic consistency) fats for the food industry, research studies on oleogelation technology have been accelerated in the past decade. Oleogel is simply defined as an oil in continuous liquid phase with macroscopic dimensions that are permanent on a time scale with immobilized networks of self-assembled molecules called organogelators (Co and Marangoni, 2012; Singh *et al*., 2017; Patel, 2018). With this technology, edible fats with various hardness and melting properties could be produced without *trans* acid formation or major changes in fatty acids or minor component compositions. Depending on the kinds and amounts of organogelators that form the gel network, totally edible, safe, and sensorially acceptable oleogels can be prepared (Bot *et al*., 2009; Co and Marangoni, 2012; Patel and Dewettinck, 2016; Patel, 2018). Based on the preparation approach and organogelators involved, various types of oleogels were developed such as directly dispersed systems, emulsion templates, structured biphasic systems and oil sorption systems (Patel and Dewettinck, 2016; Singh *et al*., 2017).

Low molecular weight gelators (LMWG) including mono-, di-, and tri-acyl glycerides, various fatty acids and alcohols, hydroxylated fatty acids, lecithin, various waxes (beeswax, shellac, sunflower, candelilla, carnauba, rice bran waxes), wax esters, ceramides, sorbitan derivatives, phytosterols, γ-oryzanol + phytosterol, sucrose esters, some amphiphiles, amino acid derivatives, and polymeric type gelators (PG) including some food proteins (gelatin, β-lactoglobulin, zein, gluten, gliadin), some hydrocolloids (xanthan gum, gum arabic, locust bean gum), ethyl cellulose, other cellulose derivatives, some carbohydrate derivatives (modified chitin, waxy starch) and combined gelator systems have been researched extensively (Terech and Weiss, 1997; Patel and Dewettinck, 2016; Patel, 2018; Sagiri *et al*., 2018). New and more convenient (food-grade, cheap, readily available, effective at low concentrations, matching desired physical properties, versatile, and permitted) organogelators for edible applications are currently under investigation (Co and Marangoni, 2012; Patel, 2018).

It was stated that a material could act as an organogelator if it poses a balanced soluble and insoluble portion on the same molecule in the oil continuous media to trigger solute-solvent and solute-solute interactions simultaneously. Through molecular interactions via H-bonding, van der Waals attractions and π-π stacking between the phases, supramolecular structures like crystal lattice, liquid crystals, micelles, bilayers, fibrils, and agglomerates result in oleogelation (Rogers, 2009; Dassanayake *et al*., 2011; Patel, 2018).

Organogelators based on tri-, di, and mono-glycerides or high melting point fractions of edible fats could provide the advantages of being food-grade, suitable for commercialization, cheap and easy to implement. Some examples of monoglyceride gels (Da Pieve *et al*., 2010; Ögütcü and Yilmaz, 2015), diglyceride gels (Rocha-Amador *et al*., 2014; Perez-Monterroza *et al*., 2014; Huang *et al*., 2018), triglyceride gels (Cerqueira *et al*., 2017), milk fat gels (Viriato *et al*., 2018), methyl cellulose coated palm stearine gels (Patel, 2017) exist in the literature. To the best of our knowledge, there has been no study on the applications of tallow fat or tallow fat-derived products used as organogelators. Since tallow is an edible and sensorially acceptable fat, it could be a feasible organogelator at possibly lower addition levels without significant enhancement of the saturated fatty acids.

Hence, the objective of this study was to evaluate the possibility of using tallow fat and partially hydrolyzed tallow fat in comparison with saturated monoglyceride and saturated monoglyceride + saturated diglyceride mixture as organogelators for structuring sunflower oil. The ultimate goal was to find cheaper, food-grade, efficient, and easily accessible new organogelators for edible applications.

2. MATERIALS AND METHODS

2.1. Materials

Refined-winterized sunflower oil (Biryağ, Trakya Birlik, Tekirdağ, Turkey), melted-filtered bovine body tallow fat (local store), saturated monoglyceride (Monomuls® 90-35 Saturated Monoglyceride, BASF Co., Illertissen, Germany), and a mono-palmitate + di-palmitate mixture (Veser Kimya, İstanbul, Turkey) were used in the study. All solvents and chemicals were of analytical grade and purchased either from Sigma Chem. Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Partial hydrolysis of tallow fat

A teflon-lined stainless steel autoclave (100 ml) was used for tallow hydrolysis. Melted tallow fat:deionized water (1:2, v/v) was put into the autoclave and then sealed. The autoclave was heated under an autogeneous pressure at 200 °C for 4 h. After cooling down to room temperature, the partially hydrolyzed mixture was analyzed for free fatty acids (FFA) according to the method of Ca 5a-40 (AOCS, 1998). The FFA was 48.5%, and the mixture was successfully washed with hot water (water: tallow, 1:1, v/v) until the FFA was reduced to 1.5%. Although not used for any purpose in this study, the free fatty acids could be valorized as soapstock or animal feed. Then, the partially hydrolyzed tallow fat was crystallized at room temperature and washed several times with cold water. Finally, it was heated to 110 °C to evaporate all water before cooling down to ambient temperature to get the partially hydrolyzed tallow fat. The initial tallow fat and partially hydrolyzed tallow fat were used as the new organogelators in this study. Both were creamy-white, odorless, solid products.

2.3. Preparation of the oleogels

Oleogels were prepared by mixing known portions of the organogelators and sunflower oil (SO) at 60 °C. First, the minimum gelling concentration (MGC) of tallow fat (TF) as the organogelator was determined by adding the serial proportion into the oil (SO:TF, from 95:5 to 60:40, w/w). It was determined that TF forms a stable gel at a 30% addition level (SO:TF, 70:30, w/w). Below that level, the oleogel was not stable and semi-liquid. Similarly, the MGC of the partially hydrolyzed tallow fat (HTF) was determined as 10%. The oleogels of monoglyceride and monoglyceride+diglyceride mixture were prepared at the same addition level (10%) for comparison. For all oleogels, after preparing the SO and organogelator mixtures, the beakers were put into a water bath (60 °C) and shaken. After complete melting, the mixtures were put into plastic cups and tubes. After cooling to ambient temperature, the prepared oleogels stayed in a refrigerator overnight. The next day, the samples were taken from the refrigerator and kept at ambient temperature. After stabilizing 24 h at room temperature, the analyses were started. During the analyses, the oleogels were kept at ambient temperature as well. The following abbreviations are used thoroughout the paper for the prepared oleogels: tallow fat oleogel (at 30% addition level) (TFO), partially hydrolyzed tallow fat oleogel (HTFO), monoglyceride oleogel (MGO), and monoglyceride+diglyceride mixture oleogel (MDGO).

2.4. Fatty acid composition analysis

The fatty acid compositions of sunflower oil (SO), tallow fat (TF), partially hydrolyzed tallow fat (HTF) and the oleogels prepared with tallow fat (TFO), hydrolyzed tallow fat (HTFO), monoglyceride (MGO), and mono- and diglyceride mixture (MDGO) organogelators were determined. Fatty acid methyl esters were prepared according to method Ce 2-66 (AOCS, 1998) and quantified by a Gas Chromatograph (Agilent Technologies 7890B, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) (Agilent Technologies, Palo Alto, CA, USA), and HP 88 capillary column (100 m×0.25mm ID×0.2µm film thickness, J&W Scientific Co, CA, USA). The oven was set to 120 °C for 1 min, 175 °C (10 °C/min) for 10 min, 210 °C (5 °C/min) for 5 min and 230 °C (5 °C/min) for 5 min. One µL injection volume, 1:50 split ratio, 2 ml/min flow rate with H_2 carrier gas, and 250 and 280 °C injector and detector temperatures, respectively, were used in the analysis. Fatty acid methyl esters were quantified by co-chromatography with FAME mixture standards (37-components, C4-C24, Supelco, Bellefonte, PA, USA).

2.5. Physico-chemical analyses of the oleogels

The gel formatin time (GFT) was determined by following the modified method of Dassanayake *et al*., (2009). First, the stock oleogels were melted completely in a water bath, and then 10 ml of each melted oleogel were placed into glass tubes. The tubes were kept for 0.5 h at 70 °C in the water bath for temperature equilibration. All the tubes were taken from the water bath to ambient temperature (set to 20 °C) for gel formation. Meanwhile, the time elapsed was recorded. The tubes were then rotated 90° and if no flow was observed, it was considered as formed gel and the time was recorded as GFT.

The method of Da Pieve *et al*., (2010) was adapted for the oil binding capacity (OBC) analysis. Empty Eppendorf tubes were weighed (a) and then 1 ml of each completely melted organogel sample was placed into the tube, and left for 1 h for organogel formation in the

refrigerator. After the formation of the oleogels, the tubes were carefully weighed (b) again and centrifuged at 9167 xg for 15 min at 20 °C. Then, the tubes were turned over on a filter paper and left for 3 min for excess oil to drain. Finally, the tubes were weighed (c). The oil binding capacity (% OBC) was calculated by the equation given below.

$$\%\text{Released Oil}=\frac{(\text{b-a})-(\text{c-a})}{(\text{b-a})}\times 100 \text{ and}$$

$$\%\text{OBC}=100\text{-Released Oil}$$

The solid fat content of the oleogels was determined following the ISO 8292 method (ISO, 2012) with a Bruker Nuclear Magnetic Resonance (NMR) spectrometer (The Minispec, Bruker Optics, Inc.). The measurements were performed at 20 and 35 °C. Instrument calibrations were done with 0, 31 and 73.5% solid fat containing standard solutions. The results were expressed as percent solid fat content.

The color of the organogel samples was measured on a Minolta CR-400 colorimeter (Konica Minolta Sensing, Osaka, Japan) with CIE lab standards, and the L, a* and b* values were recorded.

The free fatty acids (FFA) and peroxide values (PV) of the oils and oleogels were determined by the method of Ca 5a-40 and Cd 8-53 (AOCS, 1998), respectively. FFA was calculated over the major fatty acid determined from the fatty acid composition analysis for each sample, and PV was given as miliequivalent O_2 per kg sample.

2.6. Thermal analysis of the oleogels

The crystallization and melting onset and peak temperatures and enthalpies of the oleogel samples were measured with a Perkin-Elmer 4000 Series Differential Scanning Calorimetry (Groningen, The Netherlands). The instrument was calibrated with Indium and Zinc. 5-10 mg of oleogel samples were weighed into aluminum pans and sealed hermetically. The temperature program was set to heat from 20 °C to 100 °C at 10 °C/min; then cool the samples to -30 °C at 10 °C/min and hold for 3 min at that temperature for full crystal formation and a final heating to 100 °C at 10 °C/min. The Pyris 1 Manager Software of the instrument was used for the calculations. All samples were analyzed at least in duplicate (Yilmaz *et al*., 2015).

2.7. Polarized light microscopy of the oleogels

Polarized light microscope pictures (PLM) of the oleogel samples were taken with an Olympus BX51 polarized light microscope (Olympus Optical Co., Ltd., Japan) with 10x ocular and 4x lens attached, and equipped with a CCD color video camera (Canon) at room temperature (Yilmaz *et al*., 2015).

2.8. X-ray diffraction analysis of the oleogels

X-ray diffraction (XRD) patterns of the oleogels were assessed with a PANalytical Empyrean model (The Netherlands) X-ray diffractometer. Radiation was applied at a scanning rate of 0.02/0.6 (sec) within a 2.0-50° (2θ) range under 45 kV and 40 mA CuKα (λ = 1.54056 Å). Data analysis was completed with X'Pert HighScore Plus software (Malvern Panalytical Ltd., Royston, UK) (Yilmaz *et al*., 2015).

2.9. Rheological analyses of the oleogels

Rheological analyses of the samples were performed with a DHR 2 rheometer (TA Instruments, USA) using plate-plate cross hatched geometry (Φ = 40 mm, gap 0.9 ± 0.1 mm). Temperature control was maintained with a Peltier system (± 0.1 °C) under the lower plate. For each oleogel sample, amplitude sweeps (strain = 0.01 - 100%) were performed at 10 °C with 1 Hz frequency to determine the linear viscoelastic region (LVR). After determining the strain values within the LVR, frequency sweep tests were performed for each sample at 10 °C, 0.02-0.26% strain range and frequencies from 0.1 to 100 Hz, and the storage (G') ve loss (G") moduli were determined. For amplitude, frequency and time sweep tests, the 10 °C constant temperature was selected because at that temperature, the oleogels were totally solid and comparison of their rheological behaviors was easier to make. Furthermore, temperature sweep tests were carried out to observe the effect of increasing temperatures on the flow behavior.

Time sweep tests for each sample were also done at 10 °C with 1 Hz frequency and strains in the LVR, and strains below ($LVR_{strain} \geq$ Strain) and above ($LVR_{strain} \leq$ Strain) linear viscoelastic region. Time sweep was applied as 180 s for the first region, 180 s for the second region, and 900 s for the third region.

Finally, a temperature ramp test was carried out from 0 °C to 60 °C at 1 °C/min at 1 Hz frequency in LVR. The soak time was 120 s. All samples were tested three times and the results were presented as the average values.

2.10. Statistical analysis

The production of the oleogels was replicated two times, and each replicate sample was analyzed at least twice for each test. The results were presented as mean values with standard deviations. The Analysis of Variance (ANOVA) with mean comparisons by Tukey's test was completed by Minitab v.16.1 software (Minitab, 2010). The level of confidence was set at 95%.

3. RESULTS AND DISCUSSION

3.1. Physico-chemical properties

The common physico-chemical properties of the oleogels and stock fats are presented in Table 1. Gel formation times (GFT) of the oleogels were significantly different. While TFO formed gels after the longest period (35 min), the formation of MDGO took only 0.34 min. In our previous studies (Yilmaz *et al*., 2015; Öğütcü and Yılmaz, 2015), it was quite clear that different organogelator formed stable oleogels at various times. This outcome was expected since the crystalline network forming molecules were different. In MDGO and MGO, there were only mono-palmitate and mono- and di-palmitate mixtures; while in TF and HTF, there were diverse types of tri-, di-, and mono-glyceride mixtures.

The solid fat contents (SFC%) of the oleogels were measured at 20 and 35 °C (Table 1). There were slight but significant differences among the samples. The highest SFC was determined in the TFO at both temperatures, while the lowest value was in the MGO sample. Since the same stock oil (SO) was used in the creation of the oleogel, this difference was attributed to the added organogelators. Also, while creating HTFO, MGO, and MDGO, only 10% of the gelators were added to create a stable gel; whereas 30% of tallow fat was added into the TFO to get the stable gel. Hence, this proportion difference may lead to the SFC difference. Since the main advantages of the oleogels were stated as the lower content of saturated fatty acids and zero content of *trans* fatty acids (Co and Marangoni, 2012; Patel, 2018), lower SFC is preferred in oleogels. In other words, if an organogelator provides no extra saturated or *trans* fatty acids, it is preferred. In this case, TF by itself was not a good organogelator since it formed stable oleogels only at the 30% addition level, at which the saturated fatty acid content of the resulting oleogel was also increased. Partially hydrolyzed tallow fat (HTF), on the other hand, yielded stable oleogel at a 10% addition level, at which the saturated fatty acid content of the resulting oleogel was not

Table 1. Physico-chemical properties of the oleogels developed

	Gel Formation Time (min)	Oil Binding Capacity (%)	SFC (%)		L Value	a* Value	b* Value	Free Fatty Acidity (major fatty acid %)	Peroxide Value (meq O_2/kg)
			20 °C	35 °C					
SO	-	-	-	-	56.19 ± 1.1	-3.07 ± 0.2	13.47 ± 0.9	0.56 ± 0.0	2.59 ± 0.5
TF	-	-	-	-	90.06 ± 0.2	-3.23 ± 0.0	2.38 ± 0.1	1.69 ± 0.1	9.72 ± 0.0
HTF	-	-	-	-	91.35 ± 0.5	-3.10 ± 0.1	1.75 ± 0.5	1.55 ± 0.5	11.50 ± 1.4
TFO	35.00 ± 1.0^{a*}	99.61 ± 0.1^{a}	12.50 ± 1.2^{a}	7.05 ± 1.5^{a}	49.27 ± 3.8^{b}	-3.77 ± 0.2^{b}	-1.22 ± 0.5^{d}	0.85 ± 0.0^{a}	13.57 ± 1.5^{a}
HTFO	33.30 ± 1.5^{a}	99.60 ± 0.0^{a}	10.11 ± 1.5^{a}	4.87 ± 0.8^{b}	47.59 ± 2.0^{bc}	-2.15 ± 0.1^{a}	1.40 ± 0.1^{c}	0.71 ± 0.1^{b}	11.34 ± 0.0^{b}
MGO	8.33 ± 0.5^{b}	99.99 ± 0.0^{a}	8.12 ± 1.2^{b}	4.33 ± 0.9^{b}	52.01 ± 0.2^{b}	-2.52 ± 0.0^{a}	10.12 ± 0.1^{b}	0.56 ± 0.0^{c}	12.14 ± 0.7^{a}
MDGO	0.34 ± 0.0^{c}	99.90 ± 0.0^{a}	9.30 ± 0.7^{ab}	4.75 ± 0.6^{b}	67.06 ± 1.1^{a}	-3.10 ± 0.1^{b}	12.06 ± 0.6^{a}	0.56 ± 0.0^{c}	9.70 ± 1.9^{c}

SO: sunflower oil, TF: tallow fat, TFO: tallow fat oleogel, HTFO: hydrolized tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel *Small letters within each column indicate significant differences among the oleogel samples for the mean ± SD values calculated from four determinations by one-way analysis of variance and Tukey's test ($p \leq 0.05$)

significantly enhanced. Since it is cheaper, edible, legally permitted and sensorially acceptable, it might be a better alternative as organogelator.

The oil binding capacity (OBC) values of the oleogels were well above 99% for all samples, and not significantly different. This is a very important finding which indicates the presence of well-formed and stable gels. Clearly, with the organogelators used, almost all liquid oil was bound or entrapped. Quite similar results with wax gelators (Yilmaz *et al*., 2015), fatty acid and fatty alcohol gelators (Bot and Flöter, 2018), ethyl cellulose gelator (Mattice and Marangoni, 2018), and others (Toro-Vasquez *et al*., 2018; Patel, 2018) were found in the literature.

The instrumental color values of the samples were also measured (Table 1). The L values of SO and TF were significantly different, and melted tallow fat was quite luminous, but their a* values were not statistically different. The yellow color (b* value) of SO was significantly different from TF. When the oleogels were compared among themselves, the most luminous sample was MDGO. The a* value (redness-greenness) was not significantly different among the oleogels; while the b* value (yellowness-blueness) was different. MDGO and MGO were yellow-toned samples; while the TFO sample had some blueness. The oleogels can be seen in Figure 1. It is well known from the oleogel literature that the color of an oleogel is mainly determined by the stock oils used and the color and addition level of the organogelators used (Patel, 2018).

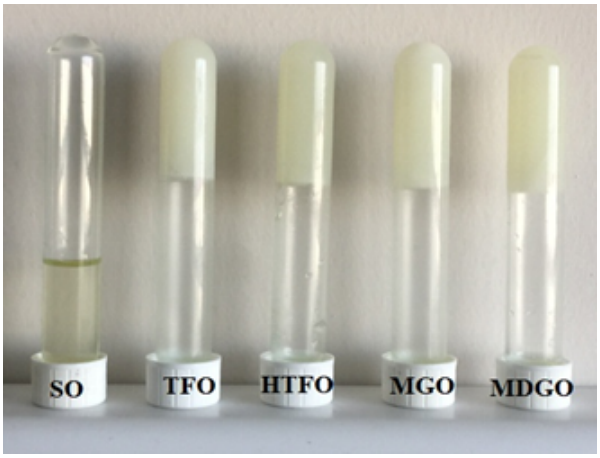

Figure 1. The stock oil (SO: sunflower oil) used in oleogel preparation, and the oleogels developed (TFO: tallow fat oleogel, HTFO: hydrolyzed tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel)

Two chemical properties, free fatty acidity (FFA) and peroxide value (PV), were also measured (Table 1). Both values were determined in the stock liquid oil (SO), tallow fat (TF), and partially hydrolyzed tallow fat (HTF), which were used in oleogel preparations. Both values were higher in TF and HTF than those in the SO, but in all samples, the values were below the edible vegetable oil codex standards (Codex, 2017), which states 0.6 mgKOH/g acid value and 10 meqO_2/kg PV as limit values. When the oleogels were compared among themselves for FFA value, TFO and HTFO had slightly higher (0.85 and 0.71%) values than those of the MGO and MDGO (0.56%), respectively. This was an expected result since the mono- and di-glycerides used as gelators were pure substances. But all oleogel samples were fairly acceptable for FFA according to the edible oil codex. On the other hand, except for the MDGO sample (9.70 meqO_2/kg), the PV of all samples exceeded the codex limit. This might be due to the presence of heat and air during oleogel preparation, which might have caused oil oxidation. Hence, vacuum or nitrogen atmospher and antioxidant addition could be suggested during oleogel production like all other oleogels reported in the literature.

The fatty acid composition of the oils/fats used as stock materials and the prepared oleogels were also measured, and the results are presented in Table 2. Four main fatty acids in the SO and five main fatty acids in TF were determined. Both composition data mainly concur with the current literature (Codex, 2017; Love, 1996). After the partial hydrolysis and consequent washing of the free fatty acids of the TF, the HTF was obtained, and its fatty acid composition analysis indicated the presence of three fatty acids. Compared to TF, the proportion of palmitic acid was enhanced significantly (77.95%) in the HTF; while myristic and linoleic acids could not be quantified. Further, significant decreases in the stearic and oleic acids were quantified in HTF. When the oleogels were compared, it was observed that the total saturated fatty acid contents of TFO (22.50%), MGO (26.66%) and MDGO (33.89%) were significantly higher than that of the HTFO (15.60%). This could be considered as a promising result in terms of nutritional perspective. Also, it is clear that the content of total saturated fatty acids is not the only factor governing oleogel formation. In fact, the presence of non-polar and polar groups on the same molecule like mono- and di-glycerides made them more efficient as organogelator

TABLE 2. Fatty acid compositions of the oil/fat used and the oleogels prepared

Fatty Acid	SO	TF	HTF	TFO (30%)	HTFO (10%)	MGO (10%)	MDGO (10%)
C14:0	-	3.34 ± 2.50	-	0.90 ± 0.20	-	-	-
C16:0	4.46 ± 1.30	30.65 ± 2.40	77.95 ± 1.50	12.50 ± 1.50	11.80 ± 1.40	24.11 ± 1.02	29.15 ± 3.23
C18:0	2.30 ± 0.20	23.40 ± 0.80	13.30 ± 1.20	9.10 ± 1.72	3.80 ± 0.80	2.55 ± 0.50	4.74 ± 0.65
C18:1	36.24 ± 1.55	38.75 ± 2.30	8.75 ± 0.50	35.07 ± 1.70	33.90 ± 1.90	26.52 ± 1.39	28.72 ± 1.60
C18:2	57.00 ± 1.00	3.05 ± 1.00	-	38.51 ± 1.50	50.50 ± 1.50	46.15 ± 2.33	36.94 ±1.67
Σ Saturated	6.75	57.39	77.95	22.50	15.60	26.66	33.89
Σ Unsaturated	93.24	41.80	22.05	73.58	84.40	72.67	65.66

SO: sunflower oil, TF: tallow fat, TFO: tallow fat oleogel, HTFO: hydrolized tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel

(Patel, 2018). HTFO, MGO, and MDGO had only 10% organogelator added, but TFO had 30% organogelator added to obtain oleogels. Hence, TFO could not be accepted as oleogel, but rather it could be claimed as a blend of sunflower oil and tallow fat. Further, there was no beefy flavor or any off odor in TFO and HTFO. Overall, partially hydrolyzed tallow fat could be an effective organogelator compared to pure commercial mono- and di-glycerides. Since it did not enhance saturated fatty acid content significantly, and it is a much cheaper and more readily available material, this proposed partial hydrolysis mechanism could be used to obtain an organogelator to be applied in foods.

3.2. Thermal properties

The thermal properties of oleogels are very important quality parameters to evaluate their suitability for edible applications. It was indicated that melting and crystallization temperatures and ranges are one of the most common quality parameters for commercial margarine, shortenings, and other semi-solid fat products (Chrysam, 1996). Different product types (table spreads, kitchen margarines, tube margarine, shortenings, cake/batter fats, etc.) are expected to have certain thermal behaviors. Furthermore, there is a usually a well-documented correlation between the thermal behavior and SFC of the known commercial solid/semi-solid fat products. High quality spreads or kitchen margarines are expected to melt quickly in the mouth to yield a cooling effect with no lingering greasiness (O'Brien, 2008; Chrysam, 1996). Oleogels could be used to prepare margarine-like products and shortenings, and could even be applied themselves as spreadable fats (Öğütcü and Yılmaz, 2015). As seen in Table 1, it was obvious that oleogel products did not have high SFC, as expected, but they certainly had plastic consistency at ambient temperature. This situation is the-well known (Co and Marangoni, 2012) main advantage of oleogels. Hence, it is essential to determine the crystallization and melting ranges, peak temperatures and ethalpies. The thermal cycling technique (Dassanayake *et al*., 2009; Öğütcü and Yılmaz, 2015) was used to get the full range of thermal behavior for both the organogelators used and the oleogels produced (Table 3).

The crystallization and melting onset and peak temperatures of the sunflower oil (SO) as stock oil for the gels, tallow fat (TF), partially hydrolyzed tallow fat (HTF), monoglyceride (MG), mono+diglyceride (MDG) as the organogelators, and the oleogels produced are presented in Table 3. SO, started to crystallize at -16 °C, and TF started at 11.67 °C. Similarly, these two organogelators had peak melting temperatures of 28.66 and 15.69 °C, respectively. Both MG and MDG had two fractions within themselves with different crystallization and melting temperatures, and both samples were similar in their thermal values. All oleogel samples (TFO, HTFO, MGO, MDGO) had two fractions presenting different thermal ranges. For crystallization and peak melting temperatures, HTFO was more similar to MGO and MDGO than to TFO. The peak melting temperatures of MGO and MDGO were 54.56 and 54.83 °C for the main fraction and 13.57 and 16.25 °C for the minor fraction, respectively. HTFO showed values of 12.39 and 47.11 °C for peak melting temperature. Clearly, HTFO totally melted at around 47 °C, which seemed suitable for food product applications. The crystallization and melting profiles presented in Table 3 showed

Table 3. Thermal properties of the oil/fat used and the oleogels prepared

	Crystallization			*Melting*		
	Onset$_c$ (°C)	**Peak (Tc, °C)**	**ΔHc (J/g)**	**Onset$_m$ (°C)**	**Peak (Tm, °C)**	**ΔHm (J/g)**
SO	-16.00 ± 0.86	-17.89 ± 0.40	-1.90 ± 0.27	22.89 ± 1.11	28.66 ± 2.06	7.05 ± 0.10
MG-Fr.1	11.93 ± 0.27	9.67 ± 0.24	-19.50 ± 1.73	10.39 ± 0.28	13.86 ± 0.23	23.93 ± 1.87
MG-Fr.2	63.69 ± 0.17	60.47 ± 0.34	-101.66 ± 0.16	61.68 ± 0.13	66.90 ± 0.23	105.45 ± 0.98
MDG-Fr.1	15.83 ± 0.76	14.11 ± 1.18	-15.74 ± 1.11	13.28 ± 0.35	16.21 ± 0.43	17.08 ± 0.94
MDG-Fr.2	65.79 ± 0.08	63.04 ± 0.41	-88.38 ± 2.23	63.52 ± 0.26	68.28 ± 0.41	82.77 ± 5.57
TF-Fr.1	11.67 ± 1.90	7.16 ± 2.88	-10.28 ± 1.29	15.70 ± 2.66	15.69 ± 0.42	15.83 ± 2.40
TF-Fr.2	31.45 ± 2.76	28.30 ± 3.46	-17.76 ± 0.50	34.08 ± 0.69	43.61 ± 1.47	29.92 ± 0.47
HTF-fr1	33.98 ± 0.53	30.44 ± 0.03	-10.15 ± 0.04	6.63 ± 3.15	12.89 ± 1.89	19.34 ± 1.06
HTF-fr2	56.19 ± 0.71	52.75 ± 1.25	-33.48 ± 0.06	57.27 ± 0.84	48.56 ± 1.68	30.07 ± 0.71
TFO-Fr.1	-0.56 ± 0.10	-3.33 ± 0.08	-2.92 ± 0.52	13.58 ± 1.82	14.37 ± 0.69	3.17 ± 0.02
TFO-Fr.2	22.77 ± 0.69	20.75 ± 1.19	-0.96 ± 0.24	33.36 ± 1.44	37.54 ± 0.93	25.37 ± 0.69
HTFO-Fr.1	11.05 ± 0.09	9.49 ± 0.18	-0.64 ± 0.02	9.67 ± 0.03	12.39 ± 0.01	3.23 ± 0.20
HTFO-Fr.2	44.19 ± 2.25	42.03 ± 1.88	-2.43 ± 0.35	32.90 ± 0.71	47.11 ± 2.07	2.31 ± 0.41
MGO-Fr.1	13.21 ± 0.27	9.32 ± 1.24	-1.86 ± 0.35	11.21 ± 0.06	13.57 ± 0.95	1.72 ± 0.08
MGO-Fr.2	52.22 ± 0.76	49.45 ± 0.70	-5.11 ± 1.63	47.02 ± 0.40	54.65 ± 0.12	5.76 ± 2.12
MDGO-Fr.1	15.76 ± 0.13	14.35 ± 0.35	-1.40 ± 0.45	14.47 ± 0.32	16.25 ± 0.01	1.73 ± 0.68
MDGO-Fr.2	52.74 ± 0.86	49.43 ± 0.23	-6.28 ± 1.80	43.79 ± 1.24	54.83 ± 0.35	7.26 ± 0.22

SO: sunflower oil, MG: monoglyceride, MDG: monoglyceride + diglyceride, TF: tallow fat, HTF: partially hydrolyzed tallow fat, TFO: tallow fat oleogel, HTFO: hydrolized tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel, Fr.1: fraction 1, Fr.2: fraction2

that HTFO could be used in margarine or spread formulations, like the monoglyceride oleogels used previously (Da Pieve *et al*., 2010; Ögütcü and Yilmaz, 2015). The SFC data presented in Table 1 for HTFO was consistent with DSC data where the sample had only around 5-10% solid fat at 25 to 35 °C, but was in solid (gelled) consistency, and completely melted at around 47 °C. The difference between melting onset and peak temperatures showed the melting range, and HTFO had a melting range of around 10-15 degrees, like MGO and MDGO. On the other hand, the peak melting temperature of HTFO (47.11 °C) was lower than those of the MGO (54.65 °C) and MDGO (54.83 °C). For margarine and similar products, a narrower melting range closer to body temperature is preferred in order to produce a cooling sensation in the mouth to help taste buds perceive food flavor (Chrysam, 1996). In this respect, HTFO seems a better candidate for edible applications.

3.3. Microstructural properties

The polarized light microscopy (PLM) images of the oleogels are presented in Figure 2. Clearly, the crystalline structures of TFO and HTFO were similar but they were different from MGO and MDGO. In fact, all images indicated the presence of some aggregates, but the aggregates were quite large in MGO and MDGO. In a previous study (Öğütcü and Yılmaz, 2015), hazelnut oil oleogels prepared with monoglyceride showed spherulitic or rosette-like crystalline patterns. Further, Kesselman and Shimoni (2007) published spherulitic crystals for corn oil-monoglyceride oleogels. The results confirmed that MGO and MDGO had spherulitic aggregate type crystals. In one study (Viriato *et al*., 2018), anhydrous milk fat was used as the organogelator to produce sunflower oil oleogels. Their PLM studies indicated crystals with long, fine needles of the spherulite type. Generally speaking, the crystal structure of an oleogel depends on the kind, purity and addition level of the organogelator, as well as the type of stock oil and process conditions (cooling rate, shear application, presence of other additives, etc.) as discussed (Mattice and Marangoni, 2018). In this study, the small, fine crystalline aggregates observed in the HTFO (Figure 2b) sample indicated that amphiphilic di- and mono-glyceride mixtures present in the HTF

could provide enough junction zones within the non-polar oil medium to yield the crystal lattice, where the mobile liquid oil was trapped. There were some aggregates in the TFO as well (Figure 2a), but since these were most probably the crystals of solid tallow triglycerides, they may not provide enough network (due to the absence of junction zones) to trap liquid oil at the 10% addition level, but could only provide enough solid surface for the oil to adhere at the 30% addition level. Overall, the partial hydrolysis of solid fat to provide higher melting fractions of di- and mono-glyceride mixtures might be quite helpful for creating potential organogelators.

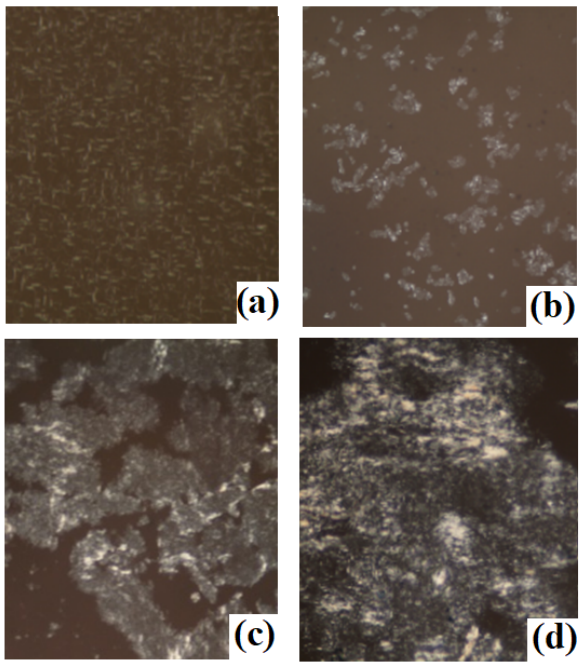

FIGURE 2. The polorized light microscopy images of the oleogels with 40-fold magnification (a: TFO, b: HTFO, c: MGO and d: MDGO)

The X-ray diffraction patterns of the oleogels are presented in Figure 3. The wide angle region peaks of all four samples were quite similar to each other and were around 4.46-4.67 Å and 3.89-3.95 Å, respectively. It was discussed (Stahl *et al*., 2017) that short spacings between fatty acid chains in solid lipids were in the α-form if they were around 4.15 Å, and the β' form were around 3.8-4.2 Å. Hence, the oleogels could be the β' type polymorph. Furthermore, small angle peaks at around 19.21-19.47 Å were found in the samples. There was a peak at around 43.29 Å for only the MDGO sample, which might indicate the interplanar distance between the mono- and di-glyceride layers with a lamella of 43 Å. The data indicated that all crystallines in the oleogel samples could be β' polymorphs with an orthorhombic-perpendicular subcell ($O_{\perp}$) structure. Similar findings were reported for cod liver oil-monoglyceride organogels (Da Pieve et al., 2010) and wax organogels (Szydłowska-Czerniak *et al*., 2005). In addition, visual inspection by eye revealed that the oleogels were very smooth, homogeneous and creamy in texture (Figure 1).

3.4. Rheological properties

All rheological measurements were carried out within the linear viscoelastic region (LVR), beyond which the structure of the sample was destroyed. To determine the LVR, each sample was first tested by an amplitute sweep test at 10 °C, 1 Hz frequency with 0.01-100% strain. The LVR strain values determined were 0.26, 0.16, 0.11 and 0.023% for TFO, HTFO, MGO and MDGO, respectively. The amplitute sweep tests also indicated that the gel structure could be destroyed at around 8, 50, 50, and 5 Pa oscillatory shear strain values for TFO, HTFO, MGO and MDGO, respectively. At those shear strain values, the cross-over points were reached, at which the gelled consistency was lost.

A frequency sweep test was performed within the LVR region at 10 °C, and frequencies from 0.1 to 100 Hz. In this test, oscillation frequency increased stepwise while keeping the amplitute constant. Frequency sweep tests can help to understand the time-dependent behavior of a sample in the non-destructive deformation range. The results of the measurements are presented in Figure 4. For all four samples, and within all the frequency ranges, storage moduli (G´) were always higher than those of the loss moduli (G´´). This means that during the storage period, the gel structure was stable in all samples. Phase segregation or settling should not develop during the storage period of these oleogels. Further storage modulus (G´) values of HTFO were between 3.000-5.000 Pa throughout the applied angular frequency region of 0-1000 rad/s and were significantly higher than those of the TFO sample, which were between 100-300 Pa. This indicates that HTFO had a stronger gel structure. This situation is probably the result of a network formation by the amphiphilic mono- and di-glycerides present in the HTF. Within the oil medium, amphiphilic molecules could create

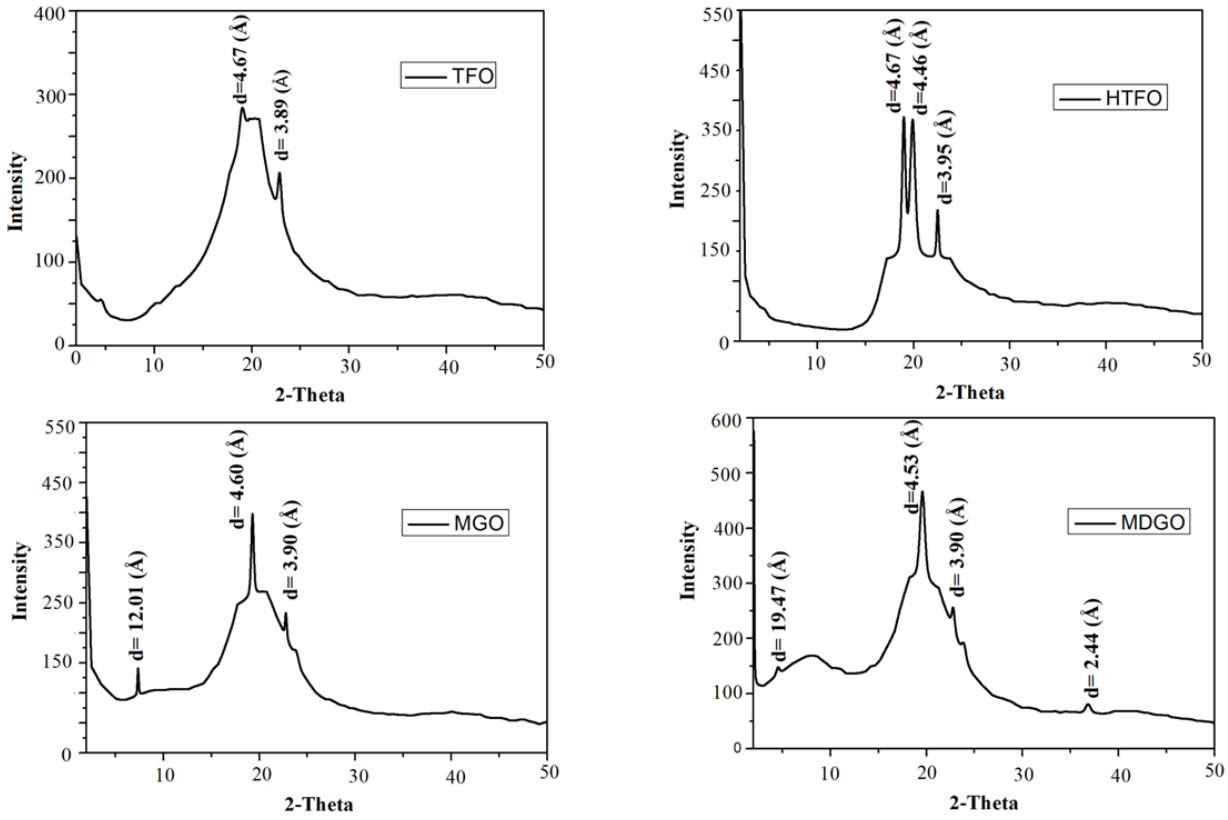

FIGURE 3. The X-Ray diffraction patterns of the oleogel samples

some junction zones for thermodynamic stability, and this yields the network that traps the liquid oil. Since TF forms oleogel due to its higher melting point triglycerides only, its gel is less strong than the HTF gel. When the storage modulus (G´) of HTFO (around 3.000 Pa) is compared to the storage modulus (G´) values of MGO (around 80.0000 Pa) and MDGO (around 110.000 Pa), it could be clearly observed that MGO and MDGO are much stronger gels. Overall, saturated mono- and di-glycerides were much better organogelators than saturated tri-glycerides. Hence, any solid fat stock could be partially hydrolyzed to its mono- and di-glycerides to get an effective organogelator.

In order to observe the time-dependent viscoelastic behavior of the oleogel samples, time-sweep tests were performed with shearing under dynamic mechanical conditions, or under constant amplitute and frequency (1 Hz) at 10 °C. Three time regions were tested with preset oscillation so as to simulate the behavior at rest (first region). A strain at LVR was applied with 1 Hz frequency for 180 sec to simulate structural breakdown or stronger shear. A strain higher than LVR (LVR_1 >>) was applied with 1 Hz frequency for 180 sec, and finally to simulate structural regeneration at rest, a very low shear (LVRs <<) was applied with 1 Hz frequency for 900 sec. The results of the measurements are presented in Figure 5. In all samples at first region (very low shear to simulate resting behavior), the storage modulus (G´) was higher than the loss modulus (G´´), and this indicates that all samples are gel structured. Once a strong shear was applied in the second region (simulate structural breakdown), both moduli decreased significantly as expected; hence, enough deformation occurred. Afterwards, in the third regeneration at the rest region with very low shear, both moduli increased again to their original levels observed in the first region. This means that a structural reformation or regeneration developed. Hence all four samples are truly gel, and all have mechanical regeneration ability. For oleogels, this ability is very important, and during product applications, for example, food formulation mixing processes, the deformed structure regenerated itself once mechanical force was removed. Similar thixotropic recovery was shown for shellac oleogels (Patel and Dewettinck, 2015).

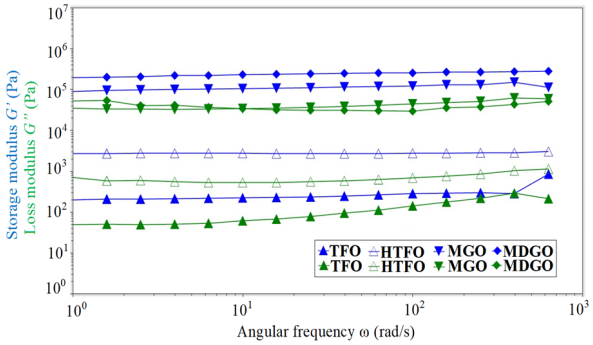

FIGURE 4. The frequency sweep test results of the oleogels developed (TFO: tallow fat oleogel, HTFO: hydrolized tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel)

Lastly, a temperature-ramp test was carried out to observe the viscoelastic behavior under constant amplitute and frequency (Figure 6). All samples kept their gelled structure until the G´ and G´´ crossed at around 40 °C for TFO, 60 °C for HTFO, 63 °C for MGO and 61 °C for the MDGO sample. The peak melting temperature values of the same samples by DSC (Table 3) were 37.54, 47.11, 54.65 and 54.83 °C, respectively. Obviously, peak melting temperatures were lower than those of the temperatures measured at the cross-over point (Figure 6). This might be due to the applied force during the rheologic measurement. There probably were some junction zones in the oleogel samples even after melting, at which the rheometer could still recognize some strength until the cross-over point reached at slightly higher temperatures. Clearly, HTFO was more stable until higher temperatures than TFO and better resembled MGO. This finding indicated that the partial hydrolysis of tallow fat yielded better properties as an organogelator. Both TFO and HTFO started to soften at around 20 and 30 °C, but retained their gelled consistency until around 40 and 60 °C, respectively. Although these temperatures were lower than those of the MGO and MDGO samples, they could still provide enough thermal stability for food applications.

4. CONCLUSIONS

Finding new, cheaper, readily available, safe and sensorially acceptable organogelators to initiate commercial production of oleogel products is still a research challange. In this context, tallow fat (TF) and partially hydrolyzed tallow fat (HTF) were investigated as organogelators in this study. They were compared to saturated monoglyceride and mono- and di-glyceride mixtures. The results showed that HTF could be a very reliable organogelator for commercial applications. It yielded stable gels at a 10% addition level without extensively enhancing saturated fatty acid contents. In contrast, TF could create oleogel at a 30% addition level, at which it can no longer be considered as an oleogel, but rather as an oil-fat blend. Furthermore, the microstructure and rheological data of the HTF oleogel were quite similar to those of the MDGO and MGO samples. HTFO showed enough stability at applied strains and frequencies, just similar to the MGO and MDGO samples. HTFO showed enough structural recovery and thermal stability abilities to be used as an organogelator for food applications. Hence, abundantly available, cheap and safe organogelators could be created with a simple partial hydrolysis process from tallow fat. This study also investigated other similar hard fat sources to be evaluated as organogelators after partial glyceride hydrolysis.

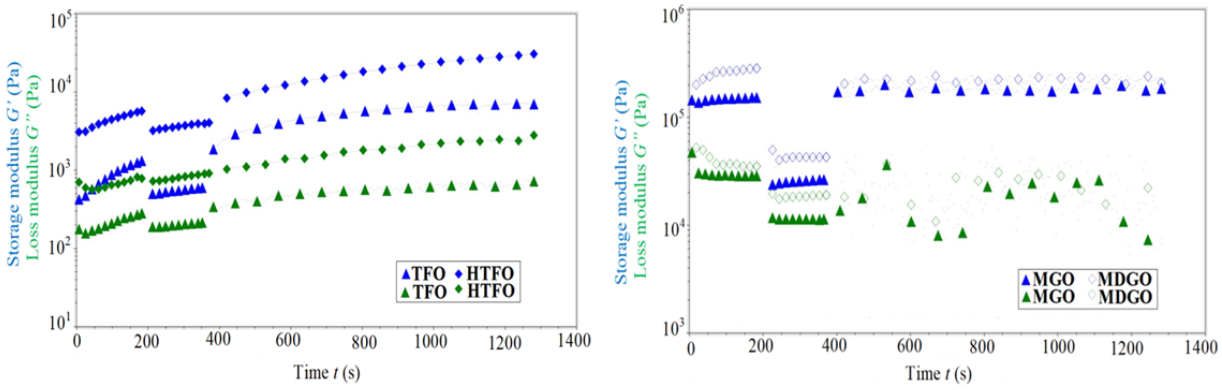

FIGURE 5. The time sweep test results of the oleogels developed (TFO: tallow fat oleogel, HTFO: hydrolized tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel)

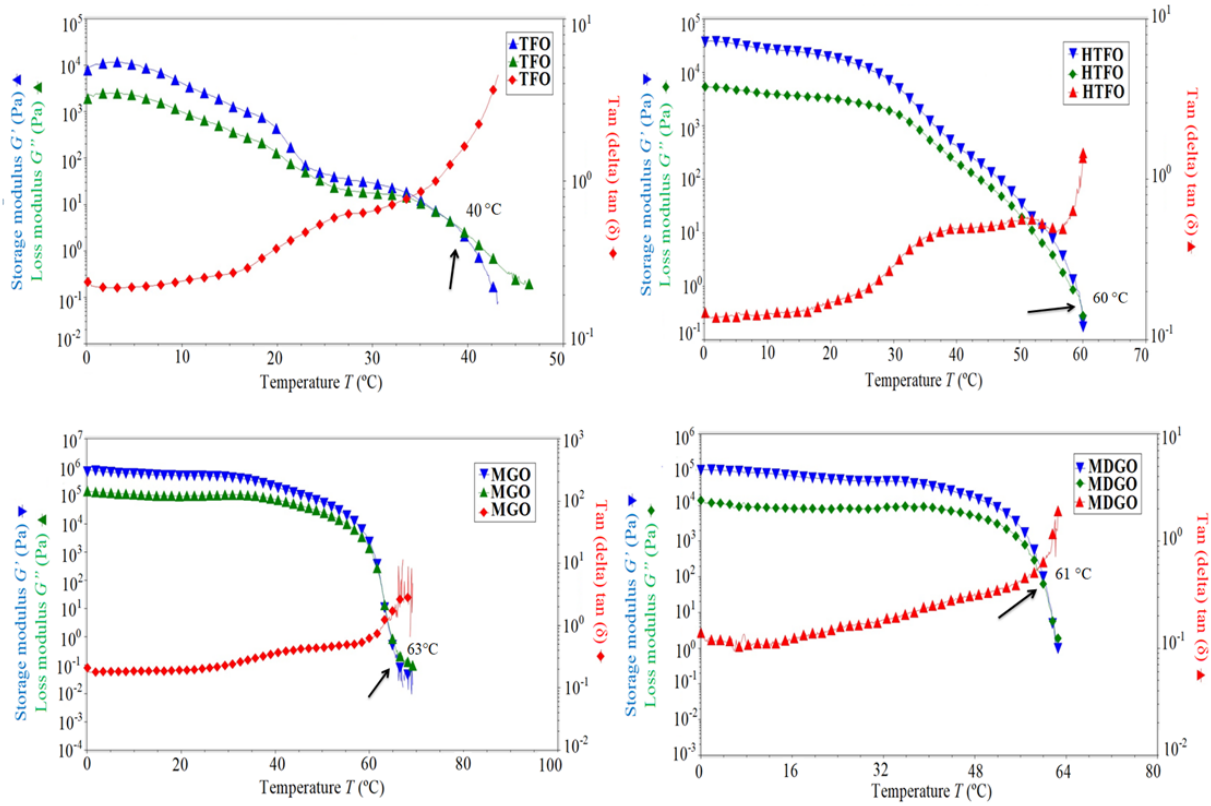

FIGURE 6. The temperature ramp test results of the oleogels developed (TFO: tallow fat oleogel, HTFO: hydrolized tallow fat oleogel, MGO: monoglyceride oleogel, MDGO: monoglyceride + diglyceride oleogel)

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REFERENCES

AOCS. 1998. Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th Edn, AOCS Press, Champaigne 1998, IL.

Bot A, Veldhuizen YSJ, den Adel R, 2009. Non-TAG structuring of edible oils and emulsions. *Food Hydrocol*. **23**, 1184-1189. https://doi.org/10.1016/j.foodhyd.2008.06.009

Bot A, Flöter E. 2018. Structuring edible oil phases with fatty acids and alcohols, in Ashok R. Patel (Ed.), *Edible Oil Structuring: Concept, Methods and Applications*, Royal Soc. Chem., Chambridge, UK, 95-105.

Cerqueira MA, Fasolin LH, Picone CSF, Pastrana LM, Cunha RL, Vicente AA. 2017. Structural and mechanical properties of organogels: role of oil and gelator molecular structure. *Food Res. Intl*. **96**, 161-170. https://doi.org/10.1016/j.foodres.2017.03.021

Chrysam MM. 1996. Margarines and Spreads, in Y.H. Hui (Ed.), *Bailey's Industrial Oil & Fat Products*, Vol. **3**, Wiley-Intersience Pub, New York, US, 65-114.

Co ED, Marangoni AG. 2012. Organogels: An alternative edible oil-structuring method. *J. Am. Oil Chem. Soc.* **89**, 749-780. https://doi.org/10.1007/s11746-012-2049-3

Codex. 2017. Codex Alimentarius, International Food Standards, Standard for named vegetable oils, Codex Stan 210-1999.

Da Pieve S, Calligaris S, Co E, Nicoli MC, Marangoni AG. 2010. Shear nanostructuring of monoglyceride organogels. *Food Biophys*. **5**, 211-217. https://doi.org/10.1007/s11483-010-9162-3

Dassanayake LSK, Kodali DR, Ueno S. 2011. Formation of oleogels based on edible lipid materials. *Current Op. Coll. Inter. Sci*. **16**, 432-439. https://doi.org/10.1016/j.cocis.2011.05.005

Dassanayake LSK, Kodali DR, Ueno S, Sato K. 2009. Physical properties of rice bran wax in bulk and organogels. *J. Am. Oil Chem. Soc*. **86**, 1163-1173. https://doi.org/10.1007/s11746-009-1464-6

Huang X, Wang J, Bi J, Hao H. 2018. Influence of diacylglycerol on physicochemical properties and crystallization behavior of palm oil. *Chem. Eng. Technol*. **41**, 1587-1593. https://doi.org/10.1002/ceat.201700550

Kesselman E, Shimoni E. 2007. Imaging of oil/monoglyceride networks by polarizing near-field scanning optical microscopy. *Food Biophys*. **2**, 117-123. https://doi.org/10.1007/s11483-007-9038-3

Love JA. 1996. Animal fats, in Y.H. Hui (Ed.), *Bailey's Industrial Oil & Fat Products*, Vol. **1**, Wiley-Intersience Pub, New York, US, 1-18.

Mattice KD, Marangoni AG. 2018. New insights into wax crystal networks in oleogels, in Ashok R. Patel (Ed.), *Edible Oil Structuring: Concept, Methods and Applications*, Royal Soc. Chem., Chambridge, UK, 71-94.

Minitab. 2010. Minitab Statistical Software (Version 16.1). Minitab, Inc., State College, Pennsylvania, US.

ISO. 2008. Animal and vegetable fats and oils-Determination of solid fat content by pulsed NMR-Part 2: Indirect method. ISO/TC 34/SC 11, On-line version.

O'Brien RD. 2008. *Fats and Oils: Formulating and Processing for Applications*. CRC Press, New York, US.

Öğütcü M, Yılmaz E. 2015. Characterization of hazelnut oil oleogels prepared with sunflower and carnauba waxes. *Int. J. Food Prop*. **18**, 1741-1755. https://doi.org/10.1080/10942912.2014.933352

Patel AR, Dewettinck K. 2015. Comparative evaluation of structured oil systems: shellac oleogel, HPMC oleogel and HIPE gel. *Eur. J. Lipid Sci. Technol*. **117**, 1772-1781. https://doi.org/10.1002/ejlt.201400553

Patel AR, Dewettinck K. 2016. Edible oil structuring: an overview and recent updates. *Food Func*. **7**, 20-29. https://doi.org/10.1039/C5FO01006C

Patel AR. 2017. Methylcellulose-coated microcapsules of palm stearine as structuring templates for creating hybrid oleogels. *Mater. Chem. Phys*. **195**, 268-274. https://doi.org/10.1016/j.matchemphys.2017.03.059

Patel AR. 2018. Oil structuring: concepts, overview and future perspectives, in Ashok R. Patel (Ed.), *Edible Oil Structuring: Concept, Methods and Applications*, Royal Soc. Chem., Chambridge, UK, 3-24.

Perez-Monterroza EJP, Marquez-Cardozo CJ, Ciro-Velasquez HJ. 2014. Rheological behavior of avocado (*Persea americana* Mill, cv. Hass) oleogels considering the combined effect of structuring agents. *LWT - Food Sci. Technol*. **59**, 673-679. https://doi.org/10.1016/j.lwt.2014.07.020

Rocha-Amador OG, Gallegos-Infante JA, Huang Q, Rocha-Guzman NE, Moreno-Jimenez MR, Gonzalez-Laredo RF. 2014. Influence of commercial saturated monoglyceride, mono-/diglycerides mixtures, vegetable oil, stirring speed, and temperature on the physical properties of organogels. *Int. J. Food Sci*. **513641**, 1-8. https://doi.org/10.1155/2014/513641

Rogers MA. 2009. Novel structuring strategies for unsaturated fats ─ Meeting the zero-trans, zero-saturated fat challenge: A review. *Food Res. Int*. **42**, 747─753. https://doi.org/10.1016/j.foodres.2009.02.024

Sagiri SS, Samateh M, John G. 2018. Biobased molecular structuring agents, in Ashok R. Patel (Ed.), *Edible Oil Structuring: Concept, Methods and Applications*, Royal Soc. Chem., Chambridge, UK, 25-52.

Singh A, Auzanneau FI, Rogers MA. 2017. Advances in edible oleogel technology ─ a decade in review. *Food Res. Int*. **97**, 307-317. https://doi.org/10.1016/j.foodres.2017.04.022

Stahl MA, Buscato MHM, Grimaldi R. 2017. Low sat-structured fats enriched in α-linolenic acid: physicochemical properties and crystallization characteristics. *J. Food Sci. Technol*. **54**, 3391-3403. https://doi.org/10.1007/s13197-017-2780-8

Szydłowska-Czerniak A, Karlovits G, Lach M, Szłyk E. 2005. X-ray diffraction and differential scanning calorimetry studies of β'-β transitions in fat mixtures. *Food Chem*. **92**, 133-141. https://doi.org/10.1016/j.foodchem.2004.07.010

Terech P, Weiss RG. 1997. Low molecular mass gelators of organic liquids and the properties of their gels. *Chem. Rev*. **97**, 3133-3159. https://doi.org/10.1021/cr9700282

Toro-Vasquez JE, Charo-Alonso MA, Alvarez-Mitre, FM. 2018. Gelation properties of gelator molecules derived from 12-hydroxystearic acid, in Ashok R. Patel (Ed.), *Edible Oil Structuring: Concept, Methods and*

Applications, Royal Soc. Chem., Chambridge, UK, 106-134.

Viriato RLS, Queiros MS, da Gama MAS, Ribeiro APB, Gigante ML. 2018. Milk fat as a structuring agent of plastic lipid bases. *Food Res. Int*. **111**, 120-129. https://doi.org/10.1016/j.foodres.2018.05.015

Yılmaz E, Öğütcü M, Güneser O. 2015. Influence of storage on physico-chemical and volatile features of enriched and aromatized wax organogels. *J. Am. Oil Chem. Soc*. **92**, 1429-1443. https://doi.org/10.1007/s11746-015-2719-z

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Analyses and evaluation of the main chemical components in different tobacco (*Nicotiana tabacum* L.) genotypes

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SUMMARY: The nicotine, reducing sugar and ion contents from the threshing of tobacco can re-used from the industry. The crude oil and fatty oil compositions of tobacco seeds can be considered as an alternative source of raw material for biodiesel. In this study, the nicotine, reducing sugar content, crude oil, fatty acid composition and ion content were determined in 29 genotypes and 1 cultivar of tobacco. The genetic diversity was determined among the tobacco cultivar and genotypes base on examined properties. The nicotine content varied between 0.10-0.87%, reducing sugar ranged from 9.70-21.30%, crude oil varied between 24.33-47.00% and fatty acid compositions was found in the range of 77.94-100%. Linoleic (13.92-75.04%) and butyric (0.33-64.98%) acids were the major components. Overall, the BSR-5 (52.56 mg/g) and ESR-5 (44.58 mg/g) genotypes exhibited the highest potassium contents and ESR-7 (6.54 mg/g) and ESR-8 (1.28 mg/g) genotypes had the lowest chlorine contents. As a result of this study, the highest nicotine content, reducing sugar and crude oil of tobacco were found in ESR-4, ESR-11 and BSR-5 genotypes, respectively. The dendrogram analysis divided the tobacco into two main groups and most of the same origin genotypes fell into the same group. The results indicated that the different tobacco leaves and seeds can be evaluated as an alternative source in the industry as cigarettes, biodiesel and different industrial applications such as cosmetic, oil paints and varnishes based on their chemical properties.

KEYWORDS: *Fatty acid; Nicotine; Sugar; Tobacco*

RESUMEN: ***Análisis y evaluación de los principales componentes químicos de diferentes genotipos de tabaco (*Nicotiana tabacum *L.).*** La nicotina, el contenido de iones y azúcares reductores pueden usarse en la selección en la industria del tabaco. La composición del aceite crudo y los ácidos grasos de las semillas de tabaco pueden evaluarse de manera alternativa para la industria de biodiesel. En este estudio, la nicotina, el contenido de azúcares reductores, el aceite crudo, la composición en ácidos grasos y el contenido de iones se determinaron en tabacos de 29 genotipos y 1 cultivar. La diversidad genética se determinó entre los cultivares de tabaco y los genotipos basándose en las propiedades examinadas. El contenido de nicotina varió entre 0,10-0,87%, el valor de azúcares reductores varió entre 9,70-21,30%, el aceite crudo osciló entre 24,33-47,00% y las composiciones de ácidos grasos oscilaron entre 77,94 y 100%. Los componentes principales fueron los ácidos linoleico (13,92-75,04%) y butírico (0,33-64,98%). En general, en los genotipos BSR-5 (52,56 mg/g) y ESR-5 (44,58 mg/g) mostraron el mayor contenido de potasio y los genotipos ESR-7 (6,54 mg/g) y ESR-8 (1,28 mg/g) el contenido más bajo de cloro. Como resultado de este estudio, se encontró un mayor contenido de nicotina, azúcares reductores y aceite crudo en los tabacos de los genotipos ESR-4, ESR-11 y BSR-5, respectivamente. El análisis mediante dendrograma mostró dos grupos principales y la mayor parte de los mismos genotipos de igual origen tuvieron lugar en el mismo grupo. Los resultados indicaron que las diferentes hojas y semillas de tabaco pueden evaluarse como una fuente alternativa en la industria como cigarrillos, biodiesel y diferentes industrias como cosmética, pinturas al óleo y barnices en función de sus propiedades químicas.

PALABRAS CLAVE: *Ácidos grasos; Azúcares reductores; Nicotina; Tabaco*

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1. INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a commercial plant and its leaves are used for cigarette production and chewing. It is most widely grown for its leaves as a non-food crop in the world. The tobacco leaf is the most important part of the plant. For this reason, it is seen as a valuable cash crop (Regassa and Chandravanshi, 2016).

Nicotine is synthesized from the root of the plant and has pleasurable properties in low doses and toxic properties in high doses. The sugar contents in tobacco ranged from 2-10% (less) to 20-25% (higher). The combustion products of reducing sugars of the acidic substances fundamentally prevent the throat from burning and the pungency that alkaloids and volatile bases generate. Therefore, reducing sugars are seen as a positive factor for cigarette tobaccos (TEA, 2018).

Tobacco seeds were seen as a waste product of the tobacco leaf industries. The nutritional value of tobacco cured oil is better than groundnut and cotton seed oils and can be compared to safflower oil. In addition, in some European countries the refined tobacco seed oil is used as edible oil (Talaqani *et al.,* 1986).

The burning quality of the tobacco is primarily dependent on the characterization and quantities of the inorganic substances in the chemical combination. Excessive amounts of ion (eg 7-9%) in the chemical combinations make it difficult to burn the tobacco, thus reducing the smoking quality of tobacco. Among the ion contents, potassium and chloride are the most influential factors for combustion. Potassium salts have a positive effect on combustion, while chloride salts have negative effects. In addition, potassium plays an important role as a catalyst in the combustion of tobacco. Equal rates of potassium and calcium or a high potassium rate facilitate burning the chemical combination of the tobacco. On the other hand, the excess amount of calcium reduces burning even though the amount of chloride in the chemical combination is low (Er and Yıldız, 2014; TEA, 2018).

The goal of this work was to determine the chemical contents, namely nicotine, reducing sugar, crude oil, fatty acid composition and ion content in tobacco genotypes and cultivar under the Bolu ecological conditions. It serves to identify the components of tobacco thought to be influential in determining the quality parameters of tobacco leaves and seeds. We also determined the fatty acid composition of the tobacco seeds because this knowledge has important taxonomic significance in plant classification and is useful for preserving seed purity in the tobacco manufacturing industry.

2. MATERIALS AND METHODS

Three tobacco cultivars (Akhisar 97, Burley 94 and Virginia 90) and 29 genotypes obtained from 9 different provinces in Turkey were used in this study. These tobaccos were grown in the 2015 and 2016 growing seasons, and except for the Burley 94 and Virginia 90 cultivars, all tobaccos adapted to the Bolu ecological conditions (Table 1).

Tobacco seeds were sown in pots (100 × 120 mm) with 3 replicates in a greenhouse. The soil of the pots was prepared with equal amounts of a sand-turf-soil mixture (1:1:1) in May of both years. During the growth period, all required agricultural practices were carried out such as weed control, monitoring for disease and pests. When the plants reached 10-15 cm height, they were transplanted to the trial area on July 3, 2015 and July 7, 2016 in open-field conditions.

The field experiments were conducted at the research area of the Department of Field Crops, Faculty of Agriculture, Bolu Abant İzzet Baysal University (BAIBU) (Turkey) (40° 44’ 44’’ N, 31° 37’ 45’’ E, 881 m altitude). Research area soils had clay-loam with 7.5 pH. The useful phosphor value, potassium ratio, organic matter content and salt content were 237.4 kg/ha, 380 kg/ha, 1.6 and 0.008%, respectively. During the vegetation period, average climatic data were recorded as 19.10 °C and 18.0 °C temperature; 259.1 mm and 208.8 mm rainfall; 71.8 and 70.86% humidity for 2015 and 2016, respectively (Yaldiz *et al.,* 2019).

The experimental design was a randomized complete block design with three replicates. Each experimental plot consisted of three rows with a row-to-row distance of 0.4 m and plant to-plant distance of 0.15 m and plot size 2.52 m^2. The distance between the blocks was one meter. During the vegetation period in the experimental years, all required agricultural practices were conducted. 40 kg/ha DAP (diammonium phosphate) were applied to the plots as a base fertilizer. After transplanting, 20 kg/ha nitrogenous fertilizer as ammonium nitrate were applied to the plants. The harvest was done between September 10 and October 8 in the first year and between September 15 and October 12

TABLE 1. List of Tobacco Genotypes and Cultivar Used in the Current Study.

No	Region-Code	Genotypes/cultivar names	Obtained place or person	Geographic coordinates
				Latitude Longitude
1	AR-1	Akhisar 97	Manisa/Cultivar	38°55'3.5904" N 27°50'11.8320" E
2	AR-2	Akhisar-Sarılar	Manisa/Akhisar/Sarılar/Ufuk Özcan	39°6'26.9100" N 28°0'8.7084" E
3	MR-1	Agonya-Yarış Village	Çanakkale/Agonya/Yarış Village/Anıl Özyurt	39°47'1.6332" N 27°15'51.7320" E
4	ESR-1	B. Çelikhan 97	Adıyaman/Çelikhan	38°1'59.0520" N 38°14'13.9128" E
5	BSR-1	Bafra Gökçeağaç	Samsun/Bafra/Gökçeağaç/Şefik Kara	41°32'45.9204" N 35°45'41.4324" E
6	BSR-2	Bafra Paşaşeyh	Samsun/Bafra/Paşaşeyh/Cemil Yüksel	41°28'50.8260" N 35°44'18.0888" E
7	MR-2	Balıkesir (AARI, 42986)	AARI	-
8	MR-3	Balıkesir (AARI, 64073)	AARI	-
9	ESR-2	Bitlis Mutki Erler Village-1	Bitlis/Mutki/Erler Village/Sait Sülün	38°28'46.7472" N 41°43'56.5536" E
10	ESR-3	Bitlis Mutki Erler Village-2	Bitlis/Mutki/Erler Village/Yusuf Kesim	38°28'46.7472" N 41°43'56.5536" E
11	MR-4	Bursa (AARI, 42884)	AARI	-
12	MR-5	Bursa (AARI, 78215)	AARI	-
13	ESR-4	Bitlis (AARI, 42076)	AARI	-
14	ESR-5	Bitlis (AARI, 80111)	AARI	-
15	ESR-6	Eski Tütün-Hatay	Hatay	36°12'1¨ N 36°10'34¨ E
16	ESR-7	Hatay (AARI, 42126)	AARI	-
17	ESR-8	Hatay (AARI, 42128)	AARI	-
18	ESR-9	Hatay (AARI, 42132)	AARI	-
19	AR-3	Manisa (AARI, 64062)	Aegean Agricultural Research Institute	38°36'50.5188" N 27°25'46.4232" E
20	ESR-10	Muş (AARI, 42094)	AARI	-
21	AR-4	Salihli Kale Village	Manisa/Salihli/Kale Village/ İbrahim Zeybek	38°43'18.6780" N 28°8'17.9700" E
22	BSR-3	Samsun (AARI, 49184)	AARI	-
23	BSR-4	Samsun (AARI, 49188)	AARI	-
24	BSR-5	Samsun (AARI, 49219)	AARI	-
25	BSR-6	Samsun (AARI, 49224)	AARI	-
26	BSR-7	Samsun Tekkeköy Hamzalı	Samsun/Tekkeköy/Hamzalı/ Mustafa Anıl	41°12'19.8756" N 36°32'15.2844" E
27	BSR-8	Samsun Terme Akçay	Samsun/Terme/Akçay/Mümin Bayram	41°8'0.7332" N 37°9'22.2264" E
28	BSR-9	Samsun Tekkeköy Balcalı	Samsun/Tekkeköy/Balcalı/Ali Doğru	41°9'14.8932" N 36°34'5.4624" E
29	BSR-10	Samsun Tekkeköy Kahyalı	Samsun/Tekkeköy/Kahyalı/ Osman Kul	41°10'21.5508" N 36°32'21.6564" E
30	ESR-11	Yayladağı Sebenoba	Hatay/Yayladağı/Sebenoba/Ayşe Şahin	36°2'48.2424" N 36°1'15.0384" E

AARI: Aegean Agricultural Research Institute, **AR**: Aegean Region, **MR**: Marmara Region, **ESR**: Eastern and Southeastern Anatolia Region, **BSR**: Black Sea Region.

(3 times) in the second year. The harvest was done in the early morning or coolness of the evening (after 17:00 pm) for both two years (Er and Yıldız, 2014). After harvesting, the leaves were dried under laboratory conditions.

2.1. Analyses of tobacco leaves

2.1.1. Nicotine extraction method

The nicotine extraction is given in Table 2a. A homogenized tobacco sample was weighed and extracted with *n*-hexane, distilled water and NaOH. Then the sample was shaken in vortex and the upper phase was removed and diluted with *n*-hexane and placed in the GC-MS instrument (ARGEFAR, 2016).

2.1.2. Extraction method of reducing sugar

Homogenized tobacco leaves were weighed and extracted with distilled water and methanol. Then the sample was shaken in a vortex and the upper phase was removed and filtered into a vial. The amount reducing sugar was calculated as g/100 g in the solution by subjecting it to the HPLC-RI system (Table 2a) (ARGEFAR, 2016).

2.1.3. Analysis of ion content

The results from the ion content analysis of tobacco genotypes are shown in Table 2b. 0.5 g of the weighed plant sample was soaked in 50 ml of sterile distilled water in an ultrasonic bath for 30 minutes. Then, the extracts were filtered using

TABLE 2. Device parameters used in nicotine and reducing sugar contents (2a) and optimum operation conditions for Dionex ics 1100 ion chromatography (2b).

TABLE 2a

	Nicotine Analysis	Reducing Sugar Analysis
Equipment	Shimadzu GC-MS	HPLC RI
Column	RTX-CL- Pesticide 2	NH2 5 µm 4.6x250 mm
Heat program	80 °C; 45 °C increase to 200 °C; 20 °C increase to 230 ° C; after 30 °C increase to 300 °C 5 min. isothermal	-
Injection type	Splittles	-
Sensor	MS	RID
Injection volume	2 mL	10 µl
Carrier gas	Helium: 1 mL/min	-
Injector heat	250 °C	-
Mobile Phase	-	HPLC purity Acetonitrile-Water
RID Detector Temperature	-	30 °C
Flow rate	-	1.500 ml/min

TABLE 2b

Operation conditions	Anion	Cation
Mobile phase	9 mM Na_2CO_3	20 mM Methanesulfonic acid
Column	Ionpac AS9-HC (250x4 mm)	Ionpac CS12-A (250x4 mm)
Guard Column	Ionpac AG9-HC (50x4 mm)	Ionpac CG12-A (50x4 mm)
Supressor	ASRS-4 mm	CSRS-4mm
Supressor current	45 mA	65 mA
Detector	Conductivity Detector	Conductivity Detector
Pressure (psi)	2000-3000	2000-3000
Oven temperature	30 °C	30 °C
Background conductance	<30 µS	0.5-2 µS
Flow Rate	1.00 mL/min	1.00 mL/min
Injection volume	500 µL	1000 µL
Rate of data transfer	5.0 Hz	5.0 Hz
Duration	30 min	15 mins

0.22 µm cellulose acetate filter paper and prepared for analysis. Before sample analysis, the standard Dionex was used for calibration. The ion contents in the samples were determined by using Dionex ICS 1100 Series ion chromatography (Wang *et al.,* 2016).

2.2. Analyses of tobacco seeds

2.2.1. Isolation of seed crude oil

Tobacco seeds were ground and measured as 5 g. Then, they were extracted at 80 °C by a Soxhlet extractor for 8 h, using n-hexane as solvent. After oil extraction the solvent was removed by a rotary evaporator (Yaldiz and Camlica, 2019).

2.2.2. Determination of fatty acid composition

Quantitative determinations of FAMEs were conducted according to Yaldiz and Çamlica (2019) using a Shimadzu GC-2010 gas chromatograph (Shimadzu Corporation, Tokyo, Japan) with a flame ionization detector (FID) and Rtx-2330 capillary column (60 m × 0.25 mm) of 0.2 µm. The detector temperature was set at 240 °C. The GC oven temperature was programmed at 140 °C for 5 min. Then the temperature was increased up to 260 °C at a rate of 4 °C/min and kept constant at 260 °C for 20 min. Helium (1 ml/min) was used as carrier gas. The FAMEs were determined by comparing their retention times with reference standards (mixture FAME Mix, SUPELCO, which included 37 FAMEs). Methyl undecanote (Sigma Aldrich Chemical Co., St. 129 Louis, MO, USA) was used for FAMEs quantity as the internal standard. The obtained total results from FAMEs were expressed as percentages.

2.3. Statistical analysis

The nicotine, reducing sugar, ion contents and seed crude oil determinations were repeated three times, and their analyses were performed within each replicate sample three times with standard deviation (SD). Significant differences among the genotypes were determined by the one-way ANOVA with means separation by the Least Significant Difference (LSD) test at the 0.01 level. dendrogram was used to show the hierarchy of clusters and to determine the genetic variability among tobacco cultivars and genotypes based on chemical components and FAC by the XLSTAT program (Yaldiz and Camlica, 2019).

3. RESULTS AND DISCUSSIONS

The data presented in Table 3 presents the significant differences in Nicotine Contents (NC), Reducing Sugar Contents (RSC) and Seed Crude Oil (SCO) among the tobacco genotypes and cultivar.

3.1. Nicotine content (NC)

NC is the most important factor in the tobacco industry especially in cigarette blends, despite the fact that it has some negative effects on the human body as healthy and protective. The quality parameter of the cigarette blend is determined by the NC. There were statistically significant differences among the tobacco genotypes and cultivar in terms of the NC (Table 3). The NC of tobacco genotypes and cultivar varied between 0.10-0.87%. The highest NC was found for the ESR-4 genotype when compared to the others, followed by ESR-5 (0.78%) and BSR-5 (0.59%). Cultivar 'Akhisar 97' was found to be the highest of the 20 genotypes at 0.41%. It was found that MR-4 (0.10%) and MR-3 (0.16%) genotypes contained lower nicotine ratios than the others. While the highest values were obtained for ESR-4 and ESR-5, the lowest values were obtained from MR-2, 3, 4, and 5 genotypes. Compared to the average NC of the tobacco-growing regions, AR, MR, ESR, and BSR were found at 0.33, 0.22, 0.46, and 0.37%, respectively. The results showed that the tobacco genotypes of ESR were higher than the others. This situation can be explained by the ecological conditions of Bolu, which are similar to a continental climate. It was noted that the characterization of quality and aroma of the tobacco depended on the soil, climate conditions and low amount of nitrogen (Bilgin *et al.,* 1993). It was also reported that Aegean tobacco had very low nicotine contents (Delibacak *et al.,* 2014). Abdallah (1986) reported that high levels of nicotine gave the hard and burner features, and low levels of nicotine led to poor taste and physiological dissatisfaction. These tobaccos can be used because of their low nicotine level and their rich flavor (Otan and Apti, 1989). Although in low doses nicotine has a stimulating effect, increasing activity, alertness and memory, it also increases the heart rate and blood pressure and causes anorexia (Bastida and Beltran, 2011).

TABLE 3. Nicotine, sugar and crude oil contents in evaluated tobacco genotypes and cultivar.

No	Genotypes/ Cultivar	Nicotine Content (%)	Reduced Sugar Content (%)	Crude oil (%)
1	**AR-1**	0.41±0.01f	16.90±0.04e	46.33±1.53ab
2	**AR-2**	0.34±0.01hıj	13.80±0.09k	33.33±1.15hı
3	**MR-1**	0.36±0.01ghı	11.80±0.08o	26.33±2.08lm
4	**ESR-1**	0.41±0.01f	14.30±0.05ıj	42.00±1.73cde
5	**BSR-1**	0.36±0.01ghı	20.80±0.05b	44.00±2.00a-d
6	**BSR-2**	0.42±0.01ef	13.80±0.01k	34.67±1.15ghı
7	**MR-2**	0.24±0.01mn	18.40±0.08d	31.67±3.51ıjk
8	**MR-3**	0.16±0.01o	19.40±0.01c	25.33±3.06lm
9	**ESR-2**	0.22±0.01n	**9.70**±0.10**q**	**24.33**±2.08**m**
10	**ESR-3**	0.36±0.01ghı	16.10±0.01f	44.67±1.15a-d
11	**MR-4**	**0.10**±0.05**p**	15.50±0.09g	41.33±2.52c-f
12	**MR-5**	0.22±0.01n	18.40±0.01d	26.33±1.53lm
13	**ESR-4**	**0.87**±0.01**a**	14.10±0.10jk	45.33±1.15abc
14	**ESR-5**	0.78±0.01b	**9.70**±0.01**q**	37.33±1.15fgh
15	**ESR-6**	0.33±0.01ıjk	15.40±0.02g	43.67±1.53a-d
16	**ESR-7**	0.46±0.01de	13.40±0.04l	29.00±3.61jkl
17	**ESR-8**	0.42±0.02ef	14.60±0.01hı	45.33±0.58abc
18	**ESR-9**	0.50±0.05d	21.00±0.10ab	35.67±0.58ghı
19	**AR-3**	0.29±0.01kl	17.20±0.03e	38.67±1.15efg
20	**ESR-10**	0.38±0.01fgh	**9.70**±0.03**q**	34.67±1.15ghı
21	**AR-4**	0.36±0.01ghı	15.30±0.11g	25.33±3.06lm
22	**BSR-3**	0.34±0.01hıj	13.00±0.04m	40.67±0.58def
23	**BSR-4**	0.40±0.05fg	10.80±0.02p	34.67±0.58ghı
24	**BSR-5**	0.59±0.01c	14.00±0.02jk	**47.00**±2.65**a**
25	**BSR-6**	0.32±0.01ı-l	12.30±0.10n	46.33±1.53ab
26	**BSR-7**	0.28±0.01lm	12.70±0.05m	46.67±1.15a
27	**BSR-8**	0.30±0.05jkl	14.90±0.01h	41.67±0.58cde
28	**BSR-9**	0.41±0.01f	14.10±0.10jk	28.33±0.58klm
29	**BSR-10**	0.23±0.01n	14.00±0.50jk	32.67±2.08ıj
30	**ESR-11**	0.34±0.01hıj	**21.30**±0.03**a**	42.33±1.53b-e
	Average	**0.37**	**14.88**	**36.85**
	SD	**0.16**	**3.22**	**7.54**
	LSD (1%)	**0.03**	**0.33**	**4.07**
	CV (%)	**5.52**	**1.02**	**5.03**

AR: Aegean Region, **MR:** Marmara Region, **ESR:** Eastern and Southeastern Anatolia Region, **BSR:** Black Sea Region.
Different letters in the same column indicate significant differences ($P < 0.01$).
SD: Standard Deviation, **LSD:** Least Significant Difference, **CV:** Coefficient Variation.

These tobaccos can be used in the tobacco industry for protect human health because of their low NC.

Ekren and Sekin (2008) noted that NC was found from 0.12 to 1.06% in tobacco from the Akhisar region. 1.8-2.1% (Kurt and Ayan, 2014), 0.73-0.95% (Yagac, 2015) were also found.

The results were similar to those of other researchers, except for Kurt and Ayan (2014). The differences can be explained by the fact that the tobacco genotypes and cultivar showed differences depending on their adaptation, genetic properties and survival in different places and the soil contents as organic matter can affect the NC of tobacco (Griesser *et al.,* 2015).

3.2. Reducing sugar content (RSC)

Reducing sugar contents can provide mildness when smoking tobacco and was found to positively affect its quality (Abdallah 1986). Differences in RSC were found to be statistically significant among the tobacco genotypes and cultivar. As seen in Table 3, the RSC of tobacco genotypes and cultivar ranged from 9.70 to 21.30%. The RSC of ESR-9 and ESR-11 were higher than the other genotypes and cultivar. The ESR-2, ESR-5 and ESR-10 genotypes were found to be lower than the others at 9.70%. When compared among the regions, the highest RSC was found in MR at 16.70% followed by AR (15.43%), ESR (14.48%) and BSR (14.04%). The cultivar was found to 16.90% above the average of the regions.

The desirable amount of RSC is between 8-13% (Camas *et al.,* 2007). The closest values to the desired ones were seen in 8 genotypes between 9.70-13.0%. The values were found in BSR genotypes (BSR-2, 3, 4, 5), followed by ESR genotypes (ESR-2, ESR-5 and ESR-10) and MR-1 in MR genotypes. Based on the RSC values (Table 3), these genotypes are different from the others. Therefore, they are suitable for the production of the RSC used in the tobacco industry for a quality product.

It is noted that the RSC of tobacco varied between 11.30-21.83% (Yagac, 2015) and 7.81-33.71 (Ekren and Sekin, 2008). Our results have been compared to those of other researchers and it was determined that they showed similar values as reported by researchers.

3.3. Ion content (IC)

Ions are non-combustible substances and are only present in the ash. It is known that, due to the increase in potassium from these substances, the ability of the tobacco to burn improves, while the chloride salt affects it negatively (Er and Yıldız 2014).

The present study was conducted for the evaluation of IM such as Potassium (K+), Magnesium (Mg^{2+}), Calcium (Ca^{2+}), Chloride (Cl^{-}), Phosphorus (PO_4^{3-}) and Sulfate (SO_4^{2-}), and in the seeds of tobacco genotypes and cultivar. Significant differences were found among tobacco genotypes and cultivar in terms of IC (Table 4).

In this study, the concentration of K^+ varied from 8.1 to 52.6 mg/g plant. The K^+ concentrations in the leaves were different among all the different genotypes. The highest value was found for BSR-5 and the lowest value was found for BSR-7. Krishnamurthy and Ramakrishnayya (1993) reported that the tobacco plant had a higher K requirement compared to other cultivated plants. They also noted that color, texture, thickness, elasticity, and burning capacity were affected by dry leaves and the high K^+ content in the dried leaves was generally considered to be the highest quality criterion. The obtained K^+ values were higher than those reported by Irget *et al.,* (1999) (0.87-2.24%). Our tobaccos were found to be rich in terms of K^+. Therefore, especially BSR-5 and ESR-5 genotypes were found to be more suitable for the burning capacity of tobacco.

The Mg^{2+} concentrations of the tobacco genotypes and cultivar varied between 6.04 and 0.61 mg/g plant (Table 4). The highest value was determined for BSR-3, and the lowest value was determined for the AR-4 genotype. Ca^{2+} content varied from 0.27 to 37.4 mg/g. While the highest values were obtained for ESR-10 (37.4 mg/g) and BSR-1 (34.7 mg/g), the lowest values were obtained for the ESR-4 (0.27 mg/g) and BSR-10 (10.7 mg/g) genotypes. Cl^- was present in the range of 1.28-32.3 mg/g. The highest concentration was present in the BSR-6 genotype, followed by the BSR-3 (29.2 mg/g) genotype. In the present study, the concentration range of PO_4^{3-} was 4.29-9.44 mg/g, as shown in Table 4. The highest level of that form of PO_4^{3-} was found in the ESR-7 genotype (9.44 mg/g), followed by the BSR-6 genotype (8.38 mg/g). SO_4^{2-} concentrations ranged from 2.61 to 7.40 mg/g. Its maximum content (7.40mg/g) was present in ESR-5, and its minimum content (2.61 mg/g) was present in BSR-9. The highest amounts of K^+, Mg^{2+} and Ca^{2+} were found in the MR genotypes, the highest PO_4^{3-} and SO_4^{2-} in the ESR genotypes and the highest

TABLE 4. Ion Contents in Tobacco Genotypes and Cultivar (mg/g).

Tobacco genotypes/ cultivar	K^+	Mg^{2+}	Ca^{2+}	Cl^-	PO_4^{3-}	SO_4^{2-}
MR-1	21.24±0.02R	5.32±0.01D	24.72±0.01M	18.2±0.01L	6.31±0.01O	3.82±0.00O
AR-1	30.38±0.03H	0.94±0.00Z	15.41±0.00a	11.97±0.01T	4.75±0.02a	2.77±0.00c
AR-2	23.84±0.01N	5.04±0.00F	32.61±0.01D	22.35±0.05E	6.15±0.01Q	3.77±0.02R
ESR-1	29.16±0.04J	1.78±0.00V	19.11±0.01T	14.13±0.03R	6.86±0.01J	4.89±0.00J
BSR-1	21.46±0.04Q	5.15±0.00E	34.74±0.03C	22.04±0.03F	8.33±0.00C	4.89±0.00J
BSR-2	14.02±0.02Y	3.63±0.00M	30.89±0.01G	14.8±0.00O	5.46±0.00S	3.3±0.00Y
MR-2	27.83±0.03L	2.48±0.00P	18.36±0.05U	9.34±0.00b	6.02±0.00R	2.64±0.01d
MR-3	28.82±0.02K	4.66±0.01H	25.08±0.03L	19.15±0.06J	5.35±0.00T	4.97±0.00I
ESR-4	-	-	0.27±0.00d	10.29±0.02a	6.42±0.00N	4.07±0.00N
ESR-5	44.58±0.02B	2.23±0.00Q	25.71±0.01J	23.78±0.02D	8.15±0.00E	7.4±0.01A
ESR-2	22.05±0.05P	5.69±0.00C	29.92±0.02H	21.69±0.01G	7.05±0.01H	5.82±0.00E
ESR-3	36.17±0.03F	0.86±0.00a	19.52±0.01R	14.26±0.05Q	6.54±0.00L	3.26±0.00Z
MR-4	18.64±0.02T	4.22±0.01K	29.88±0.01I	14.42±0.01P	6.5±0.02M	5.76±0.00F
MR-5	42.64±0.00C	3.53±0.01N	20.54±0.00P	20.75±0.00H	8.3±0.00D	6.08±0.00D
ESR-6	37.05±0.05E	1.43±0.01Y	21.48±0.01O	14.12±0.02R	7.32±0.00G	4.11±0.00M
ESR-7	16.79±0.00U	4.57±0.00J	24.15±0.00N	6.54±0.00c	9.44±0.00A	4.39±0.01K
ESR-8	24.86±0.04M	2.2±0.00R	16.00±0.01Y	1.28±0.00d	6.19±0.00P	3.73±0.00S
ESR-9	10.89±0.01b	4.6±0.00I	31.4±0.00F	10.62±0.02Z	6.72±0.00K	5.02±0.00H
AR-3	37.00±0.00E	0.75±0.00b	15.6±0.01Z	14.39±0.02P	5.34±0.00T	3.65±0.00T
ESR-10	22.42±0.01O	6.55±0.00A	37.43±0.02A	20.33±0.02I	7.02±0.00I	6.19±0.00C
AR-4	12.22±0.01Z	0.61±0.00c	16.69±0.01V	13.78±0.02S	4.92±0.00V	5.09±0.00G
BSR-3	20.91±0.01S	4.85±0.00G	19.21±0.01S	24.73±0.03C	7.57±0.00F	4.17±0.00L
BSR-4	29.58±0.02I	6.04±0.00B	35.02±0.01B	29.21±0.01B	6.3±0.00O	3.48±0.00U
BSR-5	52.56±0.07A	3.65±0.01L	20.08±0.01Q	18.52±0.02K	7.06±0.00H	3.79±0.00Q
BSR-6	-	-	-	32.31±0.01A	8.38±0.00B	7.07±0.00B
BSR-7	16.3±0V	2.51±0.00O	25.05±0.03L	11.02±0.01V	6.03±0.00R	3.8±0.00P
BSR-8	31.22±0.02G	2.13±0.00U	10.71±0.02c	17.57±0.03M	4.78±0.00Z	3.39±0.00V
BSR-9	11.79±0.01a	2.14±0.00T	31.88±0.01E	10.88±0.02Y	4.29±0.00b	2.61±0.00e
BSR-10	8.08±0.02c	2.15±0.00S	25.33±0.02K	11.37±0.02U	5.2±0.01U	3.12±0.00a
ESR-11	40.72±0.01D	-	10.98±0.02b	16.98±0.02N	4.81±0.00Y	2.82±0.00b
Average	26.19	3.32	23.03	16.36	6.45	4.33
LSD (%1)	0.05	0.01	0.03	0.05	0.01	0.01
CV (%)	0.10	0.10	0.07	0.14	0.09	0.08
SD	11.01	1.74	8.33	6.51	1.25	1.26

AR: Aegean Region, **MR:** Marmara Region, **ESR:** Eastern and Southeastern Anatolia Region, **BSR:** Black Sea Region.
Different letters in the same column indicate significant differences ($P < 0.01$).
SD: Standard Deviation, **LSD:** Least Significant Difference, **CV:** Coefficient Variation.

CI^- was seen in the BSR tobaccos. The change in the same ion contents in the tobacco genotypes among the regions can be explained by genetic differences and tobacco producers may have used the same tobacco genotype seeds in the same region.

Ca^{2+}, Mg^{2+} and CI^- contents were found between 2.08-6.32%, 0.20-0.98%, and 0.1-0.50%, respectively (Irget *et al.,* 1999). The obtained data was found somewhat similar to other researchers.

3.4. Seed crude oil (SCO)

Significant differences were found among the tobacco genotypes and cultivar in terms of SCO, as shown in Table 3. The SCO values ranged from 24.33-47.0%. The analysis showed that SCO was the highest in BSR-5 (47.0%), followed by BSR-6 (46.33%) genotypes and AR-1 cultivar (46.33%). The lowest SCO values were found for the ESR-2 (24.33%), MR-3 (25.33%) and AR-4 (25.33%) genotypes. When the SCO of the tobacco genotypes and cultivar were compared among the regions, SCO was found at 32.44% in AR, 30.20% in MR, 38.06% in ESR, and 39.20% in BSR. In general, the average SCO of the tobacco genotypes and cultivar was over 30%, with the highest average SCO determined for the BSR genotypes. So, BSR-5, BSR-6 and the AR-1 cultivar had highest crude oil contents and they can be used in biodiesel production as an alternative fuel source (Fornasier *et al.,* 2018).

Tobacco seed oil was reported as 24.56-41.93% (Mohammad and Tahir 2014), 29.82% (Abbas Ali *et al.,* 2008). The obtained data were found similarly those reported by other researchers.

3.5. Fatty acid compositions (FAC)

Table 5 shows the FAC of the tobacco genotypes and cultivar. Linoleic (18:2), oleic (18:1), palmitic (C16:0) and butyric acids (C4:0) were the four most abundant acids among the 25 FAC (13.92-75.04, 0.46-17.80, 5.55-19.11 and 0.33-64.98%, respectively).

Linoleic acid was found to be the main component and predominated the oils of tobacco (13.92-75.04%), except for the MR-5 and ESR-6 genotypes. The highest linoleic acid was found in ESR-3 (75.04%), followed by the AR-1 cultivar (67.84%) and AR-4 (67.44%) genotype. The lowest linoleic acid was determined for BSR-8 (13.92%) and ESR-9 (24.54%). The high content of linoleic acid in tobacco seed oil is very important for the production of oleo-chemicals (Abbas Ali *et al.,* 2008). It can be used as a surfactant, dispersant, bio lubricant, in cosmetics and a variety of synthetics in the formulation of protective coating and in preparations of other long chain compounds (Awola *et al.,* 2010). The content of linoleic acid was higher than that reported (4.2-9.23%) by Mohammad and Tahir (2014) and found similar to the 6.45-77.48% reported by Poltronieri (2016). Therefore, especially the ESR-3 and AR-1 genotypes are promising for linoleic acid to be obtained at high levels and these genotypes can be used in oleo-chemical production and other areas such as cosmetic and surfactant. It can also be used in making quick-drying oils such as oil paints and varnishes (Chiririwa *et al.,* 2014). In addition to this, Kirkova *et al.,* (2016) noted that the linoleic acid content must be lower to obtain better oil as quality. Therefore, the BSR-8 and ESR-9 genotypes can be evaluated for the best oil quality.

High concentrations of oleic (0.46-17.80%) and palmitic acids were detected (5.55-19.12%; except for ESR-1 genotype) in all the tobacco studied oils. The highest oleic acid was found in the BSR-3 genotype with 17.80%, followed by ESR-9 (17.35%) and AR-2 (16.35%). The lowest oleic acid was found in ESR-8 (0.46%). The highest palmitic acid was determined in MR-5 (19.12%) and the lowest palmitic acid was found in the BSR-7 genotype.

The effects of oleic acid are mediated by preventing the reduction in palmitic acid-mediated activated protein kinase (AMPK) activity, resembling the action of metformin (Palomer *et al.,* 2018). Palmitic and oleic acids were determined at 21.33-25.667% and 17.00-26.667% by Mohammad and Tahir (2014). The main fatty acids (FA) in tobacco were found to be linoleic acid (60-80%), oleic acid (10-20%) and palmitic acid (8-20%) (Giannelos *et al.,* 2002; Abbas Ali *et al.,* 2008; Stanisavljević *et al.,* 2009; Bucciarelli *et al.,* 2013; del Piano *et al.,* 2014).

The palmitic and oleic acid results were found to be different from those obtained by Mohammad and Tahir (2014); but they were close to those reported by other researchers. It has been reported that climatic conditions affect the properties of plants such as growth, yield and biochemical contents (Zandalinas *et al.,* 2018).

TABLE 5. Fatty Acid Compositions of Tobaccos (%).

Fatty acid compositions	RT (min)	AR-1	AR-2	MR-1	ESR-1	BSR-1	BSR-2	MR-2	MR-3	ESR-2	ESR-3	MR-4	MR-5	ESR-4	ESR-5	ESR-6	ESR-7	ESR-8
Butyric acid (C4:0)	4.709	11.77	14.86	5.33	7.13	17.68	27.95	16.29	6.69	20.63	0.33	24.66	58.61	3.15	17.84	64.98	20.07	23.82
Caproic acid (C6:0)	5.215	-	0.99	0.08	0.51	-	0.61	-	0.70	-	0.11	-	1.41	0.41	-	3.47	-	-
Caprylic acid (C8:0)	5.99	-	0.50	0.20	0.14	-	-	-	-	-	-	-	-	0.11	-	-	-	-
Palmitic acid (C16:0)	17.324	6.86	13.71	11.7	-	7.34	7.81	6.44	13.75	6.99	7.7	7.15	19.12	11.17	7.32	11.43	6.27	6.53
Palmitoleic acid (C16:1)	18.461	-	-	0.12	11.11	-	-	-	-	-	0.14	-	-	0.10	-	-	-	-
Heptadecanoic acid (C17:0)	19.199	0.12	-	0.23	0.18	-	0.22	-	-	-	0.13	0.12	-	0.18	-	-	-	0.12
***cis*-10-heptadecanoic acid (C17:1)**	20.15	-	-	0.51	0.29	-	-	-	-	-	0.07	-	-	-	-	-	-	-
Stearic acid (C18:0)	21.048	2.90	7.17	4.26	4.65	2.38	1.91	2.68	5.46	2.81	2.79	2.44	13.53	4.44	2.80	7.02	2.55	2.21
Elaidic acid (C18:1n9t)	21.88	-	-	0.08	-	-	0.46	-	-	-	0.06	7.22	-	0.04	-	-	-	6.87
Oleic acid (C18:1n9c)	22.02	8.18	16.35	13.22	12.57	8.58	7.00	7.85	16.26	7.59	9.02	0.51	5.53	15.98	9.25	4.81	7.48	0.46
Linoleic acid (C18:2n6c)	23.531	67.84	40.45	57.94	59.43	58.6	47.76	60.51	47.79	59.41	75.04	55.32	-	59.18	60.43	-	61.97	57.94
Arachidic acid (C20:0)	24.526	0.21	0.62	0.42	0.35	-	-	-	-	0.18	0.22	0.17	-	0.35	0.21	-	-	0.17
γ-linolenic acid (C18:3n6)	25.158	0.98	0.33	0.65	0.78	1.26	0.88	0.92	-	0.89	1.45	0.86	-	0.54	0.86	-	0.90	1.07
c*is*-11-eicosenoic acid (C20:1)	25.416	-	-	-	0.51	-	-	-	-	-	0.04	-	-	0.07	-	-	-	-
Linolenic acid (C18:3n6)	26.189	-	0.27	0.12	-	-	0.28	-	-	-	0.04	-	-	-	-	4.56	-	-
Heneicosanoic acid (C21:0)	26.765	-	0.33	0.15	-	-	0.53	-	1.32	-	0.07	-	-	0.04	-	-	-	-
***cis*-11,14-eicosadienoic acid (C20:2)**	27.752	0.14	0.4	0.92	-	-	0.66	-	0.7	-	0.26	-	1.80	0.43	-	1.79	-	-
Behenic acid (C22:0)	27.896	-	-	0.19	-	-	0.24	-	1.19	-	0.04	-	-	-	-	-	-	-
Arachidonic acid (C20:4n6)	29.198	-	0.28	0.10		-	0.66	-	0.8	-	0.04	-	-	-	-	-	-	0.14
Tricosanoic acid (C23:0)	29.683	-	-	-	0.15	-	1.13	-	-	-	0.11	-	-	0.06	-	1.95	-	-
Lignoceric acid (C24:0)	30.475	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-
***cis*-13,16-docosadienoic acid (C22:2)**	29.73	-	-	0.19		-	-	-	1.65	-	0.06	-	-	-	-	-	-	-
***cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (C20:5n3) EPA**	30.771	-	0.43	0.51	0.24	-	0.57	-		-	0.10	-	-	0.19	-	-	-	-
Nervonic aid (C24:1)	32.315	-	-	0.08	0.25	-	-	-	0.70	-	-	-	-	0.29	-	-	-	-
***cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6n3) DHA**	33.391	0.10	1.74	1.89	1.71	-	-	-		-	1.66	0.34		2.33	-	-	-	0.28
USFA (%)		77.24	60.24	76.34	86.88	68.44	58.27	69.28	67.9	67.89	87.98	64.25	7.33	79.15	70.54	11.16	70.35	66.76
TSFA (%)		21.86	38.18	22.55	13.12	27.4	40.42	25.41	29.11	30.61	11.51	34.54	92.67	19.92	28.17	88.84	28.89	32.85
MUFA (%)		8.18	16.35	13.89	13.61	8.58	7.46	7.85	16.96	7.59	9.2	7.73	5.53	16.38	9.25	4.81	7.48	7.33
PUFA (%)		69.06	43.89	62.33	62.16	59.86	50.81	61.43	50.94	60.3	78.65	56.52	1.8	62.66	61.29	6.35	62.87	59.43
Total (%)		99.1	98.42	98.89	100	95.84	98.68	94.69	97.01	98.5	99.49	98.79	100	99.07	98.71	100	99.24	99.61

TABLE 5. (Continued).

Fatty acid compositions	RT (min)	ESR-9	AR-3	ESR-10	AR-4	BSR-3	BSR-4	BSR-5	BSR-6	BSR-7	BSR-8	BSR-9	BSR-10	ESR-11	SD
Butyric acid (C4:0)	4.709	19.32	14.27	23.56	12.62	9.91	12.22	4.91	18.24	35.54	34.82	5.70	19.04	5.20	14.71
Caproic acid (C6:0)	5.215	0.52	-	-	-	0.73	-	0.06	-	-	1.24	0.58	1.71	0.88	0.83
Caprylic acid (C8:0)	5.99	0.97	-	-	-	0.27	-	0.10	-	-	-	0.20	0.90	0.20	0.32
Palmitic acid (C16:0)	17.324	18.49	6.91	6.99	7.12	14.53	6.71	10.7	5.69	5.55	12.88	11.68	19.05	10.65	4.10
Palmitoleic acid (C16:1)	18.461	-	-	-	-	-	-	0.11	-	-	-	0.12	-	0.19	4.15
Heptadecanoic acid (C17:0)	19.199	-	-	-	-	0.25	0.12	0.18	-	-	-	0.22	-	0.21	0.05
***cis*-10-heptadecanoic acid (C17:1)**	20.15	0.56	-	-	-	0.26	-	0.30	-	-	0.85	0.53	0.61	0.43	0.22
Stearic acid (C18:0)	21.048	8.88	2.98	2.48	0.12	-	2.71	4.17	1.97	1.96	8.34	4.28	6.76	4.36	2.76
Elaidic acid (C18:1n9t)	21.88	-	-	-	2.78	-	-	-	-	-	-	-	-	13.28	4.86
Oleic acid (C18:1n9c)	22.02	17.35	8.67	8.20	8.05	17.8	8.06	13.41	6.25	6.11	13.22	13.18	14.11	0.66	4.84
Linoleic acid (C18:2n6c)	23.531	24.54	64.95	56.37	67.43	45.95	67.57	60.28	45.79	48.45	13.92	57.83	32.83	56.54	13.36
Arachidic acid (C20:0)	24.526	0.73	0.19	0.17	0.20	0.44	0.18	0.40	-	-	0.82	0.40	0.69	0.54	0.21
γ-linolenic acid (C18:3n6)	25.158	-	0.91	0.93	1.00	0.43	1.03	0.59	-	0.90	-	0.66	-	0.67	0.25
***cis*-11-eicosenoic acid (C20:1)**	25.416	-	-	-	-	-	-	0.06	-	-	-	-	-	-	0.23
Linolenic acid (C18:3n6)	26.189	-	-	-	-	-	-	0.11	-	-	1.41	0.13	-	0.43	1.46
Heneicosanoic acid (C21:0)	26.765	0.43	-	-	-	-	-	0.13	-	-	1.97	0.17	-	0.26	0.61
***cis*-11,14-eicosadienoic acid (C20:2)**	27.752	1.10	-	-	-	0.69	-	0.64	-	-	4.38	0.92	1.95	1.11	1.03
Behenic acid (C22:0)	27.896	-	-	-	-	-	-	-	-	-	-	0.20	-	0.30	0.42
Arachidonic acid (C20:4n6)	29.198	0.53	-	-	-	-	-	0.10	-	-	1.60	0.11	-	0.21	0.47
Tricosanoic acid (C23:0)	29.683	0.52	-	-	-	0.21	-	0.14	-	-	2.55	-	-	0.06	0.90
Lignoceric acid (C24:0)	30.475	0.66	-	-	-	-	-	0.31	-	-	-	-	-	-	0.25
***cis*-13,16-docosadienoic acid (C22:2)**	29.73	-	-	-	-	-	-	-	-	-	-	0.19	-	0.24	0.67
***cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (C20:5n3) EPA**	30.771	0.58	-	-	-	0.39	-	0.27	-	-	2.01	0.51	0.91	0.56	0.48
Nervonic aid (C24:1)	32.315	0.48	-	-	-	0.26	-	0.08	-	-	-	0.28	-	0.57	0.21
***cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6n3) DHA**	33.391	1.46	-	-	-	2.29	-	1.40	-	-	-	1.88	1.43	1.88	0.72
USFA (%)		46.61	74.53	65.50	79.26	68.07	76.66	77.35	52.04	55.46	37.39	76.34	51.85	76.75	18.81
TSFA (%)		50.53	24.35	33.2	20.06	26.35	21.94	21.1	25.9	43.05	62.61	23.42	48.15	22.66	19.01
MUFA (%)		18.4	8.67	8.2	10.83	18.32	8.06	13.85	6.25	6.11	14.07	13.99	14.72	14.93	4.15
PUFA (%)		28.21	65.86	57.3	68.43	49.75	68.6	63.4	45.79	49.35	23.32	62.22	37.13	61.63	17.83
Total (%)		97.14	98.88	98.7	99.32	94.42	98.6	98.46	77.94	98.51	100	99.76	100	99.41	4.04

AR: Aegean Region, **MR:** Marmara Region, **ESR:** Eastern and Southeastern Anatolia Region, **BSR:** Black Sea Region, **RT:** Retention Time, **USFA:** Unsaturated Fatty Acid, **TSFA:** Total Saturated Fatty Acid, **MUFA:** Monounsaturated fatty acid, **PUFA:** Polyunsaturated Fatty Acids, **SD:** Standard Deviation.

Butyric acid can be used in different industries, and there has recently been a great interest in using it as a precursor to biofuels (Dwidar *et al.,* 2012). Moreover, it has many uses in the pharmaceutical and chemical industries. It was well known for its anticancer effects and it induces morphological and biochemical changes in various cells leading to the concomitant suppression of neoplastic properties (Cao *et al.,* 2011). Butyric acid was found between 0.33-64.98% in all the tobaccos. The highest butyric acid was found for ESR-6 (64.98%), followed by the MR-5 (58.61%) and BSR-7 (35.54%) genotypes. The lowest butyric acid was found in ESR-3 with 0.33%. ESR-6, MR-5 and BSR-7 genotypes can be cultivated to obtain high butyric acid contents for use in biofuel.

A correlation was seen between butyric acid and linoleic acid. When linoleic acid was not detected, such as in ESR-6 and MR-5, butyric acid was found at the highest levels in these genotypes. Elaidic and cis-11, 14 heneicosanoic acids were the minor fatty acids in these tobaccos, constituting 0.04-7.22 % and 0.04-1.97%, respectively. Linoleic acid and butyric acid were found at the highest FA rates among the others at 54.0% and 18.57%, respectively.

The FA of tobacco seed crude oil can be divided into two main components as saturated fatty acids (SFA) and unsaturated fatty acids (USFA). The total SFA and USFA in tobacco seed oil were found to be 33.65 and 64.26%, respectively. While the 11 FA compositions were found as SFA, 14 FA compositions were determined as USFA. SFA compositions varied from 11.51 to 88.84% in ESR-3 and ESR-6 from the ESR genotypes. The USFA compositions were found between 11.16-87.98% in ESR-3 and ESR-6 in the ESR genotypes. Among the USFA, linoleic acid (54.0%) and oleic acid (9.52%) were the most abundant while palmitic and butyric acids (18.71%) were most abundant among the SFA (Table 5).

The total FA compositions ranged from 77.94-100% in the tobacco genotypes and cultivar. The highest total FA compositions were found in the ESR-1 and BSR-8 genotypes (100%), followed by MR-5, ESR-6 and BSR-10 with 99.99%. The lowest total FA compositions were seen in BSR-6 (77.94%), followed by the BSR-3 (94.42%) and MR-2 (94.69%) genotypes.

The amounts of unsaturated and saturated fatty acids reported in the present work are close to those reported as 73.95-26.1% (Zalatanov *et al.,* 2000) and 85.2 and 14.8% (Giannelos *et al.,* 2002), respectively.

3.6. Genetic diversity of tobacco cultivar and genotypes

Dendrogram analyses were carried out among the chemical components as NC, RSC, SCO and 25 FACs in two different figures in 29 tobacco genotypes and 1 cultivar. The dendrogram analyses were divided into two main groups in terms of NC, RSC, SCO and FAC as A and B (Figures 1a and b). All figures consisted of 2 sub-groups as A1 and B1. While group A was formed of 16 genotypes and one cultivar, group B contained 13 genotypes. Sub-group A1 comprised 11. including cultivar (AR-1), 4 ESR, 4 BSR, 1 MR and 1 AR genotypes. Sub-group A2 included 6 genotypes (5 ESR, 5 BSR, 2 AR and 1 MR genotypes). 3 genotypes fell into sub-group B1 (ESR 4, ESR 5 and BSR 5) and sub-group B2 had 10 genotypes as 4 BSR, 3 ESR, 2 AR and 1 AR genotypes (Figure 1a). When figure 1b was examined, it was seen that the FACs of the tobacco cultivar and genotypes were divided into four sub-groups as A1, A2, B1 and B2. Sub-group A1 and B1 had only one FAC as lignoceric acid and arachidic acid, respectively. While group A2 had 13 FACs including one of the major acids, butyric and linoleic acid; B2 had FACs. Figures 1a and 1b were evaluated together and the major FACS were determined in group A and B1 subgroup and minor FACs were observed in B2 in Figure 1b was formed based on linoleic acid. Most of the saturated acids were found in group A and unsaturated acids were detected in group B in Figure 1b.

4. CONCLUSIONS

This study focused on the chemical components of 29 tobacco genotypes and 1 cultivar as nicotine, sugar, crude oil, fatty acid composition and ion content. The cultivar ‘AR-1’ was found to be higher than the other genotypes in terms of NC, RSC and SCO. Among the genotypes, ESR-4, ESR-5 and BSR-5 had better results for the examined properties except for IC. IC as K^+ was found in high contents in the genotypes, especially the BSR-5 genotype. This genotype can be characterized as having high quality because high K^+ and low Cl^- contents in tobaccos have a positive effect. Generally, the BSR-5 genotype was determined to have high quality in terms of the chemical components of tobacco genotypes. From the quality perspective, tobacco seed fatty

acid compositions classified as butyric, oleic, palmitic and linoleic acid were found as the major components in the tobacco genotypes and cultivar. They can be used in paint industries and cosmetics as potential raw materials. For human use, the highest values in terms of butyric and linoleic acid were found in ESR-6 and ESR-3. Hence, this study would be helpful for tobacco producers and users to know the chemical contents in tobacco products and to determine the chemical contents in tobacco genotypes and cultivar.

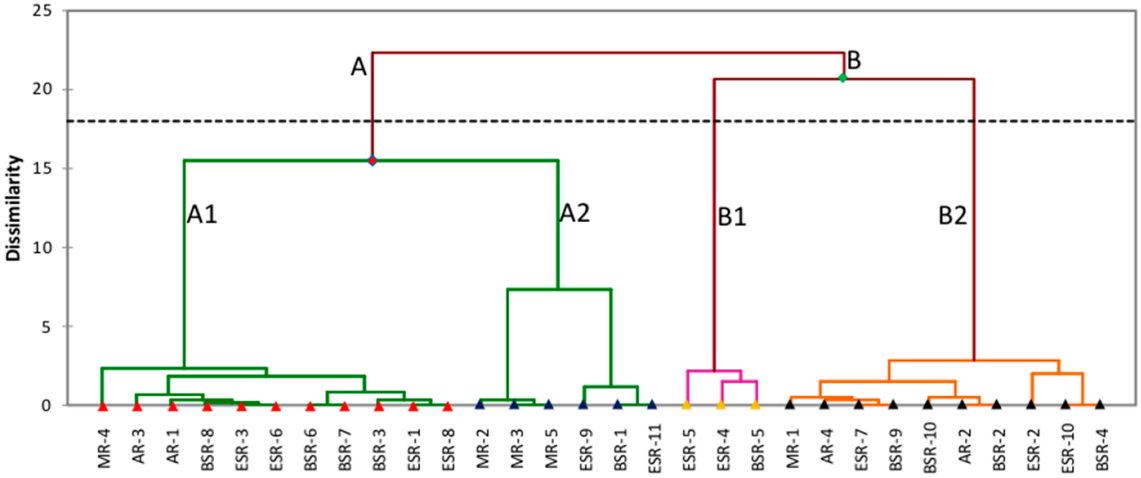

FIGURE 1a. AR: Aegean Region, MR: Marmara Region, ESR: Eastern and Southeastern Anatolia Region, BSR: Black Sea Region.

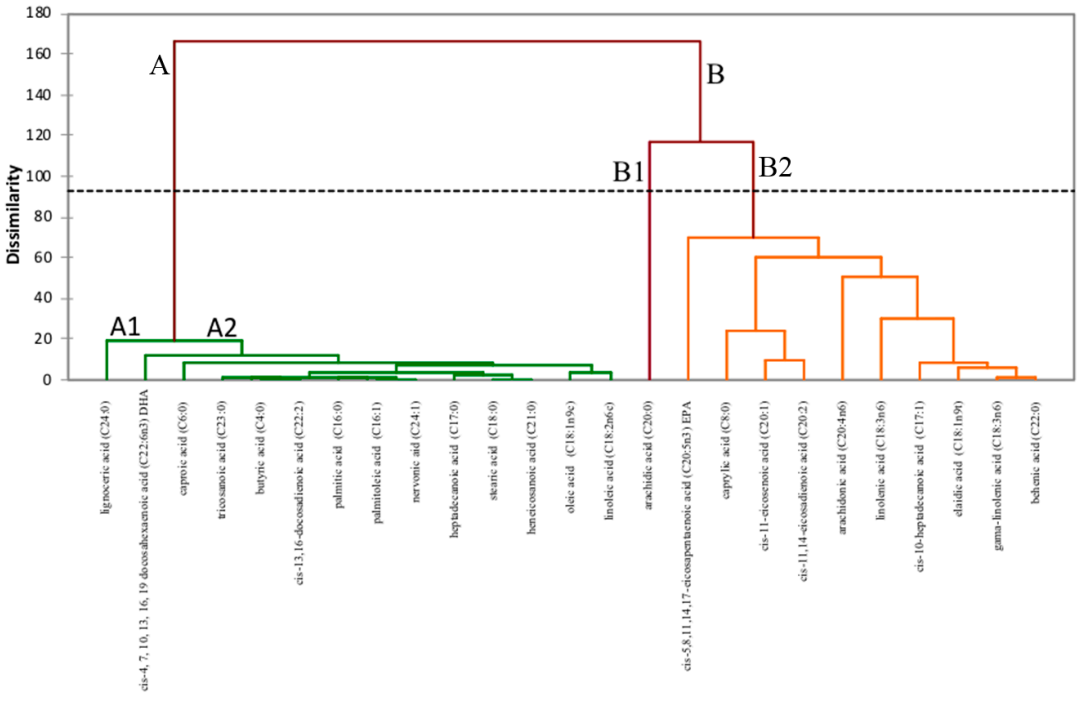

FIGURE 1b

FIGURE 1. Dendrogram analysis of tobacco cultivar and genotypes based on nicotine, reducing sugar contents and crude oil (Figure 1a) and fatty acid compositions (Figure 1b)

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REFERENCES

Abbas Ali M, Abu Sayeed M, Roy RK. 2008. Comparative study of on characteristics of Seeds oils and Nutritional composition of seeds from different varieties of tobacco cultivated in Bangladesh. *Asian J. Biochem.* **3** (4), 203-212. https://doi.org/10.3923/ajb.2008.203.212

Abdallah F. 1986. Is Tobacco Quality Measurable? Trans. K. Ketenci. Monopoly Institute Istanbul.

ARGEFAR. 2016. Center for Drug Research & Development and Pharmaceutical Applications, Ege University. https://argefar.ege.edu.tr/eng-3641/.html, 21.08.2018.

Awola VG, Ogunniyi DS, Odetoye TE. 2010. Refining, modification and characterization of tobacco seed oil for improved potentials for industrial use. *Nigerian J. Pure Appl. Sci.* 2168-2174.

Bastida G, Beltran B. 2011. Ulcerative colitis in smokers, non-smokers and ex- smokers. *World J. Gastroenterol.* **17**, 2740-2747. https://doi.org/10.3748/wjg.v17.i22.2740

Bilgin AE, Müftüoğlu Y, Ustralı A. 1993. Calibration of Commercial Tobacco Requirements and Phosphorus-Potash Analysis Methods of Oriental Tobacco Under Field Conditions in Aegean Region. T. C. Ministry of Agriculture and Rural Affairs General Directorate of Rural Services Menemen Research Institute Publications. Publication No: 1995, İzmir. 1993.

Bucciarelli S, Civitella D, Fecondo G, Ghiann G, Sorrentino C, Del Piano L, Raimo F, Stanisci V. 2013. Influence of plant density and leaf priming on seed and seed oil yield tobacco. Proc. CORESTA Meeting, Agronomy/ Phytopathology, Brufa di Torgiano (PG), Italy.

Cao Y, Li HQ, Zhang J. 2011. Homogeneous synthesis and characterization of cellulose acetate butyrate (CAB) in 1-Allyl-3-methylimidazolium chloride (AmimCl) ionic liquid. *Indust. Engineer. Chem. Res.* **50** (13), 7808-7814. https://doi.org/10.1021/ie2004362

Camas N, Karaali H, Ozcan H. 2007. Effects of different fertilizer doses on yield, quality and technological properties of pressed tobacco genotype in Erbaa-Taşova Conditions. 2007 TTL Foreign Trade. Research Report.

Chiririwa H, Hapanyengwi A, Muzenda E. 2014. Tobacco seed oil as an economically viable alternative for the tobacco ındustry. International Conference on Chemical Engineering and Advanced Computational Technologies, November, 24-25, 36-39. https://doi.org/10.15242/IIE.E1114017

Delibacak S, Ongun AR, Ekren S. 2014. Influence of soil properties on yield and quality of tobacco plant in Akhisar region of Turkey. *Eurasian J. Soil Sci.* **3**, 286-292.

Dwidar M, Park JY, Mitchell RJ, Sang B. 2012. The future of butyric acid in ındustry. *Scient. World J.* 1-9. https://doi.org/10.1100/2012/471417

Ekren S, Sekin S. 2008. Investigation of chemical and expertise characteristics of Akhisar region tobaccos and relations with the leaf yield. *Ege J. Agric. Res.* **45** (3), 165-173.

Er C, Yıldız M. 2014. Pleasure plant. Ankara University Publications, No: 419.

Fornasier F, Gomez JFC, de Carvalho FS, Schneider RdCdS, Costa ABd, Moraes JAR, Bravo dCAG. 2018. Biodiesel production from energy tobacco. *Orbital: Electronic J. Chem.* **10** (2), 123-132.

Giannelos PN, Zannikos F, Stourna S, Lois E, Anastopoulos G. 2002. Tobacco seed oil as an alternative diesel fuel: physical and chemical properties. *Ind. Crops Prod.* **16**, 1-9. https://doi.org/10.1016/S0926-6690(02)00002-X

Griesser M, Weingart G, Schoedl-Hummel K, Neumann N, Becker M, Varmuza K, Liebner F, Schuhmacher R, Forneck A. 2015. Severe drought stress is affecting selected primary metabolites, polyphenols, and volatile metabolites in grapevine leaves (*Vitis vinifera* cv. Pinot noir). *Plant. Physiol. Biochem.* **88**, 17-26. https://doi.org/10.1016/j.plaphy.2015.01.004

Irget ME, Oktay M, Hakerlerler H, Atil H, Cakici H. 1999. A research on the nutrition and soil-plant relationships of Virginia (flue-cured) tobacco grown in Düzce. *J. AARI* **9** (2), 125-142.

Kirkova S, Srbinoska M, Ivanova S, Georgieva A. 2016. Determination of fatty acid composition

of seed of oriental tobacco. *Tobacco* **66** (1-6), 53-58. https://doi.org/10.13140/RG.2.2.18885.14560

Krishnamurthy V, Ramakrishnayya BV. 1993. Yield and quality of fcv tobacco as affected by potassium nutrition. pp. 78-102. In: Plant nutrition effects on production and quality of tobacco. Potash and Phosphate Inst. of Canada. India Programme Sector-19, Haryana.

Kurt D, Ayan AK. 2014. Effect of the different organic fertilizer sources and doses on yield in organic tobacco (*Nicotiana tabacum* L.) production. *J. Agric. Faculty Gaziosmanpasa Univer.* **31** (2), 7-14.

Mohammad MT, Tahir NA. 2014. Evaluation of chemical compositions of tobacco (*Nicotiana tabacum* L) genotypes. *Seeds Annual Res. Review Biol.* **4** (9), 1480-1489. https://doi.org/10.9734/ARRB/2014/8211

Otan H, Apti R. 1989. Tobacco. T.C. T.O.K.İ.B. Ege Agricultural Research Institute Publications, No: 83. Menemen-İzmir.

Palomer X, Pizarro-Delgado J, Barroso E, Vázquez-Carrera M. 2018. Palmitic and oleic acid: The yin and yang of fatty acids in type 2 diabetes mellitus. *Trends Endocrinol. Metabol.* **29** (3), 178-190. https://doi.org/10.1016/j.tem.2017.11.009

Piano L del, Abet M, Raimo F, Modestia F, Sicignano M, Enotrio T. 2014b. Caraterizzazione morfologica e qualitativa di accessioni di *Nicotiana tabacum* L. per la produzione di olio da seme. Proc. X National Congress on Biodiversity, Rome, Italy.

Poltronieri P. 2016. Tobacco seed oil for biofuels. Pp. 161-185. In: Poltronieri P and D'Urso O.F. Biotransformation of Agricultural Waste and By-Products. The Food, Feed, Fibre, Fuel (4F) Economy. Elsevier, Oxford, UK. 2016. ISBN: 9780128036228. https://doi.org/10.1016/B978-0-12-803622-8.00006-9

Regassa R, Chandravanshi BS. 2016. Levels of heavy metals in the raw and processed Ethiopian tobacco leaves. *Springer Plus* **5**, 232 https://doi.org/10.1186/s40064-016-1770-z

Stanisavljević IT, Veličković DT, Todorović ZB, Lazić ML, Veljković VB. 2009. Comparison of techniques for extraction of tobacco seed oil. *Europ. J. Lipid Sci. Technol.* **111** (5), 513-518. https://doi.org/10.1002/ejlt.200800232

Talaqani TE, Shafik J, Mustafa FK. 1986. Fatty acid composition of the seed oil of certain tobacco varieties cultivated in Northern Iraq. *Indian J. Agric. Chem.* **19**, 147-154.

TEA. 2018. Tobacco Experts Association. Chemical structure of tobacco leaf in terms of blending. http://www.tutuneksper.org.tr, 21.08.2018.

Wang M, Fu Y, Liu H. 2016. Nutritional quality and ions uptake to PTNDS in soybeans. *Food Chem.* **192**, 750-759. https://doi.org/10.1016/j.foodchem.2015.07.002

Yagac C. 2015. Determination of yield and quality characters of tobacco cultivars of Aegean Region in Denizli conditions. MsC Namık Kemal University Graduate School of Natural and Applied Sciences Department of Field Crops.

Yaldiz G, Çamlica M. 2019. Variation in the fruit phytochemical and mineral composition, and phenolic content and antioxidant activity of the fruit extracts of different fennel (*Foeniculum vulgare* L.) genotypes. *Ind. Crops Prod.* **142**, 111852. https://doi.org/10.1016/j.indcrop.2019.111852

Yaldiz G, Camlica M, Ozen F. 2019. Biological value and chemical components of essential oils of sweet basil (*Ocimum basilicum* L.) grown with organic fertilization sources. *J. Sci. Food Agric.* **99**, 2005-2013. https://doi.org/10.1002/jsfa.9468

Zalatanov M, Menkov N. 2000. Phospholipid and fatty acid composition of tobacco seeds. *Rostlinna Vyroba* **46**, 439-441.

Zandalinas SI, Mittler R, Balfagón D, Arbona V, Gómez-Cadenas A. 2018. Plant adaptations to the combination of drought and high temperatures. *Physiol. Plant.* **162**, 2-12. https://doi.org/10.1111/ppl.12540

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Common Kilka oil and its primary and secondary oxidative dynamics stabilized by different variants of clove essential oil

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SUMMARY: The objective of this study was to investigate the properties of clove essential oil extracted by different microwave-assisted methods and to evaluate its effects on the stability of common Kilka oil. Each of these methods was hypothesized to yield a clove essential oil that would have a distinguishable composition and effect when added to common Kilka oil by maintaining its oxidative stability. The oxidation of common Kilka oil was examined by accelerated oxidation using the active oxygen method and Rancimat test. The clove essential oil extracted by microwave-assisted hydrodistillation showed the highest induction period according to the active oxygen method (16.56 h) and the Rancimat induction period (3.64 h) in common Kilka oil and its antioxidant activity was comparable to that of BHT (16.59 h and 4.34 h, respectively) and tocopheryl acetate (16.30 h and 4.02 h, respectively). Furthermore, the microwave-assisted hydrodistillation method resulted in the amount of eugenol that exhibited the highest antioxidant capacity for preserving PUFA in common Kilka oil. Ultimately, clove essential oil can become an efficient natural antioxidant for the oxidative stability of common Kilka oil.

KEYWORDS: *Antioxidant activity; Clove essential oil; Common Kilka oil; Microwave; Oxidation*

RESUMEN: ***Aceite de Kilka común y su dinámica oxidativa primaria y secundaria estabilizada por diferentes variantes de aceite esencial de clavo.*** El objetivo de este estudio fue investigar las propiedades de los aceites esenciales de clavo que se extrajeron utilizando diferentes métodos asistidos por microondas y evaluar los efectos de estos aceites esenciales en la estabilidad del aceite de Kilka común. Se hipotetizó que cada uno de los métodos produce un aceite esencial de clavo que tendría una composición y un efecto distintivo cuando se agrega al aceite de Kilka común manteniendo su estabilidad oxidativa. La oxidación del aceite de Kilka común se determinó mediante oxidación acelerada utilizando el método de oxígeno activo y Rancimat. El aceite esencial de clavo extraído por hidrodestilación asistida por microondas logró en el aceite de Kilka común un período de inducción, mediante el método de oxígeno activo, más alto (16,56 h) y un período de inducción mediante Rancimat de 3,64 h y su actividad antioxidante fue comparable a la del BHT (16,59 h y 4,34 h, respectivamente) y a la del acetato de tocoferol (16,30 h y 4,02 h, respectivamente). Además, el método de hidrodestilación asistido por microondas influyó en la cantidad de eugenol que presentó una mayor capacidad antioxidante para preservar los PUFAs del aceite de Kilka común. Por último, el aceite esencial de clavo puede convertirse en un antioxidante natural eficiente para la estabilidad oxidativa del aceite de Kilka común.

PALABRAS CLAVE: *Aceite de Kilka común; Aceite esencial de clavo; Actividad antioxidante; Microondas; Oxidación*

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1. INTRODUCTION

The common Kilka (*Cluponella cultriventris*), the anchovy (*C. engraulifermis*), and the large-eyed Kilka (*C. grimmi*) are the most important fish in the Caspian Sea. Common Kilka is the most abundant species in the southern Caspian, comprising 97% of the total catch of clupeids there (Jorjani, 2014). It has been reported that fish oil has many health benefits such as reducing the risk of inflammatory and cardiovascular diseases (Kromhout *et al*., 2011; Wall *et al*., 2010). The quality of fish oil is primarily due to the presence of long-chain ω-3 polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Wang *et al*., 2011). However, unsaturated ω-3 PUFAs are highly prone to oxidation, leading to the development of free radicals, reactive aldehydes, and off-flavors, which ultimately reduce the shelf-life of fish oil (Jacobsen *et al*., 2008). Therefore, the use of potent antioxidants in fish oil is required in order to reduce oxidation. To that end, there is a considerable amount of focus on exploiting antioxidant compounds which can be found in medicinal plants (Golmakani *et al*., 2017a; Golmakani *et al*., 2018; Shahbazi *et al*., 2018).

Clove (*Syzygium aromaticum*) is a member of the *Myrtaceae* family and an important aromatic spice. The majority of compounds found in clove essential oil (CEO) are grouped as phenyl-propanoids, such as eugenol and eugenyl acetate, and sesquiterpenes such as β-caryophyllene and α-humulene (Chaieb *et al*., 2007; Guan *et al*., 2007). In this context, Gülçin (2011) reported that eugenol is capable of exhibiting a high antioxidant activity in a linoleic acid emulsion system. Furthermore, eugenol has a significant reducing power, can scavenge reactive oxygen species and reduce lipid peroxidation (Ogata *et al*., 2000). In addition, β-caryophyllene is known to have high radical scavenging activity (Mishra *et al*., 2013).

Hydrodistillation (HD) and steam distillation (SD) are two conventional methods for essential oil extraction. These methods are generally time consuming, degrade thermolabile compounds, and require large amounts of solvents for their performance (Wang and Weller, 2006). Recently, microwave-assisted extraction methods have become popular and more convenient compared to conventional extraction procedures (Mazidi *et al*., 2012). The variants of microwave-assisted extraction such as microwave-assisted hydro-distillation (MAHD) and microwave-assisted steam distillation (MASD) have proven to be successful methods for the extraction of essential oil (Chemat *et al*., 2012). It has been reported that the extraction time of essential oil in microwave-assisted extraction methods is shorter than the conventional methods. Furthermore, the vigor of radical scavenging activities, staged by the extracted essential oils, remains quite untouched when using the microwave radiation techniques (Mazidi *et al*., 2012).

So far, many studies have focused on the antioxidant activity of different herbs and spices in edible oils. For instance, Olmedo *et al*., (2018) reported that *Aloysia triphylla* and *Minthostachys mollis* essential oils were effective in reducing sunflower oil oxidation. Golmakani *et al*., (2018) showed that common Kilka oil (CKO) oxidation can be reduced by *Ocimum sanctum* essential oil nearly as much as it can be reduced by the synthetic BHA. One report claimed that *Zataria multiflora* essential oil can reduce the oxidation of virgin olive oil to the same extent that BHT can. *Zataria multiflora* essential oil proved to be more effective than β-carotene (Golmakani *et al*., 2017a).

This study was designed to evaluate the quality of CEO when extracted by different methods (i.e. HD, SD, MAHD, and MASD) and to assess its effects on the oxidative stability of CKO in comparison to the effects produced by synthetic BHT, natural α-tocopherol and β-carotene, and semi-natural tocopheryl acetate, which are all considered to be effective antioxidants. The assessments were performed with the help of Rancimat and AOM (active oxygen method). In addition, changes that may occur in the fatty acid profile of CKO samples were investigated during accelerated storage.

2. MATERIALS AND METHODS

2.1. Materials

Dried clove buds (*Syzygium aromaticum*) were purchased from a local market, Shiraz, Iran. The genus and species of the plant were confirmed by elite taxonomists from the Herbarium of Biology Department, Shiraz University, Shiraz, Iran. The CKO was provided by the Pars Kilka Company (Babolsar, Iran). The α-Tocopherol, β-carotene, tocopheryl acetate, and BHT were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany).

2.2. CEO extraction

Four different extraction methods were used in order to extract the CEO: two conventional methods (HD and SD) and two relatively new methods (MAHD and MASD). Briefly, 40 g of the dried clove buds were processed by HD with a Clevenger-type apparatus. The extraction was performed in the presence of water (400 mL) and took 4 h. The ratio of clove-to-water was 1:10 (w/w). The SD method was similar to HD, but the clove was placed in a glass column, of which the bottom and top parts were connected to a water flask and a condenser, respectively. The structures of MAHD and MASD were similar to HD and SD, respectively. However, a domestic microwave oven (ME3410W, Samsung, Malaysia) was used for MAHD and MASD instead of the Electromantle heater of HD and SD. The microwave operated at a frequency of 2.45 GHz with a maximum output power of 1000 W for 80 min. The extracted CEOs were then dehydrated with anhydrous sodium sulfate, collected in amber-colored vials, and were kept at -18 °C until further experiments were performed (Golmakani and Moayyedi, 2015; Golmakani *et al*., 2017b).

2.3. Gas chromatography analysis of CEOs

The chemical composition of CEO was determined by a gas chromatography system (Agilent technologies 7890A, Santa Clara, CA) coupled to a mass spectrometer (Agilent Technologies 5975C, Santa Clara, CA), operating at 70 eV ionization energy, 0.5 s/scan, and at the mass range of 35-400 amu. The HP-5MS capillary column was characterized by 5% Phenyl Polysilphenylene-siloxane, 30m length, 0.25mm internal diameter and 0.25 µm film thickness (Agilent Technologies, Santa Clara, CA) (Golmakani *et al*., 2017a).

2.4. Physicochemical properties and fatty acid composition of CKO

2.4.1. Fatty acid composition by gas chromatography

To determine fatty acid profiles, a gas chromatography/flame ionization detector (B420A, BEIFEN, China) was used. The film thickness, length and inner diameter of the BPX70 GC column were 0.25 mm, 60 m, and 0.25 mm, respectively. The stationary and carrier phases were Polymer Biscyanopropylsiloxane silphenylene and nitrogen gas, respectively (Keramat and Golmakani, 2016).

2.4.2. Peroxide value (PV)

The PV was quantified according to the Official Method (Cd 8-53) of American Oil Chemists' Society and was expressed as meq O_2 per kg oil (AOCS, 2000).

2.4.3. Free fatty acid content

Reported as a percentage of oleic acid, the free fatty acid content was measured according to the AOCS official method (Cd 3d-63) (AOCS, 2000).

2.4.4. Density measurement

The density of CKO was measured following the AOCS official method (Cc10a- 25) whereby a pycnometer was used (AOCS, 2000).

2.4.5. Solid fat content (SFC)

Measuring the CKO was assisted by Nuclear Magnetic Resonance (NMR, Bruker, NMF 100, Karlsruhe, Germany). Prior to NMR analysis, the samples were melted at 100 °C and then cooled to 80 °C for 5 min. After that, the temperature was decreased to 60 °C and held for 5 min. Finally, the samples were rapidly cooled to 0 °C and t held at this temperature for 1 h. The samples were stabilized at 0, 5, 10, 20, 25, and 30 °C and were kept for 30 min at each temperature prior to NMR analysis *(Nejadmansouri et al*., 2016).

2.4.6. Refractive index

The refractive index of CKO was determined using a refractometer (RX7000a; Atago, Japan) at 20 °C.

2.5. Oxidative stability of CKO

CEOs were added to the CKO at 1000 ppm concentration. The β-carotene, α-tocopherol, tocopheryl acetate and BHT were added to the CKO at 100 ppm concentration. For the control, a pure sample was used without any antioxidant.

2.5.1. Active oxygen method (AOM)

The AOM analysis was carried out according to the AOCS official method (Cd 12-57). Briefly, 20 mL portions of CKOs were poured into the reaction tube and placed in a constant-temperature heater which kept the temperature constant at 97.8 ± 0.2 °C. The total flow rate was adjusted to 2.33 mL/min for each tube. The AOM induction period (IP) (i.e. the time required to reach a PV of 100 meq O_2/kg oil) was calculated according to the standard method of AOM (AOCS, 2000).

The protection factor (PF) was calculated according to eq. (1).

$$PF=\frac{\text{AOM IP}_a}{\text{AOM IP}_c} \quad \text{eq. (1)}$$

Where AOM IP_a is the AOM induction period (IP) of the CKO samples containing antioxidants (BHT, β-carotene, α-tocopherol, tocopheryl acetate and CEO) and AOM IP_c is the AOM IP of the control sample (Hraš *et al*., 2000).

2.5.2. Rancimat method

The oxidative stability was estimated by measuring the Rancimat IP, using a rancimat 743 apparatus (Metrohm, Switzerland) according to the AOCS method (Cd 12b-92). The tests were carried out with 3.0 ± 0.1 g of CKO. All samples were studied at 80 °C. The temperature of the conductivity tube was kept constant at 21 °C and the air flow rates of motion were set at 20 L/h for each experiment. The Rancimat IP was printed automatically by the apparatus software with a precision of two decimals (AOCS, 2000).

2.6. Statistical analysis

All experiments were performed in triplicate. The results were reported as the mean value ± standard deviation. Analysis of variance (ANOVA) was performed using the SPSS software (ver. 22, IBM, New York, NY), and the Duncan's multiple range test was used so as to compare the data with the mean values. A *P*-value of < 0.05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Gas chromatography analysis of CEO

The chemical compositions of the CEOs extracted by HD, SD, MAHD, and MASD showed variations (Table 1). Even though there were similarities among the compositions of the CEOs extracted by the different methods, the relative concentrations of the identified compounds were apparently different.

According to Table 1, phenylpropanoids such as eugenol (78.44-88.14%) and eugenyl acetate (8.11-18.26%) were the main components of the CEOs. Vanin *et al*., (2014) reported that eugenol and eugenyl acetate are capable of exhibiting high antioxidant activity. Also, Mishra *et al*., (2013) reported that the antioxidant activity of eugenol tends to be higher than that of other phenolic compounds in essential oil - compounds such as thymol and carvacrol.

In the current study, the CEOs obtained by the SD-based methods contained lower percentages of eugenol, and higher amounts of eugenyl acetate. The CEOs obtained by HD-based methods had higher amounts of eugenol and lower eugenyl acetate. The eugenol/eugenyl acetate ratios in CEOs obtained by the HD and MAHD methods (8.59 and 10.87, respectively) were higher than those obtained by the SD and MASD methods (4.56 and 4.54, respectively). This may be due to the hydrolysis of eugenyl acetate and its conversion to eugenol when HD-based methods were employed (Golmakani *et al*., 2017b). The CEO obtained by MAHD contained the highest percentage of eugenol (88.14%). Similar to our findings, Golmakani *et al*., (2017b) reported that MAHD is capable of extracting CEO with the highest percentage of eugenol, whereas the CEO extracted by the SD reportedly contained the lowest percentage of eugenol.

3.2. Physicochemical properties and fatty acid composition of CKO

According to Table 2, the values relating to the refractive index, density, PV, and acid content of CKO were 1.4711, 0.934 g/mL, 2.02 meq O_2/kg, and 1.134 mg KOH/g, respectively, at the beginning of the experiment. The PV and acid value were lower than the maximum permitted level recommended by Codex (10 meq O_2/kg for PV and 4 mg KOH/g for acid value) (Codex Alimentarius, 2015).

The SFC of CKO decreased from 4.29 to 0%. This decrease occurred parallel to the increase in temperature from 0 to 30 °C. Nejadmansouri *et al*., (2016) also reported that the SFC of fish oil decreased from 11.96 to 2.48% by increasing the temperature from 0 to 25 °C.

TABLE 1. Chemical composition of clove essential oil obtained by different extraction methods

No.	Compound	Retention time (min)	Retention index	Releative peak area (%)			
				HD*	SD	MAHD	MASD
1	Benzaldehyde	7.5	893	0.07±0.02^{a}	0.03±0.00^{a}**	0.05±0.02^{a}	0.05±0.03^{a}
2	2-Nonanone	11.5	988	0.04±0.00^{b}	0.03±0.02^{b}	0.08±0.02^{a}	0.03±0.00^{b}
3	Benzyl acetate	13.1	1087	0.05±0.01^{ab}	0.06±0.01^{ab}	0.10±0.03^{a}	0.04±0.00^{b}
4	Ethyl benzoate	13.6	1084	0.17±0.00^{b}	0.28±0.01^{a}	0.19±0.04^{b}	0.26±0.01^{a}
5	Methyl salicylate	16.7	1082	0.07±0.00^{a}	0.05±0.01^{a}	0.07±0.01^{a}	0.06±0.01^{a}
6	Chavicol	18.9	1179	0.03±0.01^{ab}	0.07±0.03^{a}	0.02±0.01^{b}	0.01±0.01^{b}
7	Eugenol	22.6	1276	86.49±1.94^{a}	78.56±0.84^{b}	88.14±2.8^{a}	78.44±0.59^{b}
8	(E)-Caryophyllene	25.1	1374	1.97±0.53^{a}	2.40±0.19^{a}	2.33±1.05^{a}	1.97±0.70^{a}
9	α-Humulene	26.1	1373	0.84±0.32^{a}	0.96±0.08^{a}	0.76±0.47^{a}	0.67±0.32^{a}
10	Eugenyl acetate	28.3	1470	10.07±1.13^{b}	17.24±0.62^{a}	8.11±1.18^{b}	18.26±0.53^{a}
11	Caryophyllene oxide	31.0	1467	0.09±0.03^{b}	0.17±0.02^{a}	0.07±0.02^{b}	0.14±0.93^{ab}
12	Benzyl benzoate	36.7	1692	0.10±0.04^{a}	0.14±0.02^{a}	0.07±0.02^{a}	0.06±0.04^{a}
Eugenol/Eugenyl acetate				8.59	4.56	10.87	4.54

*HD, hydro-distillation; MAHD, microwave-assisted hydro-distillation; SD, steam-distillation; MASD, Microwave- assisted steam-distillation.
**Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's test; Degree of significance: $P < 0.05$

The fatty acid composition of CKO is presented in Table 2. The major SFA and MUFA were palmitic acid and oleic acid, respectively. DHA was the most abundant PUFA and EPA was the second major PUFA in CKO. Our results on the major fatty acids of CKO are in agreement with previous reports (Golmakani *et al*., 2017a; Hosseini *et al*., 2019a). Here, the PUFA/SFA ratio in the CKO (0.99) was higher than the minimum level recommended by the UK Department of Health (0.45) (HMSO, 1994) which indicates that CKO is highly susceptible to deterioration by oxidation. The ω-3/ω-6 ratio in CKO was 10.99. Similarly, Hosseini *et al*., (2018) reported that the ω-3/ω-6 ratio in CKO was 7.62.

3.3. Oxidative stability of CKO

3.3.1. Active oxygen method (AOM).

The PVs of CKO samples were worthy of documentation during accelerated storage (Figure 1). The PVs of all CKO samples increased at the early stages of the storage period. Even though the PV of the control group began to decrease after 12 h, the PVs of the samples containing antioxidants began to decrease after 18 h. The PV of the control sample increased much faster and to a higher level than that of the CKO samples as they contained antioxidants and reached 107.93 meq O_2/kg after 12 h. In going beyond the mentioned time, and especially after 18 h, the PVs of samples which contained CEOs extracted by HD, SD, MAHD and MASD reached 89.94, 82.18, 80.20, and 88.45 meq O_2/kg, respectively (Figure 1a). This indicated that the CEOs can delay the rate of hydroperoxide formation. The CEO extracted by MAHD functioned more effectively in reducing the PV of CKO, compared to the function of CEOs extracted by the HD, SD and MASD methods ($P < 0.05$). This may be attributed to the presence of higher amounts of eugenol in the CEO extracted by the MAHD method. Also, Golmakani *et al*., (2017b) reported that a higher antioxidant activity of CEO extracted by HD-based methods is a result of higher eugenol contents. The antioxidant activity of phenolic compounds comes from their chemical structure and reducing functions. They quench the singlet oxygen, neutralize free radicals and chelate transitional metals (Hosseini *et al*., 2019b). It has been reported that eugenol can prevent the creation of iron and OH radicals, two known chemical agents which contribute to lipid peroxidation (Nagababu *et al*., 2010).

TABLE 2. Physicochemical properties and fatty acid profile of common Kilka oil

Characteristic	Amount
Refractive index (20 °C)	1.4711±0.0001*
Density (20 °C)	0.934±0.004
Peroxide value (meq O_2/kg oil)	2.02±0.08
Acid value (mg KOH/g oil)	1.13±0.01
Free fatty acid (% oleic acid)	0.57±0.01
Solid fat content (%)	
at 0 °C	4.29±0.01
at 5 °C	2.48±0.01
at 10 °C	2.21±0.01
at 20 °C	1.90±0.01
at 25 °C	1.29±0.01
at 30 °C	0.00±0.00
Fatty acid (%)	
Myristic acid (C14:0)	4.86±0.07
Palmitic acid (C16:0)	20.39±0.05
Palmitoleic acid (C16:1 ω-7)	6.95±0.01
Stearic acid (C18:0)	3.78±0.04
Oleic acid (C18:1 ω-9 cis)	28.88±0.34
Linoleic acid (C18:2 ω-6 cis)	2.05±0.01
α-Linolenic acid (C18:3 ω-3)	2.12±0.02
Arachidic acid (C20:0)	3.16±0.03
Docosatetraenoic acid (C22:4 ω-6)	0.57±0.01
Eicosapentaenoic acid (C20:5 ω-3)	8.43±0.26
Docosapentaenoic acid (C22:5 ω-3)	0.56±0.01
Docosahexaenoic acid (C22:6 ω-3)	18.26±0.23
SFA (saturated fatty acid)	32.18
UFA (unsaturated fatty acid)	67.82
MUFA (monounsaturated fatty acid)	35.83
PUFA (polyunsaturated fatty acid)	31.99
PUFA/SFA	0.99

* Mean ± SD (n=3).

Tocopheryl acetate, α-tocopherol, and β-carotene reduced the PV of CKO to a similar extent that BHT did after 8 h of storage (Figure 1b). After 12 h, however, tocopheryl acetate, α-tocopherol, and β-carotene were less effective in their functions than BHT was. Also, Keramat *et al.*, (2017) reported that β-carotene and BHT were equally effective in reducing the PV of virgin olive oil after 21 days of storage. However, β-carotene showed a pro-oxidant activity after 42 days of storage. They concluded that the differences among the antioxidant activities of different antioxidants can be clearly determined at the latter stages of oxidation. The effect of BHT in reducing the PV of CKO was similar to that of the CEO extracted by the MAHD method. After 12 h of storage at 97.8 °C, BHT and the CEO extracted by the MAHD method reduced the PV of CKO by 31.49 and 25.69%, respectively. Furthermore, Golmakani *et al.*, (2018) reported that *O. sanctum* essential oil is able to inhibit the oxidation of CKO, quite similar to the action of BHA.

The AOM induction period and protection factor of the CKO samples are presented in Table 3. There was no significant difference ($P < 0.05$) among the AOM IP values of CKO samples containing the different variants of CEOs. Among different CEO samples, the one extracted by MAHD showed the highest PF. The PFs of CKO samples containing BHT and tocopheryl acetate showed no significant differences compared to the PF of the CEO extracted by MAHD. The AOM IPs of the samples containing the MAHD-extracted CEO, BHT, and tocopheryl acetate were 1.53, 1.54 and 1.51 times higher than that of the control sample. This indicates that the CEO extracted by MAHD can replace the function of BHT. Furthermore, α-tocopherol was observed to be less effective than tocopheryl acetate in extending the AOM IP of CKO. A better performance of tocopheryl acetate was observed, which increased the AOM IP of CKO more sharply than the action of *α*-tocopherol. This can be due to the fact that the esters of *α*-tocopherol become less susceptible to degradation at elevated temperatures. According to Wüstenberg *et al.*, (2011), the esterification of natural antioxidants can improve their chemical stability, oxidative stability, and heat tolerance, especially in systems which are characterized by the presence of lipids. Here, the AOM IPs and PFs of CKO samples containing β-carotene and *α*-tocopherol were similar to those which contained CEOs extracted by the HD, SD, and MASD methods.

3.3.2. Rancimat method

The role of any antioxidant in retarding the formation of secondary oxidation products in the CKO can be monitored by the Rancimat method.

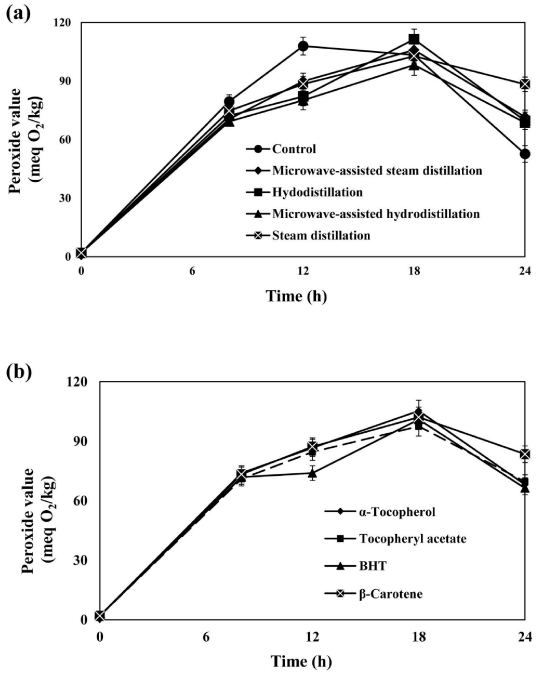

FIGURE 1. Changes in peroxide values of common Kilka oil samples containing (a) different variants of clove essential oil and (b) natural, semi-natural, and synthetic antioxidants during accelerated storage at 97.8 °C (Mean ± standard deviation; Number of replicates for each analysis: 3).

The Rancimat IP of the CKO samples was automatically recorded and taken as the breaking point of the plotted curves (i.e. the intersection point of the two extrapolated parts of the curve). Per antioxidant, the Rancimat IP values of the CKO samples varied when quantified by the Rancimat method (Figure 2). The Rancimat IP of the control sample was significantly lower (2.79 h) which can be attributed to the presence of high amounts of PUFAs in the CKO, hence the susceptibility to oxidation. All antioxidants that were used herein proved to be effective in increasing the Rancimat IP of CKO in comparison to the control. In this regard, Viuda-Martos *et al*., (2010) reported that oregano, thyme, clove, sage, and rosemary essential oils were effective in increasing the Rancimat IP of lard. Here, CKO samples that contained CEOs experienced increases in their Rancimat IP values, especially when the CEO had higher amounts of eugenol. For example, the Rancimat IP was extended by 30.11% in the case of the CKO sample enriched with the CEO extracted by MAHD (which had a eugenol content of 88.14%). On the other hand, the Rancimat IP was extended by 18.28% in the case of the sample containing the CEO extracted by MASD (which had a eugenol content of 78.44%). This shows a slower oxidation rate in samples with higher eugenol contents. The CEOs extracted by the HD and SD methods increased the Rancimat IP of CKO by 22.22 and 20.43%, respectively. BHT and tocopheryl acetate were the most effective antioxidants in reducing the production of secondary oxidation products in CKO. Accordingly, BHT and tocopheryl acetate increased the Rancimat IP of CKO by 55.55 and 44.08%, respectively. The Rancimat method revealed that the Rancimat IP values of samples containing CEO (extracted by MAHD) were significantly lower than those of samples enriched with the BHT and tocopheryl acetate. However,

TABLE 3. Oxidative stability of common Kilka oil samples during accelerated storage at 97.8 °C

Sample	Active oxygen method induction period (h)	Protection factor
Control	10.80 ± 0.56^{c*}	1.00 ± 0.00^{c}
HD**	15.56 ± 0.81^{ab}	1.44 ± 0.08^{b}
SD	15.38 ± 1.69^{ab}	1.42 ± 0.01^{b}
MAHD	16.56 ± 0.94^{a}	1.53 ± 0.01^{a}
MASD	15.59 ± 0.81^{ab}	1.44 ± 0.00^{b}
β-Carotene	15.57 ± 0.90^{ab}	1.44 ± 0.01^{b}
α-Tocopherol	15.28 ± 0.87^{ab}	1.41 ± 0.01^{b}
Tocopheryl acetate	16.30 ± 0.94^{a}	1.51 ± 0.01^{a}
BHT	16.59 ± 0.94^{a}	1.54 ± 0.01^{a}

* Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's test; Degree of significance: $P < 0.05$.

**HD, hydro-distillation; MAHD, microwave-assisted hydro-distillation; SD, steam-distillation; MASD, Microwave- assisted steam-distillation.

through the AOM assay, no significant differences were found between the AOM IP of samples containing CEO (extracted by MAHD) and those containing BHT and tocopheryl acetate. A strong and significant correlation was not found between the AOM IP values of CKO samples through the AOM and their Rancimat IP values obtained by the Rancimat method (R^2 of 0.48). These results may be related to the fact that the Rancimat method measures the changes in electrical conductivity caused by the generation of volatile compounds by thermal oxidation (Velasco *et al*., 2004). Thus, in CKO samples containing CEO, the volatile compounds in the CEO could possibly be measured as secondary oxidation products through the Rancimat method. The Rancimat method is not a reliable method for measuring the antioxidant activity of CEO.

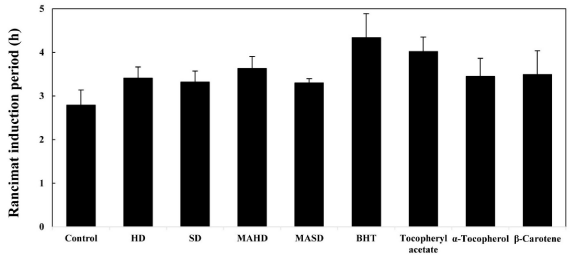

FIGURE 2. Rancimat induction period of common Kilka oil samples containing clove essential oils extracted with hydrodistillation (HD), steam distillation (SD), microwave-assisted hydrodistillation (MAHD), and microwave-assisted steam distillation (MASD) (Mean ± standard deviation; Number of replicates for each analysis: 3).

The α-tocopherol and β-carotene increased the Rancimat IP of CKO by 22.94 and 25.09%, respectively, indicating that they were less effective than BHT, tocopheryl acetate, and CEO extracted by MAHD. Similarly, a previous study showed that BHT, *Zataria multiflora* essential oil, and *Thymus vulgaris* essential oil were more effective than α-tocopherol and β-carotene in reducing virgin olive oil oxidation (Keramat and Golmakani, 2016; Keramat *et al*., 2017).

3.3.3. Changes in fatty acid composition of CKO samples

There were significant differences among the fatty acid profiles of the CKOs before AOM (untreated) and after AOM (Table 4). The PUFA content of all CKO samples, particularly EPA and DHA, decreased under accelerated oxidation conditions. Regarding the control group, the amount of PUFA in the CKO was 31.11%, but it decreased to 17.13% at the end of the accelerated storage period. Furthermore, the amounts of EPA and DHA of the control sample were reduced by 49.32 and 49.97%, respectively, in comparison to the untreated CKO. Natural and synthetic antioxidants markedly reduced the loss in PUFA in CKO samples during accelerated storage. CEOs extracted by HD, SD, MAHD, and MASD methods reduced the PUFA contents in the CKO samples by 40.63, 47.03, 29.67, and 42.46%, respectively. On the other hand, the use of BHT, tocopheryl acetate, α-tocopherol, and β-carotene in CKO samples reduced their PUFA contents by 42.14, 40.50, 39.18, and 39.15, respectively. The CEO extracted by MAHD was the most effective antioxidant, followed by β-carotene and α-tocopherol. The effects of BHT and tocopheryl acetate, as revealed by the AOM assay, were similar to the effect of CEO extracted by MAHD, and they were better than the effects of β-carotene and α-tocopherol. Nonetheless, BHT and tocopheryl acetate were less effective when compared to the CEO extracted by MAHD, and also when compared to β-carotene and α-tocopherol, regarding the preservation of the PUFA in CKO. Similar to our findings, Luther *et al*., (2007) reported that although the extract of black raspberry seed flour was more effective than the extract of Chardonnay grape seed flour in preserving the PUFA of fish oil, the extract of grape seed flour exhibited a stronger capacity for reducing the overall lipid oxidation in fish oil. These results indicate that the capacity of an antioxidant for preserving a selected fatty acid may not be the same as its ability to reduce the overall lipid oxidation of an oil sample. Here, the CEOs extracted by the HD and SD methods protected the PUFAs of the CKO to the same extent that the BHT and tocopheryl acetate did. Similarly, Golmakani *et al*., (2018) showed that the PUFA contents of soybean oil samples containing *O. sanctum* essential oil were similar to the PUFA contents of samples containing BHA during storage at both 45 and 60 °C.

4. CONCLUSIONS

In this study, CEOs were extracted by four methods, namely HD, SD, MAHD and MASD. The effects of these CEOs were evaluated on CKO oxidation in comparison to the function of synthetic (BHT), natural (α-tocopherol and β-

carotene), and semi-natural (tocopheryl acetate) antioxidants. CEOs and other natural or synthetic antioxidants that were used here caused increases in the oxidative stability of CKO when analyzed through the AOM assay and the Rancimat method. Among the four types of CEOs, the CEO extracted by MAHD showed the best performance in increasing the AOM IP and Rancimat IP of the CKO, and this can be attributed to the higher yield of eugenol obtained by this extraction method. The role of CEO in reducing CKO oxidation was similar to the functional strength of BHT and tocopheryl acetate. Furthermore, CEO performed better than α-tocopherol and β-carotene. Specifically, the CEO extracted by MAHD was stronger than BHT and tocopheryl acetate in preserving the PUFA of CKO. In conclusion, CEO extracted by MAHD can be suggested as a suitable natural antioxidant for improving the oxidative stability of CKO and for preserving its nutritional value during storage.

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TABLE 4. Fatty acid composition (%) of common Kilka oil as a function of different variants of clove essential oil during accelerated storage at 97.8 °C

Fatty acid	Untreated oil	Control	HD*	SD	MAHD	MASD	β- Carotene	α- Tocopherol	Tocopheryl acetate	BHT
C8:0	0.00±0.00^{g**}	0.01±0.00$^{g\ de}$	0.01±0.00^{e}	0.02±0.00^{b}	0.01±0.00^{f}	0.02±0.00^{a}	0.02±0.00^{b}	0.01±0.00^{cd}	0.01±0.00^{c}	0.01±0.00^{c}
C10:0	0.01±0.00^{g}	0.01±0.00$^{g\ f}$	0.02±0.00^{cde}	0.02±0.00^{b}	0.02±0.00^{ab}	0.01±0.00^{e}	0.02±0.00^{cde}	0.02±0.00^{bc}	0.02±0.00^{de}	0.02±0.00^{a}
C12:0	0.07±0.01^{ab}	0.07±0.00$^{g\ b}$	0.07±0.00^{b}	0.07±0.00^{ab}	0.07±0.00^{b}	0.07±0.00^{b}	0.07±0.00^{ab}	0.07±0.00^{b}	0.07±0.00^{ab}	0.08±0.00^{a}
C13:0	0.10±0.00^{bcd}	0.10±0.01$^{g\ cd}$	0.10±0.01^{d}	0.13±0.01^{a}	0.08±0.00^{e}	0.11±0.01^{b}	0.09±0.01^{d}	0.08±0.00^{e}	0.11±0.01^{bc}	0.10±0.01^{bcd}
C14:0	4.74±0.09^{d}	5.25±0.26$^{g\ bcd}$	5.36±0.27^{abc}	5.27±0.26^{abcd}	4.92±0.25^{cd}	5.30±0.27^{abcd}	5.42±0.27^{abc}	5.33±0.27^{abcd}	5.53±0.28^{ab}	5.69±0.28^{a}
C15:0	0.88±0.01^{b}	1.10±0.10$^{g\ a}$	1.07±0.05^{a}	1.11±0.06^{a}	1.04±0.05^{a}	1.19±0.05^{a}	1.11±0.06^{a}	1.12±0.06^{a}	1.08±0.05^{a}	1.09±0.05^{a}
C16:0	19.77±0.25^{a}	25.62±1.28$^{g\ a}$	23.92±1.20^{a}	25.93±1.30^{a}	24.37±1.22^{a}	25.51±1.28^{a}	24.91±1.25^{a}	24.23±1.27^{a}	24.31±1.22^{a}	24.58±1.23^{a}
C16:1ω-7	6.75±0.08^{b}	8.08±0.40$^{g\ a}$	7.60±0.37^{a}	8.09±0.04^{a}	7.60±0.38^{a}	7.98±0.40^{a}	7.73±0.39^{a}	7.55±0.38^{a}	7.73±0.39^{a}	7.80±0.39^{a}
C17:0	0.54±0.00^{d}	0.69±0.03$^{g\ a}$	0.60±0.03^{cd}	0.66±0.03^{ab}	0.61±0.03^{c}	0.67±0.03^{ab}	0.67±0.03^{ab}	0.60±0.03^{cd}	0.61±0.03^{bc}	0.66±0.03^{abc}
C18:0	3.68±0.08 ^{d}	4.53±0.23$^{g\ bc}$	5.02±0.26^{a}	4.99±0.25^{a}	4.06±0.20^{cd}	5.13±0.26^{a}	5.06±0.25^{a}	4.94±0.25^{a}	4.83±0.24^{ab}	4.77±0.24^{ab}
C18:1ω-9	28.12±0.18^{c}	33.71±1.69$^{g\ ab}$	33.38±1.67^{ab}	33.74±1.69^{ab}	31.16±1.56^{bc}	33.26±1.68^{ab}	33.32±1.62^{ab}	33.29±1.66^{ab}	33.66±1.68^{ab}	33.78±1.69^{ab}
C18:2ω-6	1.99±0.04^{a}	1.86±0.09$^{g\ a}$	2.10±0.11^{a}	1.94±0.10^{a}	2.01±0.01^{a}	1.94±0.10^{a}	1.92±0.10^{a}	2.01±0.10^{a}	1.66±0.08^{b}	1.99±0.10^{a}
C18:3ω-3	2.06±0.02^{a}	1.65±0.08$^{g\ bcd}$	1.69±0.08^{cd}	1.66±0.08^{cd}	1.87±0.09^{b}	1.63±0.08^{cd}	1.72±0.09^{cd}	1.78±0.09^{bc}	1.71±0.09^{cd}	1.66±0.08^{cd}
C20:0	3.07±0.01^{a}	2.17±0.11$^{g\ cd}$	2.04±0.01^{cd}	2.01±0.10^{d}	2.47±0.12^{b}	1.97±0.10^{de}	2.13±0.11^{cd}	2.23±0.11^{c}	2.11±0.11^{cd}	2.08±0.10^{cd}
C21:0	0.39±0.10^{d}	0.41±0.02$^{g\ de}$	1.33±0.07^{a}	0.39±0.02^{de}	0.71±0.03^{b}	0.34±0.02^{ef}	0.32±0.02^{f}	0.60±0.03^{c}	0.41±0.02^{de}	0.42±0.02^{de}
C22:0	0.21±0.05^{b}	0.18±0.01$^{g\ cd}$	0.16±0.01^{d}	0.16±0.01^{d}	0.20±0.01^{bc}	0.16±0.01^{d}	0.18±0.01^{cd}	0.18±0.01^{cd}	0.18±0.01^{cd}	0.17±0.01^{cd}
C20:5ω-3	8.19±0.29^{a}	4.15±0.21$^{g\ de}$	4.76±0.24^{cd}	4.26±0.21^{ef}	5.69±0.28^{b}	4.19±0.21^{ef}	4.93±0.25^{c}	4.76±0.24^{cd}	4.87±0.24^{c}	4.69±0.23^{cd}
C23:0	0.03±0.00^{g}	0.04±0.00$^{g\ ef}$	0.05±0.00^{de}	0.05±0.00^{bc}	0.04±0.00^{ef}	0.05±0.00^{ab}	0.05±0.00^{de}	0.04±0.00^{def}	0.05±0.00^{cd}	0.04±0.00^{f}
C22:4ω-6	0.55±0.00^{a}	0.26±0.01$^{g\ c}$	0.36±0.02^{cde}	0.33±0.02^{e}	0.46±0.02^{b}	0.33±0.02^{e}	0.37±0.02^{cd}	0.42±0.02^{b}	0.35±0.02^{cde}	0.34±0.02^{de}
C24:0	0.48±0.01^{e}	0.80±0.04$^{g\ a}$	0.74±0.04^{abc}	0.79±0.04^{a}	0.70±0.03^{cd}	0.80±0.04^{a}	0.74±0.04^{abc}	0.71±0.04^{bcd}	0.70±0.03^{bcd}	0.66±0.03^{d}
C24:1ω-9	0.02±0.00^{e}	0.04±0.00$^{g\ ab}$	0.03±0.00^{d}	0.04±0.00^{ab}	0.03±0.00^{d}	0.04±0.00^{ab}	0.04±0.00^{bc}	0.03±0.02^{cd}	0.04±0.00^{ab}	0.03±0.00^{d}
C22:5ω-3	0.54±0.00^{a}	0.32±0.02$^{g\ cd}$	0.38±0.02^{cde}	0.39±0.02^{cde}	0.44±0.02^{b}	0.37±0.02^{def}	0.39±0.02^{cd}	0.34±0.02^{cde}	0.41±0.02^{bc}	0.35±0.02^{ef}
C22:6ω-3	17.77±0.47^{a}	8.89±0.45$^{g\ b}$	9.19±0.46^{bc}	7.91±0.39^{d}	11.44±0.57^{a}	8.95±0.45^{c}	9.60±0.48^{bc}	9.58±0.48^{bc}	9.53±0.48^{bc}	8.96±0.45^{c}
PUFA	31.11±0.81^{a}	17.13±0.86^{cde}	18.47±0.92^{c}	16.48±0.82^{de}	21.88±1.09^{b}	17.39±0.84^{cde}	18.93±0.95^{c}	18.92±0.95^{c}	18.51±0.93^{c}	18.00±0.90^{cd}
UFA	66.00±1.64^{a}	58.96±4.12^{b}	59.49±4.14^{b}	58.35±3.44^{b}	62.68±4.14^{ab}	58.68±4.02^{b}	59.19±3.94^{b}	59.80±4.21^{b}	59.94±4.24^{b}	59.60±4.32^{b}

*HD, hydro-distillation; MAHD, microwave-assisted hydro-distillation; SD, steam-distillation; MASD, Microwave- assisted steam-distillation.

**Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's test; Degree of significance: $P < 0.05$.

REFERENCES

AOCS. 2000. *Official Methods and Recommended Practices of the American Oil Chemists' Society*. AOCS Press, Champaign, IL.

Chemat F, Abert-Vian M, Fernandez X. 2012. Microwave-assisted extraction of essential oils and aromas, in Chemat F, **Cravotto** G. (Eds.) *Microwave-assisted extraction for bioactive compounds.* Springer, Boston, MA, pp. 53-68. https://doi.org/10.1007/978-1-4614-4830-3_1

Chaieb K, Hajlaoui H, Zmantar T, Kahla-Nakbi AB, Rouabhia M, Mahdouani K, Bakhrouf A. 2007. The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. *Phytother Res.* **21**, 501-506. https://doi.org/10.1002/ptr.2124

Codex Alimentarius. 2015. Standard for edible fats and oils not covered by individual standards. CODEX STAN 19-1981. https://www.fao.org/input/download/standards/74/CXS_019e_2015.pdf

Golmakani MT, Moayyedi M. 2015. Comparison of heat and mass transfer of different microwave-assisted extraction methods of essential oil from *Citrus limon* (Lisbon variety) peel. *Food Sci. Nutr.* **3**, 506-518. https://doi.org/10.1002/fsn3.240

Golmakani MT, Keramat M, Moosavi-Nasab M, Moosavian B. 2017a. Oxidative stability of common Kilka (*Clupeonella cultriventris* caspia) oil supplemented with microwave extracted Ghure (unripe grape) marc extract. *J. Aquat. Food Prod. Technol*. **26**, 1022-1031. https://doi.org/10.1080/10498850.2017.1375589

Golmakani MT, Zare M, Razzaghi S. 2017b. Eugenol enrichment of clove bud essential oil using different microwave-assisted distillation methods. *Food Sci. Technol. Res.* **23**, 385-394. https://doi.org/10.3136/fstr.23.385

Golmakani MT, Keramat M, Niakousari M, Khosravi H. 2018. Changes in fatty acid profile and oxidation indices of soybean oil supplemented with *Ocimum sanctum* essential oil during accelerated storage. *J. Essent. Oil Res*. **30**, 214-224. https://doi.org/10.1080/10412905.2018.1433084

Guan W, Li S, Yan R, Tang S, Quan C. 2007. Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chem.* **101**, 1558-1564. https://doi.org/10.1016/j.foodchem.2006.04.009

Gülçin İ. 2011. Antioxidant activity of eugenol: A structure-activity relationship study. *J. Med. Food.* **14**, 975-985. https://doi.org/10.1089/jmf.2010.0197

HMSO. 1994. Nutritional aspects of cardiovascular disease (report on health and social subjects No. 46). UK, London.

Hosseini H, Ghorbani M, Jafari SM, Sadeghi Mahoonak A. 2018. Investigating the effect of lipase from *Candida rugosa* on the production of EPA and DHA concentrates from *Kilka* fish (*Clupeonella cultiventris* caspia). *LWT Food Sci. Technol.* **93**534-541. https://doi.org/10.1016/j.lwt.2018.03.066

Hosseini H, Ghorbani M, Jafari SM, Sadeghi Mahoonak A. 2019a. Encapsulation of EPA and DHA concentrate from *Kilka* fish oil by milk proteins and evaluation of its oxidative stability. *J. Food Sci. Technol.* **56**, 59-70. https://doi.org/10.10 07/s13197-018-3455-9

Hosseini H, Tajiani Z, Jafari SM. 2019b. Improving the shelf-life of food products by nano/micro-encapsulated ingredients, in Galanakis CM (Ed.). *Food quality and shelf life*, Academic Press, Massachusetts, United States, pp. 159-201. https://doi.org/10.1016/B978-0-12-817190-5.00005-7

Hraš AR, Hadolin M, Knez Ž, Bauman D. 2000. Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chem.* **71**, 229-233. https://doi.org/10.1016/S0308-8146(00)00161-8

Jacobsen C, Let MB, Nielsen NS, Meyer AS. 2008. Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with *n*-3 polyunsaturated lipids: a comparative evaluation. *Trends Food Sci. Technol*. **19**, 76-93. https://doi.org/10.1016/j.tifs.2007.08.001

Jorjani S. 2014. Chemical composition and fatty acid profile of common kilka, *Clupeonella cultriventris* caspia. *Caspian J. Environment. Sci.* **12**, 119-128.

Keramat M, Golmakani M. 2016. Effect of *Thymus vulgaris* and *Bunium persicum* essential oils on the oxidative stability of virgin olive oil. *Grasas Aceites* **67**, e162. https://doi.org/10.3989/gya.0337161

Keramat M, Golmakani MT, Aminlari M, Shekarforoush S. 2017. Oxidative stability of virgin olive oil supplemented with *Zataria*

multiflora Boiss. and *Rosmarinus officinalis* L. essential oils during accelerated storage. *J. Food Process. Preserv*. **41**, e12951. https://doi.org/10.1111/jfpp.12951

Kromhout D, Yasuda S, Geleijnse JM, Shimokawa H. 2011. Fish oil and omega-3 fatty acids in cardiovascular disease: do they really work?. *Eur. Heart J.* **33**, 436-443. https://doi.org/10.1093/eurheartj/ehr362

Luther M, Parry J, Moore J, Meng J, Zhang Y, Cheng Z, Yu LL. 2007. Inhibitory effect of Chardonnay and black raspberry seed extracts on lipid oxidation in fish oil and their radical scavenging and antimicrobial properties. *Food Chem.* **104**, 1065-1073. https://doi.org/10.1016/j.foodchem.2007.01.034

Mazidi S, Rezaei K, Golmakani M, Sharifan A, Rezazadeh S. 2012. Antioxidant activity of essential oil from Black Zira (*Bunium persicum* Boiss.) obtained by microwave-assisted hydrodistillation. *J. Agric. Sci. Tech*. **14**, 1013-1022

Mishra PK, Singh P, Prakash B, Kedia A, Dubey NK, Chanotiya C. 2013. Assessing essential oil components as plant-based preservatives against fungi that deteriorate herbal raw materials. *Int. Biodeterior. Biodegradation* **80**, 16-21. https://doi.org/10.1016/j.ibiod.2012.12.017

Nagababu E, Rifkind JM, Boindala S, Nakka L. 2010. Assessment of antioxidant activity of eugenol in vitro and in vivo, in **Uppu** RM, **Murthy** SN, **Pryor** WA, **Parinandi** NL (Eds.) *Free Radicals and Antioxidant Protocols.* Humana Press, NY, USA, 165-180. https://doi.org/10.1007/978-1-60327-029-8_10

Nejadmansouri M, Hosseini SMH, Niakosari M, Yousefi GH, Golmakani MT. 2016. Physicochemical properties and storage stability of ultrasound-mediated WPI-stabilized fish oil nanoemulsions. *Food Hydrocoll.* **61**, 801-811. https://doi.org/10.1016/j.foodhyd.2016.07.011

Ogata M, Hoshi M, Urano S, Endo T. 2000. Antioxidant activity of eugenol and related monomeric and dimeric compounds. *Chem. Pharm. Bull.* **48**, 1467-1469.

Olmedo R, Ribotta P, Grosso NR. 2018. Antioxidant activity of essential oils extracted from *Aloysia triphylla* and *Minthostachys mollis* that improve the oxidative stability of sunflower oil under accelerated storage conditions. *Eur. J. Lipid Sci. Technol.* **120**. https://doi.org/10.1002/ejlt.201700374

Shahbazi H, Hashemi Gahruie H, Golmakani MT, Eskandari MH, Movahedi M. 2018. Effect of medicinal plant type and concentration on physicochemical, antioxidant, antimicrobial, and sensorial properties of kombucha. *Food Sci. Nutr.* **6**, 2568-2577. https://doi.org/10.1002/fsn3.873

Vanin AB, Orlando T, Piazza SP, Puton BM, Cansian RL, Oliveira D, Paroul N. 2014. Antimicrobial and antioxidant activities of clove essential oil and eugenyl acetate produced by enzymatic esterification. *Appl. Biochem. Biotechnol*. **174**, 1286-1298. https://doi.org/10.1007/s12010-014-1113-x

Velasco JN, Andersen ML, Skibsted LH. 2004. Evaluation of oxidative stability of vegetable oils by monitoring the tendency to radical formation. A comparison of electron spin resonance spectroscopy with the Rancimat method and differential scanning calorimetry. *Food Chem*. **85**, 623-632. https://doi.org/10.1515/chempap-2015-0102

Viuda-Martos M, Ruiz Navajas Y, Sánchez Zapata E, Fernández-López J, Pérez-Álvarez JA. 2010. Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour Fragr. J.* **25**, 13-19. https://doi.org/10.1002/ffj.1951

Wall R, Ross RP, Fitzgerald GF, Stanton C. 2010. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr. Rev.* **68**, 280-289. https://doi.org/10.1111/j.1753-4887.2010.00287.x

Wang L, Weller CL. 2006. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **17**, 300-312. https://doi.org/10.1016/j.tifs.2005.12.004

Wang H, Liu F, Yang L, Zu Y, Wang H, Qu S, Zhang Y. 2011. Oxidative stability of fish oil supplemented with carnosic acid compared with synthetic antioxidants during long-term storage. *Food Chem.* **128**, 93-99. https://doi.org/10.1016/j.foodchem.2011.02.082

Wüstenberg B, Stemmler RT, Létinois U, Bonrath W, Hugentobler M, Netscher T. 2011. Large-scale production of bioactive ingredients as supplements for healthy human and animal nutrition. *Chimia International Journal for Chemistry* **65**, 420-428.

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Effect of the damages caused by the green shield bug (*Palomena prasina* L.) on the qualitative traits of hazelnuts

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SUMMARY: This study was conducted in 2018 to determine the effects of green shield bug damage (GD) on the chemical properties of the hazelnut cultivar "Tombul". The proximate composition, protein, total lipid (TL), carbohydrate, total ash ratio (TA), vitamin E (VE), total phenolics, energy values (EV), color value, fatty acid composition, total fatty acids, lipid oxidation, and nutritional quality index properties of the kernel were detected in relation to the "bug damage". The level of TL, TA, VE, EV, monounsaturated fatty acids (MUFA), and unsaturated/saturated fatty acids (UFA/SFA) were found to be lower in GD kernels than in good kernels (GK). Although the GD kernels had higher iodine, free fatty acidity, and peroxide levels, they showed lower oleic/linoleic acid levels, and rancimat values. In addition, the GD kernels contained lower PUFA/SFA and hypocholesterolemic/hypercholesterolemic ratios but higher atherogenicity and thrombogenicity index values.

KEYWORDS: *Bug damage;* Corylus avellana *L.; Fatty acid profile; Oil oxidation; Proximate composition*

RESUMEN: ***Efecto de los daños causados por el insecto escudo verde (*Palomena prasina *L.) en las características cualitativas de la avellana.*** Este estudio se realizó en 2018 para determinar el efecto del daño del insecto escudo verde (DV) en las propiedades químicas del cultivar de avellana "Tombul". La composición proximal, proteína, lípidos totales (LT), carbohidratos, relación total de cenizas (CT), vitamina E (VE), fenólicos totales, valores de energía (E), color, composición de ácidos grasos, ácidos grasos totales, oxidación de lípidos e índice de calidad nutricional se determinaron en relación con los daños causados por el insecto. Se encontró que el nivel de LT, CT, VE, E, ácidos grasos monoinsaturados (MUFA) y ácidos grasos insaturados/saturados (UFA/SFA) fue menor en los granos de DV que en los granos buenos (GB). Aunque los granos de DV tienen niveles más altos de yodo, acidez, grasa libre y peróxidos, tienen niveles más bajos de la relación ácido oleico/linoleico y de los valores de rancimat. Además, los granos de DV tienen una relación más baja PUFA/SFA y de hipocolesterolémica/hipercolesterolémica, pero tienen valores de índice de aterogenicidad y trombogenicidad más altos.

PALABRAS CLAVE: *Composición proximal;* Corylus avellana *L.; Daño por insecto; Oxidación de aceite; Perfil de ácidos grasos*

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1. INTRODUCTION

Among nuts, the hazelnut is the richest in terms of vitamin E and oleic acid (C18:1) contents. In addition, it is a good source of bioactive compounds (Alaşalvar *et al*., 2010). 100-g portion of hazelnut kernels containing on average 10%-24% protein meets 22% of the daily protein intake, and contains 50-65% fat, with the oleic acid as the primary fatty acid, followed by linoleic, palmitic, stearic, and linolenic acid (Köksal *et al*., 2006; Seyhan *et al*., 2007). Parcerisa *et al*., (1995) reported that the Spanish hazelnut (*Corylus avellana* L.) contained 60.23% fat, 5.79% palmitic, 0.28% palmitoleic, 1.97% stearic, 79.1% oleic, and 12.58% linoleic acids. In addition, Cristofori *et al*., (2015) reported that the Italian hazelnut contained 47.06%-49.65% fat, 5.29-7.06% palmitic, 79.78-83.66% oleic acid, and 7.48-10.52% linoleic acids.

Hazelnuts are a good source of monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), tocopherol, phytosterol, polyphenol, and phytochemicals (Alaşalvar *et al*., 2006; Shahidi *et al*., 2007; Seyhan *et al*., 2007). The high level of unsaturated fatty acids (UFA) not only increases the nutritional quality of the hazelnut but also makes it more sensitive to oil oxidation. A high level of MUFA tends to increase HDL cholesterol; whereas LDL has a tendency to lower cholesterol (Olivera *et al*., 2008). Therefore, hazelnuts are highly beneficial as they prevent the vascular occlusion associated with cholesterol.

Fatty acids do not remain constant but vary based on genetic, ecological, morphological, and physiological properties and cultural practices. As a result of the lack of accurate and timely agricultural measures associated with cultural practices, damage to hazelnuts by bugs causes large economic losses in the form of kernel abortion, malformation, and the occurrence of necrotic tissues (Memoli *et al*., 2017). Numerous bug species that cause such damage and affect cultivar quality are found in Turkish hazelnut orchards (Bosco *et al*., 2018). There are more than 15 detrimental bug species, and the green shield bug (*Palomenaprasina* L.) is known to be the most harmful (Erper *et al*., 2016; Ak *et al*., 2018). The damage to hazelnuts which is caused by green shield bugs, called kernel spot, is not detectable by appearance. Therefore, manufacturers can sell the products without any problem. However, there is no management strategy to prevent this damage. Spotted kernel damage is a serious concern, particularly for hazelnut exporters. The kernel spot damage negatively affects hazelnuts in terms of appearance and taste and causes problems for their use in chocolate production and as dried nuts (Saruhan and Tuncer, 2010).

It has been reported that cimiciate damage reduces the total fat, saturated fatty acids (SFA), and single SFA levels in the Italian hazelnut cultivar Tonda di Giffoni (Memoli *et al*., 2017). Neverthless, information regarding the effects of pests on the chemical properties of Turkish hazelnut cultivars is extremely limited. Therefore, this study was conducted to determine the effect of GD on the qualitative traits and kernel composition on "Tombul", the most widely used hazelnut cultivar in Turkey.

2. MATERIALS AND METHODS

2.1. Kernel samples

The study was conducted on “Tombul” hazelnuts in 2018 from a single orchard, and nut samples were provided by Yavuz Gıda Sanayi ve Ticaret AŞ (Giresun, Turkey) (40°54′37.33″N, 38°26′17.23″E, and altitude 12 m). The average kernel moisture content was ~27.5% at the time of harvest (August 4 - August 5, 2018). The clusters were spread on the grass ground and dehydrated for 3 days (August 9 to August 12, 2018) to allow moisture loss (~21.52%). Then, the nuts were separated from their husks by hand. The samples were dried in the sun and the drying process continued until the moisture content was 6.45%.The samples (unshelled) were kept in a 2 kg vacuum polyethylene package (150 ± 8 µm thickness, 0.029 gm^{-2}/day oxygen permeability, 5 gm^{-2}/day water vapor permeability), and stored in a refrigerator (Bosch KDN53NW22N A, No-Frost, Germany) at 60-65% relative humidity and −5 °C temperature until oil extraction and further analysis.

2.2. Oil extraction

Hazelnut oil was extracted using a Ceselsan cold press oil extraction system (AISI3004, Ceselsan, Giresun, Turkey) (compression force: 10,000 kgf, pressure: 34.7 MPa, temperature: -5 °C to +45 °C, and capacity: 250 g kernel) (Turan, 2018a). The extracted hazelnut oil was stored in the freezer at −18 °C until analysis (Bosch KDN53NW22N A, No-Frost, Germany).

2.3. Proximate analysis

2.3.1. Moisture, protein, total lipid, ash, carbohydrate, and energy value

Moisture content is based on the Turkish Standards Institute (EN ISO 65-2000) -TS 3075/T1 hazelnut kernel standard (Turan, 2018b). Shredded hazelnuts (Fakir Motto 800 w, Germany) were dried at 105 °C until constant weight (Refsan RK 55, Kutahya, Turkey). The protein level was determined according to the AOAC standard method (N×6.25) with 0.5 g of sample using the macro Kjehldahl method (method 945.18B) (Velp UDK 149, Europe).,The fat level was determined according to the AOAC method (AOAC, 2000) with 5 g sample using Soxhlet extraction (110 °C) with petroleum ether (method 960.39) (Velp Ser 148, Milan, Italy). The total ash content was determined by gradual temperature increase (250-650 °C) and constant weight maintenance (AOAC, 923.03). The total carbohydrate content was calculated by subtracting other contents from 100% (Rezai *et al*., 2014) (1).

$$\text{Total carbohydrate} = 100\ \% - (\%\ \text{moisture} + \%\ \text{protein} + \%\ \text{fat} + \%\ \text{ash}) \quad (1)$$

The energy value was calculated using the following formula (Fernandes *et al*., 2019): (2).

$$\text{Energetic value}\left(\frac{\text{kcal}}{100\text{g}}\right) = 4\ \text{x}\ [(\%)\text{carbohydrate} + (\%)\text{protein}] + 9\ \text{x}\ [(\%)\text{lipid}] \quad (2)$$

2.4. Vitamin E

The vitamin E (tocopherol) composition of the samples was determined using the standard method described in AOCS Ce 8-89 (AOCS, 1997). One gram of extracted hazelnut oil was diluted with 10 mL hexane and the resulting mixture was injected into the HPLC instrument using a 0.45 µm PTFE syringe filter. It was analyzed by Shimadzu-Prominence LC-20A under the following HPLC conditions: column: C8 (250×4 mm) 5 µm, flow rate: 1 mL min, mobile phase: Hexane:Isopropyl alcohol (99:1), wavelength: 295 nm, column temperature: 25 °C.

2.5. Total phenolics

The total phenolic content was determined by modifying the Folin-Ciocalteu colorimetric method with a UV-visible spectrophotometer (Singleton *et al*., 1965). For analysis, 20 µL of sample extract was taken in a micro cuvette and 1.58 mL of purified water and 100 µL of Folin-Ciocalteu reagent were added. After 5 min, 300 µL of a saturated Na_2CO_3 solution were added. The solution was stored in a dark place for 2 h. After 2 h, the absorbance of the samples was determined at 760 nm simultaneously in triplicate. A calibration curve was created by using a set of solutions with 40, 80, 120, 160, 200, 250, and 300 mg gallic acid/L concentrations to calculate the results. The results were expressed as gallic acid equivalent (GAE).

2.6. Color ordinates

The color ordinates of the hazelnut kernels were determined by Hunter Lab Color Flex Ez color instrument (HunterLab, USA) as L* (lightness), a* (redness), and b* (yellowness). The colors of the samples were read following calibration to X:79.05, Y:84.02 and Z:89.03 (Mexis and Kontominas, 2009). The browning index (BI) was measured based on CIE L*a*b* coordinates, using the following formula (Marzocchi *et al*., 2017):

$$\text{BI} = 100 \times \left(\frac{\text{x} - 0.31}{0.17}\right) \quad (3)$$

$$\text{X} = \frac{(\text{a}^* + 1.75\text{L})}{(5.645\text{L} + \text{a}^* - 3.021\text{b}^*)} \quad (4)$$

2.7. Fatty acid analysis

To obtain fatty acid methyl esters (Turan, 2018a), 0.5 g oil was weighed in Erlenmeyer flasks, and 4 mL of iso-octane and 2 mL of methanolic KOH solution were added, followed by agitation for 30 s. The mixture was stored in a sealed container in a dark place for 6 min; 2 drops of 1% methyl orange indicator were added; and the mixture was then titrated with 1 M HCl solution until a pink color was developed. After it was kept for 15 min, the colorless layer formed on top of the mixture was put into glass vials and analyzed by GC. The composition of fatty acids was determined by gas chromatography with a flame ionization detector and TR-CN100 column (60 m × 0.25 mm I.D., 0.20 µm; Shimadzu GC-2010, Japan). Both injector temperature and detector temperature were set at 250 °C. The sample (1.0 µL) was injected and helium was used as the carrier gas at 200 kPa. The injection was made at a ratio of 1:100. The column

temperature was held at 90 °C for 7 min and then increased to 240 °C at 5 °C/min. Finally, it was maintained at 240 °C for 15 min. Fatty acids were characterized by comparison of the FAME mixture consisting of 37 standard components (Supelco 37 Component FAME Mixture, Cat. No. 18919-1AMP, Bellefonte PA, USA) based on their elution times (Turan, 2018b). SFA (saturated fatty acid), UFA (unsaturated fatty acid), MUFA (monounsaturated fatty acid), and PUFA (polyunsaturated fatty acid) were calculated by the following equations:

$$SFA = C14{:}0 + C16{:}0 + C17{:}0 + C18{:}0 + C20{:}0 + C22{:}0 \quad (5)$$

$$MUFA = C16{:}1 + C17{:}1 + C18{:}1 + C20{:}1 + C24{:}1 \quad (6)$$

$$PUFA = C18{:}2 + C183 \quad (7)$$

$$UFA = MUFA + PUFA \quad (8)$$

2.8. Oil oxidation parameters

Free fatty acidity (method Ca 5a-40) was determined by the AOCS Standard Method (AOCS, 2004), peroxide level (method Cd 8-53) by AOCS (AOCS, 2004) (Metrohm, Dosimat 799, Switzerland), and the Rancimat value by the Rancimat 743 device (Metrohm, Switzerland) (Velasco *et al*., 2004). The iodine value (IV) was calculated using the percentage of fatty acids (Belviso *et al*., 2017; Turan, 2019) (9).

$$IV = (C16{:}1 \times 1.901) + (C18{:}1 \times 0.899) + (C18{:}2 \times 1.814) + (C18{:}3 \times 2.737) \quad (9)$$

2.9. Oil quality indices

Data from the fatty acid profile analyses was used to evaluate the nutritional composition of the lipid fraction. Three oil quality indices were used: Index of atherogenicity (IA), index of thrombogenicity (IT) and hypocholesterolemic/ hypercholesterolenic fatty acid ratio (H/H). The IA (10) and IT (11) were calculated as described by Bezerra *et al*., (2017).

$$AI = \frac{C12{:}0 + 4 \times C14{:}0 + C16{:}0}{\sum MUFA + \sum FA\omega 6 + \sum FA\omega 3} \quad (10)$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{(0.5 \times \sum MUFA) + (0.5 \times \sum FA\omega 6) + (3 \times FA\omega 3)} \quad (11)$$

The H/H (12) index was determined by Fernandez *et al.* (2019).

2.10. Statistical analysis

The experiment was conducted with three replicates based on the randomized block design. Descriptive statistics were determined by SPSS v. 22.0 (Armonk, New York: IBM Corp.). Statistical tests were performed using SAS-JAMP v. 10.0 (SAS Institute Inc., Cary, North Carolina, USA). Statistical differences were determined using the *t*-test. The differences among the results were determined at the levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

Tombul hazelnut takes the first place in terms of quality among 18 hazelnut cultivars in Turkey; whereas the other cultivars are regarded secondary with respect to quality (Alaşalvar *et al*., 2010). The kernel of the Tombul cultivar content was determined to comprise 15.01% protein, 59.83% fat, 22.77% carbohydrate, and 2.39% ash by Seyhan *et al*., (2007); 4.63% moisture, 64.60% fat, 17.5% protein, 383.60 mg/100 g vitamin E, and 726.5 mg/100 g total phenolic substance by Köksal *et al*., (2006); 15.35% protein, 61.21% fat, 17.30% carbohydrate, 3.90% moisture, 2.24% ash, and 631 kcal/100g energy by Alaşalvar *et al*., (2009); and 61% fat, 16% carbohydrate, 14.9% protein, and 5.3% moisture by Memoli *et al*., (2017). In our study, the good kernel (GK) content was determined to contain 4.70% moisture, 54.21% fat, 15.00% protein, 23.91 g/100 g carbohydrate, 2.39% ash, 64.30 mg/kg vitamin E, 196.68 mg GAE/100 g total phenols, and 613.27 kcal/100 g energy (Table 1). Differences between bug-damaged and good kernel values were found to be statistically significant except for humidity ($p < 0.001$; Table 1). While total fat, ash, vitamin E, and energy values were higher, protein, carbohydrate, and total phenolic contents were lower in GK than in GD samples. Memoli *et al*., (2017) reported that damage caused by bugs affects the nutrient content of hazelnuts and cimiciate causes significant damage to hazelnuts. This damage is predictably caused by secretion during feeding in the developmental stages of the kernel and delay in growth (Figure 1A). Oil oxidation begins (Figure 1B) in the deformed kernel and changes in nutrient content occur.

$$\frac{H}{H} = \frac{C18{:}1 + C18{:}2 + C20{:}4 + C18{:}3 + C20{:}5 + C22{:}5 + C22{:}6}{C14{:}0 + C16{:}0} \quad (12)$$

TABLE 1. Effect of green shield bug damage on proximate composition, energetic value, and color ordinates of hazelnuts.

Parameters	Nut samples		Significant level
	Green shield bug damage	Good kernel	
Moisture (%)	4.77±0.29	4.70±0.17	ns
Total lipid (%)	46.23±0.01	54.21±1.41	**
Crude protein (%)	16.79±0.53	15.00±0.03	*
Total carbonhyrate (g/100 g)	30.48±0.01	23.91±0.01	***
Total ashes (%)	2.31±0.01	2.39±0.01	***
Vitamin E (mg/kg)	61.20±0.01	64.30±0.01	***
Total phenolics (mg GAE/100g)	262.42±0.01	196.68±0.01	***
Energetic value (kcal/100g)	576.31±1.62	613.27±11.27	**
Colour ordinates			
L* (Lightness)	58.46±0.01	58.85±0.01	***
a*(Redness)	2.95±0.01	3.16±0.01	***
b*(Yellowness)	11.23±0.01	10.99±0.01	***
Browning index (BI)	24.63±0.01	24.21±0.01	ns

Data represent the mean ± standard deviation of triplicate analyses (n=3). Differences were determined using the *t*-test. Significant level; *, **, *** and "ns" mean significance at p < 0.05, 0.01, 0.001 and "not significant", respectively, between green shield bug damage and good kernels.

Color is known to be an important parameter in the evaluation of hazelnuts (Marzocchi *et al*., 2017; Deng *et al*., 2018). The differences among the color ordinates were found to be statistically significant ($p < 0.001$) except for the browning index (BI), which is presented in Table 1 in detail. It was found that L* and a* levels were higher in the good kernel, whereas the b* level was higher in the bug-damaged nuts. The reason is that oil oxidation begins and progresses at places where bug damage occurs. Therefore, an increase in yellowness (Figure 1B), an indicator of oxidation in nuts, was noted.

3.2. Fatty acid profiles

Alaşalvar *et al*., (2010) reported that there is a generally high level of MUFA (78.10-87.26%), moderate level of PUFA 83.92-13.86%), and low level of SFA (7.46-9.59%) in hazelnuts, which is consistent with the reports of other studies (Alaşalvar *et al*., 2006; Turan, 2018a; Turan, 2019). It is known that hazelnut oil is preferred over olive, corn, and sunflower oil as it contains a higher level of UFA (Köksal *et al*., 2006; Alaşalvar *et al*., 2010) based on the scientific evidence that these fatty acids have a protective

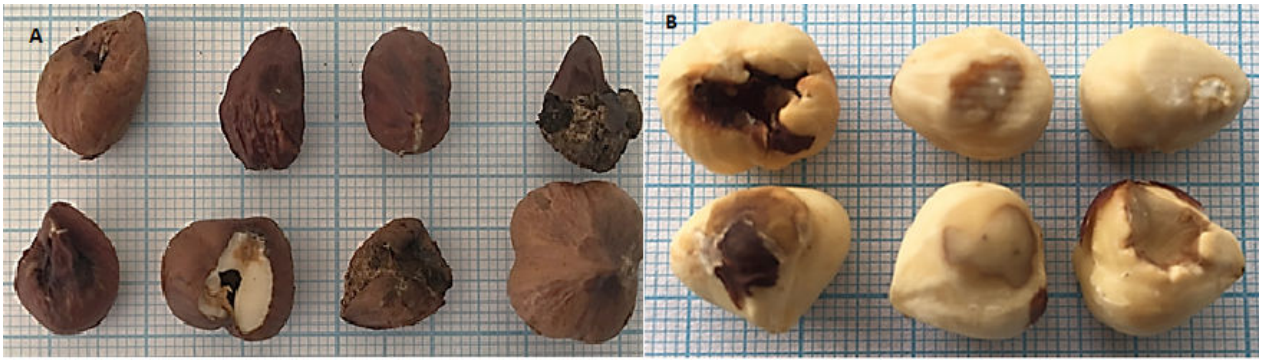

FIGURE 1. Effect of green sheld bug (*Palomena prasina* L.) damage on kernels (A, tumor, and/or spot kernel), and initial degree of oxidation (B, yellowing) of hazelnut oil

effect on the cardiovascular system (Uribe *et al*., 2018).

In our study, a total of 13 fatty acids were determined in the "Tombul" hazelnut, although 8 fatty acids were under the limit of detection (< 0.001%; Table 2). Palmitic, stearic, oleic, and linoleic fatty acids formed the major group; whereas myristic, margaric, arachidic, behenic, palmitoleic, heptadecanoic, eicosenoic, nervonic, and linolenic fatty acids formed the minor group. The fatty acids in the major group formed approximately 99.39% of the total fatty acids; whereas those in the minor group formed approximately 0.6% (Table 2). The effect of bug damage on fatty acid composition was found to be statistically significant except for margaric, behenic, and nervonic fatty acids ($p < 0.001$), which is presented in Table 2.

The effect of bug damage on SFA was found to be significant ($p < 0.001$) at 7.27% in the good kernels and 7.71% in the damaged kernels. As expected, there was a difference in the levels of palmitic and stearic major fatty acids which form the SFA. The primary fatty acid level of MUFA was 81.19% in the good kernels and 80.32% in the bug-damaged kernels. It is known that the primary fatty acid in the PUFA is linoleic acid. The effect of bug damage on linoleic acid was found to be statistically significant ($p < 0.001$; Table 2), with 11.12% in the good kernels and 11.60% in bug-damaged kernels. Telahigue *et al*., (2019) indicated that pathogens caused a decrease in fatty acids and SFA ranged between 1.82 and 0.41%, MUFA between 0.70 and 0.11% and PUFA between 2.67 and 0.38%. It was reported that this difference in PUFA is caused by the oxidation of linoleic and linolenic fatty acids (Memoli *et al*., 2017). Therefore, it should not be consumed in specific diets (Mostafavi *et al*., 2019). The effect of bug damage on UFA levels was found to be significant ($p < 0.001$). It was higher in the good kernels than in the bug-damaged kernels (92.65 and 92.29%, respectively, Table 2). It was reported that diseases and pests have an effect on the UFA/SFA ratio in hazelnuts, with 14.82% in good kernels, 16.13% in cimiciate and 15.96% in mold-effected kernels (Memoli *et al*., 2017). In our study, it was also found that bug damage has an impact on the UFA/SFA ratio ($p < 0.001$). However, it was found to be higher in the good kernels (12.74-11.98%). This difference has probably resulted from the different nutrient contents and/or various types of damage. In addition, it was stated that these differences may have been caused by the interaction of several factors such as altitude, latitude, longitude, temperature, precipitation, cultural practices, harvest time, and drying method (Koyuncu *et al*., 1997; Amaral *et al*., 2006; Cristofori *et al*., 2008; Alaşalvar *et al*., 2010; Turan *et al*., 2018b; Mostafavi *et al*., 2019).

3.3. Oxidation of hazelnut kernel oil

The oleic/linoleic acid ratio (O/L) is one of the essential characteristics used to evaluate the quality of hazelnut kernels, and linoleic acid is more sensitive to oxidation than oleic acid (Turan, 2019). Therefore, the high O/L ratio indicates resistance to oxidation (Belviso *et al*., 2017; Turan, 2018a). The effect of bug damage on the O/L ratio was found to be statistically significant ($p < 0.001$, Table 2), and O/L was higher in the good kernels (7.30%). Based on this, it can be concluded that bug damage (Figure 1A) causes oxidation in hazelnuts (Figure 1B). The iodine value (IV) is known as a measure of degree of unsaturation in fats and expressed as the amount of iodine absorbed (Belviso *et al*., 2017; Turan, 2018b). In addition, a high value of IV indicates that the content is unstable and more sensitive to oil oxidation. The effect of bug damage on IV was not found to be significant ($p > 0.05$). However, it was shown that good kernels had a lower level (93.59). In conclusion, it appears that good kernels have a longer shelf-life. Free fatty acids (FFA) are considered to be the first indicator of the lack of quality, and their level above FFA ≥1% indicates spoilage. In our study, there was a remarkable difference between FFA values in the good and bug-damaged kernels (oleic acid: 0.49, 2.62%, respectively, Table 2). Therefore, it can be concluded that bug damage results in oxidation in the nut, and these nuts cannot be purchased. The peroxide value (PV) is one of the crucial characteristics used to indicate the quality of products stored in the hazelnut industry (Turan, 2018a), and is also considered to be the most important indicator of PV oil oxidation. The effect of bug damage on PV was found to be statistically significant ($p < 0.001$), which is presented in Table 2 in detail. PV levels were noted as 21.58 $meqO_2 \cdot kg^{-1}$ in the bug-damaged kernels and 15.18 $meqO_2 \cdot kg^{-1}$ in the good kernels. It was highlighted that bug damage increased oil oxidation in nuts (Figure 1B). The rancimat value (RV) is a characteristic used to determine the shelf-life of hazelnuts (Turan, 2019).

TABLE 2. Effect of green shield bug damage on the fatty acid profiles, sum of fatty acids and oil oxidation of hazelnuts.

Fatty Acids (FA, %)	Nut samples		Significant level
	Green sheild bug damage	Good kernels	
Caproic acid (C6:0)	nd	nd	
Caprylic acid (C8:0)	nd	nd	
Capric acid (C10:0)	nd	nd	
Lauric acid (C12:0)	nd	nd	
Myristic acid (C14:0)	0.04±0.01	0.03±0.01	*
Palmitic acid (C16:0)	5.23±0.03	5.03±0.05	**
Margaric acid (C17:0)	0.06±0.01	0.05±0.00	ns
Stearic acid (C18:0)	2.24±0.01	1.99±0.02	***
Arachidic acid (C20:0)	0.09±0.00	0.12±0.00	**
Behenic acid (C22:0)	0.04±0.01	0.05±0.01	ns
Lignoceric acid (C24:0)	nd	nd	
Total saturated FA (ΣSFA)	7.71±0.03	7.27±0.05	***
Palmitoleic acid (C16:1)	0.10±0.00	0.05±0.00	***
Heptadecanoic acid (C17:1)	0.08±0.07	0.05±0.00	*
Oleic acid (C18:1)	80.32±0.12	81.19±0.09	***
Eicosenoic acid (C20:1)	0.05±0.00	0.08±0.01	*
Erucic acid (22: 1),	nd	nd	
Nervonic acid (C24:1)	0.04±0.01	0.05±0.01	ns
Total monounsaturated FA (ΣMUFA)	80.59±0.02	81.41±0.09	***
Linoleic acid (C18:2)	11.60±0.02	11.12±0.08	***
Linolenic acid (C18:3)	0.10±0.01	0.12±0.00	**
Eicosadienoic acid (20: 2)	nd	nd	
Docosadienoic acid (22: 2)	nd	nd	
Total polyunsaturated FA (ΣPUFA)	11.70±0.03	11.24±0.08	**
Unsaturated FA (UFA)	92.29±0.04	92.65±0.05	***
Unsaturated/saturated FA (UFA/SFA)	11.98±0.05	12.74±0.09	***
Oil oxidation parameters			
Oleic to linoleic acid (O/L)	6.93±0.01	7.30±0.06	***
Iodine value (IV)	93.71±0.07	93.59±0.07	ns
Free fatty acid (FFA; %, Oleic acid)	2.62±0.33	0.49±0.04	***
Peroxide value (PV, meq $O_2 \cdot kg^{-1}$)	21.58±0.22	15.18±0.20	***
Rancimat value (RV, h)	5.53±0.01	8.21±0.01	***

Data represent the mean ± standard deviation of triplicate analyses (n=3). nd: Not detected (< 0.001%). Differences were determined using the *t*-test. Significant level; *, **, *** and "ns" mean significance at $p < 0.05$, 0.01, 0.001 and "not significant", respectively, between green shield bug damage and good kernels.

Table 2 shows that the RV value is higher (8.21 h) in the good kernels. Thus, the rancimat value of the nuts is decreased due to bug damage and the shelf-life is expected to be shortened.

3.4. Quality indices

Generally, the effect of bug damage on the quality index was found to be significant, which is presented in Table 3 in detail. The effect of bug damage on the PUFA/MUFA ratio was not found to be significant ($p > 0.05$, Table 3). Consumption of low levels of SFA and high levels of PUFA/SFA is associated with a low risk of heart attack (Chan and Matanjun, 2017); therefore, this characteristic is used to determine the quality of the fat fraction in foods. The PUFA/SFA ratio is generally considered to indicate the quality of fats in a diet program (Telahigue *et al*., 2019), and values lower than 0.45 are not desirable owing to their ability to increase blood cholesterol. In our study, although the bug damage decreased the PUFA/SFA level, it was determined to be above the threshold (1.52-1.55) and higher in the good kernels (1.55, Table 3). This characteristic was reported to be 1.46 in fish (Telahigue *et al*., 2019); therefore, it can be confirmed that its amount is lower in fish than in hazelnuts. In fact, this aspect of the evaluation of hazelnuts suggested that they are a valuable nutrition source for humans. Atherogenicity (AI) and thrombogenicity index (TI) levels should approach zero (Bezerra *et al*. 2017) because this trend represents an increase in anti-atherogenic fatty acids, which has an effect on preventing heart disease. The AI (0.16) and TI (0.15) levels in the good kernels were determined to be lower than those in the bug-damaged kernels. It has been indicated that the H/H ratio is associated with the cholesterol mechanism (Fernandes *et al*., 2019) and a higher level of this ratio has a positive effect on human health. In our study, the H/H ratio (18.27) was found to be higher in the good kernels than that in the bug-damaged kernels. Based on the findings of this study, it is suggested that bug damage has a negative effect on the nut quality index values. Therefore, in case of bug-damaged hazelnuts being consumed, their expected effect on the cardiovascular system and cholesterol mechanism would not be observed.

4. CONCLUSIONS

To our knowledge, this is the first report in the literature regarding the effect of GD on nutrient content, fatty acid composition, oil oxidation, and food quality index of "Tombul" hazelnuts. In this study, the effect of bug damage was found to be statistically significant. Bug damage caused decreased UFA and UFA/SFA ratio levels. In addition, it resulted in lipid oxidation, thereby leading to decreased O/L and RV values and increased IV, FFA, and PV levels. Moreover, this oxidation also caused increased AI and TI levels and a decreased H/H ratio.

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TABLE 3. Effect of green shield bug damage on oil quality indices of hazelnuts.

Indices	Nut samples		Significant level
	Green shield bug damage	Good kernels	
Polyunsaturated (PUFA)/ Monounsaturated (MUFA)	0.15±0.00	0.14±0.00	ns
Polyunsaturated (PUFA)/ Saturated (SFA)	1.52±0.00	1.55±0.01	*
Atherogenicity index (AI)	0.23±0.02	0.16±0.02	***
Thrombogenivity index (TI)	0.16±0.00	0.15±0.00	*
Hypocholesterolemic/ Hypercholesterolemic (H/H)	17.45±0.11	18.27±0.16	**

Data represent the mean ± standard deviation of triplicate analyses (n=3). Differences were determined using the *t*-test. Significant level; *, **, *** and "ns" mean significance at $p < 0.05$, 0.01, 0.001, and "not significant", respectively, between green shield bug damage and good kernels.

REFERENCES

Ak K, Tuncer C, Baltacı A, Eser Ü, Saruhan İ. 2018. Indicence and severity of stink bug damage on kernel in Turkish hazelnut orchards. *Acta Hortic.* **1226**, 407-412. https://doi.org/10.17660/ActaHortic.2018.1226.62

Alaşalvar C, Amaral JS, Shahidi F. 2006. Functional lipid characteristics of Turkish Tombul hazelnut (*Corylus avellana* L.). *J. Agric. Food Chem.* **54**, 10177-10183. https://doi.org/10.1021/jf061702w

Alaşalvar C, Pelvan E, Topal B. 2010. Effect of roasting oil and fatty acid composition of Turkish hazelnut varieties (*Corylus avellana* L.). *Int. J. Food Sci. Nutr*. **61**, 630-642. https://doi.org/10.3109/09637481003691820

Alaşalvar C, Shahidi F, Amaral JS, Oliviera BPP. 2009. Tree Nuts: Compositional characteristics and health effect of hazelnut (*Corylus avellana* L.): An overview. *Nut. Sci. Tech.* **9**, 185-214.

Amaral JS, Casal S, Seabra RM, Olivera BPP. 2006. Effects of roasting on hazelnut lipids. *J. Agric. Food Chem*. **54**, 1315-1321. https://doi.org/10.1021/jf052287v

AOAC. 2000. Official Methods of Analysis of AOAC International 17th ed. 40:1-3.

AOCS. 1997. Official Method Ce 8-9. Determination of Tocopherols and Tocotrienols in Vegetable Oils and Fats by HPLC. Campaign, IL: AOCS

AOCS. 2004. Official Methods and Recommended Practices of the American Oil Chemist's Society, 5th ed. American Oil Chemist Society, US

Belviso S, Bell BD, Giacosa S, Bertolino M, Ghirardello D Giordano M, Rolle L, Gerbi V, Zeppa G. 2017. Chemical, mechanical and sensory monitoring of hot air and infrared roasted hazelnuts (*Corylus avellana* L.) during nine months of storage. *Food Chem*. **217**, 398-408. https://doi.org/10.1016/j.foodchem.2016.08.103

Bezerra CV, Rodrigues AMC, Olivera PD, Silva DA, Silva LHM. 2017. Technological properties of amazonian oil and fats and their applications in the food industry. *Food Chem*. **221**, 1466-1473. https://doi.org/10.1016/j.foodchem.2016.11.004

Bosco L, Moraglio ST, Tavella L. 2018. *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. *J. Pest Sci*. **91**, 661-670. https://doi.org/10.1007/s10340-017-0937-x

Chan PT, Matanjun P. 2017. Chemical composition and physicochemical properties of tropical red seaweed, *Gracilaria changii*. *Food Chem*. **221**, 302-310. https://doi.org/10.1016/j.foodchem.2016.10.066

Cristofori V, Bertazza G, Bignami C. 2015. Changes in kernel chemical composition during nut development of three Italian hazelnut cultivars. *Fruits* **70**, 311-322. https://doi.org/10.1051/fruits/2015025

Cristofori V, Ferramondo S, Bertazza G, Bignami C. 2008. Nut and kernel traits and chemical composition of hazelnut (*Corylus avellana* L.) cultivars, *J. Sci. Food Agric*. **88**, 1091-1098. https://doi.org/10.1002/jsfa.3203

Deng LZ, Yang XH, Mujumdar AS, Zhao JH, Wang D, Zang Q, Wang J, Gao ZJ, Xiao HW. 2018. Red pepper (*Capsicum annuum* L.) drying: Effect of drying methods on drying kinetics, physical properties, antioxidant capacity, and microstructure. *Dry. Technol*. **36**, 893-907. https://doi.org/10.1080/07373937.2017.1361439

Erper İ, Saruhan İ, Akça İ, Aksoy HM, Tuncer C. 2016. Evaluation of some entomophogenetic fungi for controlling to green shield bug, *Palomena prasina* L. (Heteroptera: Pentatomidae). *Egyp. J. Bio. Pest. Cont.* **26**, 573-578

Fernandes I, Fernandes T, Cordeiro N. 2019. Nutritional value and fatty acid profile of two wild edible limpets from the Madeira Archipelago. *Eur. Food Res. Technol*. **245**, 895-905. https://doi.org/10.1007/s00217-019-03234-y

Koyuncu MA, Bostan SZ, Islam A. 1997. Change of fat content and fatty acid composition during the fruit development period in the hazelnuts Tombul and Palaz cultivars grown in Ordu. *Acta Hortic*. **445**, 229-233. https://doi.org/10.17660/ActaHortic.1997.445.32

Köksal Aİ, Artik N, Şimşek A, Güneş N. 2006. Nutrient composition of hazelnut (*Corylus avellana L.*) varieties cultivated in Turkey. *Food Chem*. **99**, 509-515. https://doi.org/10.1016/j.foodchem.2005.08.013

Marzocchi S, Pasini F, Verardo V, Ciemniewska-Zytkiewicz H, Caboni MF, Romani S. 2017. Effects of different roasting conditions on physical-chemical properties of Polish hazelnuts (*Corylus avellana* L. var. Kataloński). *LWT Food Sci. Technol.* **77**, 440-448. https://doi.org/10.1016/j.lwt.2016.11.068

Memoli A, Albanese D, Esti M, Lombardelli C, Crescitelli A, Miatteo MD, Benucci I. 2017. Effect of bug damage and mold contamination on fatty acids and sterols of hazelnut oil. *Eur. Food Res. Technol.* **243**, 651-658. https://doi.org/10.1007/s00217-016-2778-x

Mexis SF, Kontominas MG. 2009. Effect of γ-irradiation on the physicochemical and sensory properties of cashew nuts (*Anacardium occidentale* L.). *LWT Food Sci. Technol*. **42**, 1501-1507. https://doi.org/10.1016/j.lwt.2009.03.023

Mostafavi S, Asadi-Gharneh HA, Miransari M. 2019. The phytochemical variability of fatty acids in basil seeds (*Ocimum basilicum* L.) affected by genotype and geographical differences. *Food Chem*. **276**, 700-706. https://doi.org/10.1016/j.foodchem.2018.10.027

Oliveira I, Sousa A, Morais JS, Ferreira ICFR, Bento A, Estevinho L, Pereira JA. 2008. Chemical composition, and antioxidant and antimicrobial activities of three hazelnut (*Corylus avellana* L.) cultivars. *Food Chem. Tox*. 46, 1801-1807. https://doi.org/10.1016/j.fct.2008.01.026

Parcerisa J, Boatella J, Codony R, Rafecas M, Castellote AI, Garcia J, Lopez A, Romero A. 1995. Comparison of fatty acid and triacylglycerol composition of different hazelnut varieties (*Corylus avellana* L.) cultivated in Catalonia (Spain). *J. Agric. Food Chem.* **43**, 13-16.

Rezai F, Bakhshi D, Ghazvini RF, Majd DJ, Pourghayoumi M. 2014. Evaluation of fatty acid content and nutritional properties of selected native and important hazelnut (*Corylus avellana* L.) varieties grown in Iran. *J. Ap. Bot. Food Quality* **87**, 104-107.

Saruhan İ, Tuncer C. 2010. Research on damage rate and type of green shield bug (*Palomena prasina* L. Heteroptera: Pentatomidae) on hazelnut. *J. Agric. Sci*. **25**, 75-83

Seyhan F, Özay G, Saklar S, Ertaş E, Satır G, Alaşalvar C. 2007. Chemical changes of three native Turkish hazelnut varieties (*Corylus avellana* L.) during fruit development. *Food Chem*. 105, 590-596. https://doi.org/10.1016/j.foodchem.2007.04.016

Shahidi F, Alaşalvar C, Liyana-Pathırana CM. 2007. Antioxidant phytochemicals in hazelnut kernel (*Corylus avellan*a L.) and hazelnut by products. *J. Agric. Food Chem.* **55**1212-1220. https://doi.org/10.1021/jf062472o

Singleton VL, Rossi JJA. 1965. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic*. **16**, 144-158

Telahigue K, Rabeh I, Chetoui I, Bejaoui S, Cafsi ME, Hajji T. 2019. To what extent are hake fat and its oil quality affected by the parasite *Lernaeocera lusci*? *Grasas Aceites* **70**, e297. https://doi.org/10.3989/gya.0697181

Turan A. 2018a. Effect of drying methods on fatty acid profile and oil oxidation of hazelnut oil during storage. *Eur. Food Res. Technol*. **12**, 2181-2190. https://doi.org/10.1007/s00217-018-3128-y

Turan A. 2018b. Effect of drying methods on nut quality of hazelnuts (*Corylus avellana* L.). *J. Food Sci. Technol.* **55**, 4554-4565. https://doi.org/10.1007/s13197-018-3391-8

Turan A. 2019. Effect of drying on the chemical composition of Çakıldak (cv) hazelnuts during storage. *Grasas Aceites.* **70**, e296. https://doi.org/10.3989/gya.0693181

Uribe E, Vega-Galvez A, Garcia V, Pasten A, Lopez J, Goni G. 2018. Effect of drying methods on phytochemical content and amino acid and fatty acid profiles of the green seaweed, *Ulva* spp. *J. Appl. Pycol*. **31**, 1967-1979. https://doi.org/10.1007/s10811-018-1686-9

Velasco J, Anderson ML, Skibsted LH. 2004. Evaluation of oxidative stability of vegetable oils by monitoring the tendency to radical formation. A comparison of electron spins resonance spectroscopy with the rancimat method and differential scanning calorimetry. *Food Chem*. **85**, 623-632. https://doi.org/10.1016/j.foodchem.2003.07.020

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Preliminary investigation of possible effects of mineral clay treatment applied to oils produced from olives: focus on moisture removal and compositional changes

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SUMMARY: In this preliminary study, two non-filtered virgin olive oils (one freshly produced VOO-N; one VOO-O stored for one year) were subjected to moisture removal with mineral clay (raw or activated) and analyses were performed to attest possible effects on the quality of the product. The results demonstrated that the treatment of oil with mineral clay at 36-38 °C had no negative effect on the basic quality parameters or on the volatile comound profile. On the other hand, a significant decrease in the water amount as well as in pigments was observed in the samples subjected to this kind of treatment, in particular with raw clay. Regarding the colour measurement, the lightness (*L*) as well as the consumers' acceptability exhibited a marked increase when oils were treated with raw clay.

KEYWORDS: *Colorimetric measurement; Consumers' acceptability; Mineral clay; Moisture; pigments; Quality parameters; Virgin olive oil; Volatile compounds*

RESUMEN: ***Investigación preliminar sobre los posibles efectos del tratamiento con arcilla mineral aplicado a aceites producidos a partir de aceitunas: enfoque sobre la eliminación de humedad y cambios en la composición.*** En este estudio preliminar, dos aceites de oliva vírgenes no filtrados (uno VOO-N recién producido y el otro, VOO-O almacenado durante un año) fueron sometidos a la eliminación del contenido de humedad con arcilla mineral (cruda o activada) y se realizaron análisis para atestiguar posibles efectos sobre la calidad del producto. Los resultados demostraron que el tratamiento del aceite con arcilla mineral, realizado a 36-38 °C, no tuvo consecuencias negativas en los parámetros básicos de calidad y en el perfil de los compuestos volátiles. Por otro lado, se observó una disminución significativa en la cantidad de agua y en los pigmentos en las muestras sometidas a este tipo de tratamiento, en particular con arcilla cruda. Con respecto a la medida del color, luminosidad (L) y aceptabilidad de los consumidores mostraron un aumento notable cuando los aceites se trataron con arcilla cruda.

PALABRAS CLAVE: *Aceite de oliva virgen; Aceptabilidad de los consumidores; Arcilla mineral; Compuestos volátiles; Humedad; Medida colorimétrica; Parámetros de calidad; Pigmentos*

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1. INTRODUCTION

Extra virgin olive oil (EVOO) is the highest quality category for virgin olive oils (VOO) as exhibited by the International Olive Council (IOC, 2015). EVOO is produced from fresh and intact olives using only mechanical/physical extraction processes and without heating (less than 30 °C), the addition of food additives or adjuvants or blending with other edible oils (IOC, 2015). For this reason, it deserves the qualification of "olive juice". EVOO exhibites a high shelf life in comparison to other oils and fats thanks to its characteristic composition in fatty acids, and, as recognized in many studies, the presence of peculiar antioxidants (Bendini *et al*., 2007; Issaoui *et al*., 2009). Yet, storage conditions are important factors that can affect the conservation period of the VOO and several factors, such as temperature, light, oxygen moisture and presence of microorganisms have to be carefully controlled (Stefanoudaki, 2010). According to the main international standards (Codex Alimentarius, International Olive Council (IOC), European Commission (EC)) the quality of VOO is based on several parameters, such as the percentage of free fatty acids, the peroxide value, the spectrophotometric absorbances in the UV region, the sensory analysis and the fatty acid ethyl ester content (Conte *et al*., 2019). In order to evaluate olive oil quality, the Codex Alimentarius and IOC have also included the determinations of insoluble impurities, moisture percentage, some metals and unsaponifiable matter (CAC, 2003; Boskou 2006; IOC, 2015). In general, for food safety and shelf life validation of the food product, information about water activity can be considered very useful. Water activity (Aw) is a measurement of the free water in a sample and ranges between 0 (bone dry) to 1.0 (pure water), providing valuable information about the possible effects of chemical-physical modifications as well as microbial growing. Together, water activity and moisture content give a more complete picture of the food stability status (Isengard, 2001).

The color of the foodstuff is one of the first sensory attributes evaluated by consumers (Pagliarini *et al*., 1994). VOO has a typical color, which ranges from the different shades of yellow to deep green depending on the olive variety, the agricultural practices such as irrigation, the maturation index and the oil extraction process (Escolar *et al*., 2007; Lazzerini and Domenici, 2017).The VOO color is linked to the presence of two main classes of pigments which are transferred from the olive fruit to the VOO during the extraction process: the green chlorophylls and the yellow and orange carotenoids (Cerretani *et al*., 2008). Thus, the consumer may feel attracted by green oils, richer in chlorophylls, or more yellowish oils, richer in carotenoids and pheophytins. The color determination is not included among the criteria used to determine VOOs quality grade but it could represent one of the major purchase drivers among consumers.

Clarification and/or filtration are the latter steps of the production of VOO before bottling which can be applied to eliminate water and suspended particles that still remain in the oily layer. The mechanical process, mainly vertical centrifugation, is the most commonly used clarification method. This system requires the use of lukewarm water, in a ratio variable from 1:1 to 1:2 v\v oil\water. The addition of water to the centrifuge system leads to the production of an effluent with high contaminant activity (from 20 to 40 L of liquid by-product per 100 kg of fruits). The second disadvantage of this system is its impact on the olive oil quality by changing its aromatic bouquet and decreasing its amount of phenolic molecule contents and consequently its sensory attributes (mainly bitterness) and shelf life (Uceda, Jimenez and Beltran, 2006; Garcia *et al*., 2001; Masella *et al*., 2009; Gila *et al*., 2017). However, at the industrial level, a filtration process is generally applied to cloudy VOO for removing suspended solids and humidity before storage or bottling steps. Conventional filtration systems use organic or inorganic filter aids, such as diatomaceous earth and cellulose fibers, in conjunction with filtration equipment (tanks or presses) (Lozano-Sanchez *et al*., 2010; Masella *et al*., 2011). Previous studies (Lozano-Sánchez *et al*., 2007; Gómez-Caravaca *et al*., 2007) remarked that the filtration step may either positively or negatively affect the stability, phenolics, volatiles, pigments and the sensory proprieties of olive oil and discussed the possible use of different types of filtering systems.

Clay minerals, thanks to specific absorbtion properties, are used in the purification and clarification of vegetable oils (Mosbahi *et al*., 2017; Mosbahi *et al*., 2008). Based on the recent research of Mosbahi *et al.,* (2017), satisfactory bleaching of 100 mL of soybean oil was obtained with 0.75% of mineral clay under stirring at 110-130 °C. The activated clay used presented a higher capacity to clarify soybean oil than those

given by commercialized products used for the same purpose (Mosbahi *et al*., 2017).

The main aims of the present research were: (i) to study the the application of raw and activated mineral clay for removing the moisture in oils produced from olives (ii) to investigate the possible effects of this kind of treatment performed at 36-38 °C in terms of basic quality parameters (such as free acidity and peroxide value), oxidative stability, volatile compounds, pigments and color (iii) to evaluate consumer acceptance in terms of the appearance characteristic (color).

2. MATERIAL AND METHODS

2.1. Mineral material

The raw clay used for the basic activation of clay and the clarification processes of the oil were carried out as previously described (Mosbahi *et al*., 2017). The clay from Chouabine formation (Ypresian) was collected from the OuedErrizgui section at Jebel Meheri El Jebbes, Central Tunisia. The origin, specific surface, porosity and other chemical compositions of the clay have been described in detail by Mosbahi *et al*., (2017). The raw clays were dispersed in water for a few days and screened through a sieve of 40 µm to remove impurities, and then dried at 60 °C for several days. For the clay activation, a mixture of 15 g of sieved clay and 5% Na_2CO_2 was soaked in 150 mL of distilled water and stirred continuously for 30 minutes.

2.2. VOO samples

The present study was applied to two samples of unfiltered virgin olive oil. The first sample, coded VOO-N, was produced by the extraction of Chemlali olives using a continuous system equipped with a three-phase decanter (freshly produced VOO-N). The second sample, coded VOO-O, was a virgin olive oil which had been stored for one year, produced during the previous season and purchased in bulk. These samples were submitted to clarification with raw clay (C) and with activated clay (A), stored at -20 °C and kept in the dark before analysis. Control samples (T) for each untreated VOO sample were also analyzed. Analytical determinations were performed in triplicate. The treatment was carried by the introduction of 0.75 g of clay (raw or activated) mixed with 100 mL of olive oil. The mixture was continuously stirred for 30 min at 36-38 °C and then filtered. The procedure was repeated many times until obtaining the amount needed for analysis (around 2 L).

2.3. Basic quality parameters

Free acidity (FA, given as the % of oleic acid) and peroxide value (PV), expressed as milli equivalents of active oxygen per kilogram of oil, meq O_2/kg, were evaluated according to the official methods (EEC 2568/91 and following amendments). A sensory analysis was performed by the professional panel of the Office national de l'huile (ONH), composed of a fully trained assessors trained and recognized by the International Olive Council (IOC). Sensory analysis was carried out as described in IOC, 2018.

2.4. Water activity and moisture content

Water activity is defined as the ratio between the vapor pressure of a food system and the saturation vapor pressure of distilled water under identical conditions. Water activity (Aw) was determined at 25 °C using a NOVASINA aw Sprint TH-500 apparatus (Novasina, pfäffikon, Switzerland).

Moisture and volatile matter contents were determined according to the method described in ISO 662:1998 (heating at 103 °C ± 2 °C until stable weight).

2.5. Oxidative stability index

The evaluation of the oxidative stability index was carried out by the Rancimat apparatus (Mod. 743, Metrohm Ω, Switzerland). Briefly, 3 g of each sample were heated to 120 °C and submitted to an air flow of 20 $L \cdot h^{-1}$. Stability was expressed as induction time in hours (h).

2.6. Color measurement

The color of the different samples was measured using a chromometer (Lovibond PFX-195 Tintometer). CIE 1976 L*a*b* (CIELAB), based on the opponent color theory, was applied as the color scale to study changes in the color: L* (lightness), refers to the lightness of the samples and ranges from black = 0 to white = 100; a negative value for a* (redness) indicates

green, while a positive one indicates a red-purple color. Positive b* (yellowness) indicates yellow, and a negative blue color. Oil color was measured by placing olive oil in quartz cuvettes in the color instrument. The instrument was standardized with a white and black ceramic plate. Hue angle (Equation 1) and chroma (Equation 2) were calculated using L*, a*, and b* values:

$$\text{Hue angle} = \frac{b^*}{a^*} \qquad \text{(Equation 1)}$$

$$Chroma = \sqrt{a^{*2} + b^{*2}} \qquad \text{(Equation 2)}$$

The chromameter was used for measuring reflected color and color differences among the samples subjected or not to treatment with mineral clay. Three readings were taken for each sample. Characterization of olive oil color was carried out as described previously by Gámbaro *et al.*, (2014). Based on the value for lightness, the degree of clarity (L value between 50-60 gives a medium clarity appearance; L value between 65 and 75 gives a high clarity appearance; L value higher than 80 gives a very high clarity appearance) was determined.

2.7. Determination of pigments

Olive oil pigments represtented mainly by carotenoids and chlorophylls (mg/kg oil) were determined at 470 nm and 670 nm, respectively, in cyclohexane, using specific extinction values, according to the method of Minguez-Mosquera *et al.,* (1991).

The total content in pigments was determined as the sum of the chlorophyll and carotenoid concentrations in the oil, as previously described by Gámbaro *et al.,* (2014).

2.8. Volatile compound analysis

The aromatic profile of the samples was studied and analysed according to the protocol previously detailed in Issaoui et *al.,* (2019). A Supelco Solid Phase Micro Extraction (SPME) fiber coated with polydimethylsiloxane (PDMS, 100 µm) was used. 2 mL of sample were placed in a 5 mL glass vial and, after equilibration (30 min), the fiber was inserted into the headspace of the sample for 50 min at room temperature. Once sampling was finished, the fiber was withdrawn into the needle and transferred and desorbed in the injection port of the GC-MS system. SPME sampling was performed using the same new fiber, preconditioned according to the manufacturers' instructions for all the analyses. SPME sampling and desorption conditions were identical for all the samples. Blanks were performed before each first SPME extraction and randomly repeated during each series. Quantitative comparisons of the relative peaks areas were made between the same chemicals in the different samples. A GC-EIMS analysis was carried out with a Varian CP 3800 gas-chromatograph equipped with a DB-5 Capillary column (30 m x 0.25 mm, 0.25 µm coating thickness) and a Varian Saturn 2000 ion trap mass detector. Injector and transfer line temperatures were 250 °C and 240 °C, respectively; oven temperature was programmed from 60 °C to 240 °C at 3 °C min^{-1}; carrier gas, helium at 1 mL min^{-1}; splitless injection. Identification of compounds was based on comparison of the retention times with those of pure standards, comparing their linear retention indices relative to the series of *n*-hydrocarbons, using the information from the National Institute of Standards and Technology library (NIST 2014 and ADAMS) and homemade library mass spectra built from pure substances and components of known mixtures and MS literature data (Adams, 1995; Jennings and Shibamoto, 1980; Davies, 1990; Massada, 1976; Stenhagen, Abrahamsson, and MC Lafferty, 1974; Swigar and Maestri, 2006).

2.9. Acceptance test

A total of 33 subjects participated in the study and were interviewed to investigate the color liking of the samples. The group of participants came from different regions of Tunisia and was selected using predetermined screening criteria based on level of education (high or incomplete), purchasing and consumption frequency, as well as familiarity with VOOs in Tunisia. Participants were asked to evaluate the samples according to their preference before and after the clarification process with raw and activated clay by checking the color of the oil and to express their degree of liking using a 9-point hedonic scale (scores: like extremely: 9; like very much: 8; like moderately: 7; like slightly: 6; neither like nor dislike: 5; dislike slightly 4; dislike moderately: 3; dislike very much: 2; dislike extremely: 1) (Peryan and Girardot, 1952). Each consumer had to complete a questionnaire on personal data and other information (age, gender, region of origin, socio-professional category, and consumption frequency

of EVOO). Samples (50 mL) were served at room temperature in transparent glass bottles coded with three-digit numbers and presented to consumers randomly, under blind conditions.

2.10. Statistical analysis

Data were processed by the SPSS statistical package (Version 12.00 for Window, SPSS Inc. Chicago, Illinois, 2003). The significance of difference at a 5% level among means was determined by one-way ANOVA, using Tukey's test. For the acceptance sensory test, to check whether a difference among samples existed, ANOVA and the F-test were used. Duncan's multiple range test was used to obtain all pair wise comparisons among sample means. A correlation analysis was performed by employing Person's test.

3. RESULTS AND DISCUSSION

Results related to basic quality parameters (free acidity, peroxide value and sensory analysis) of the two samples, freshly produced VOO-N and the one stored for one year, VOO-OT, before and after the treatment with raw and activated mineral clay, are shown in Table 1. The freshly produced oil treated with raw and activated clay (VOO-NC and VOO-NA) remained practically unchanged in comparison to the non-treated one (VOO-NT) and the oil stored for one year after treatment (VOO-OC and VOO-OA) showed only very slight differences compared to the control (VOO-OT). All samples, before and after the treatment with raw and activated mineral clay, belonged to the same quality grade, virgin olive oil, due to the presence of a mean perceived sensory defect with intensity below 3.5 and of a percentage of free acidity between 0.8% and 2% (peroxide number was below 20 meq per kg of oil in all samples).

The sensory analysis by panel test was applied to the samples before and after the treatment in order to evaluate the possible effect of the clay treatment on the presence of positive and negative attributes (EU regulations, 2011 and IOC, 2015). The freshly produced oil VOO-N had a higher fruitiness perception compared to the sample stored for one year VOO-O (4*vs* 1). However, the two samples were similar in terms of low intensities of bitterness and pungency. The VOO-N and the VOO-O, before and after treatment, were characterized by the presence of a rancid defect, perceived by lower intensity in the case of the freshly produced sample. The oil stored for one year also showed fusty-muddy as a secondary defect (Table 1). The obtained results exhibited that the clay treatment (raw and activated) had no significant effect on sensory characteristics.

Water activity is considered one of the most important parameters in food preservation. In fact, water is very important for the life of microrganisms and the major enzymatic activities, thus influencing the stability and the shelf-life of food products (Mathlouthi, 2001; FAO, 2003). This parameter is also interesting from the commercial point of view and it is legally regulated (Isengard, 2001). Humidity is indeed among VOO's worst enemies. It negatively affects the stability of the product against hydrolytic and oxidative degradations, the sensory profile and consequently its shelf-life. The IOC (2015) sets the moisture content at lower or equal to 0.2% (m/m) for edible virgin olive oil.

As expected, the highest water content in all samples (moisture, Table 1) was recorded in non-treated oils. In particular, the samples exhibited elevated moisture contents ranging from 2 to 0.9% (m/m), respectively, for (VOO-NT) and (VOO-OT). After the process with raw and activated mineral clay, values markedly decreased: the moisture content significantly lowered for both kinds of oils in the case of the treatment with raw clay (VOO-N moved from 2 to 0.3% and VOO-O decreased from 0.9 to 0.5%). A significant reduction was also observed when the freshly produced VOO-N was treated with activated clay (from 2% to 0.2%) but not in the case of sample stored for one year VOO-O. The Aw of VOO-N and VOO-O, when samples were treated with raw clay, significantly decreased from 0.601 to 0.449, and from 0.626 to 0.422, respectively; whereas the activated clay did not evidence the same effect.

No significant differences in terms of oxidative stability were shown after the treatment with mineral clay (for both types of clay) compared to the untreated one (Table 1). However, it has to be considered that the values were very low, in agreement with the fact that the oils presented sensory rancidity and low intensities of bitter and pungent positive attributes that, as known, are strictly related to the phenolic content.

Consumers perceive VOO color and may dislike oil only based on this parameter, even though the rest of its sensory attributes are suitable (Gámbaro *et al*., 2014). Color seems to be an important factor in consumers' choice of virgin

TABLE 1. Basic quality parameters (free fatty acid FA, peroxide value PV, sensory analysis), colorimetric measurements (L* lightness, a* redness and b* yellowness), water activity, moisture content, oxidative stability index and pigments for untreated freshly produced VOO (VOO-NT) and one after a year of storage VOO (VOO-OT).

Samples		**VOO-N**			**VOO-O**		
Treatment		**T**	**C**	**A**	**T**	**C**	**A**
FA (%)		1.5^{a}	1.3^{a}	1.5^{a}	1.1^{x}	0.8^{y}	0.8^{y}
PV (meqO_2/kg)		3.7^{a}	4.6^{a}	3.2^{a}	12.5^{x}	9.3^{y}	9.3^{y}
	Fruity	4.0^{a}	4.0^{a}	4.0^{a}	1.0^{x}	1.0^{x}	1.0^{x}
	Bitter	2.0^{a}	2.0^{a}	2.1^{a}	2.5^{x}	2.0^{x}	1.0^{y}
Sensory Analysis	*Pungent*	1.0^{a}	1.0^{a}	1.0^{a}	1.0^{x}	1.0^{x}	1.0^{x}
	Fusty/muddy	$n.p^{a}$	$n.p^{a}$	$n.p^{a}$	0.6^{x}	0.9^{x}	$n.p.^{y}$
	Rancid	0.9^{a}	0.6^{a}	0.8^{a}	2.0^{x}	1.9^{x}	2.1^{x}
	L	63.6^{b}	90.1^{a}	89.1^{a}	88.0^{y}	91.0^{x}	90.7^{x}
Colorimetric parameters	a*	-8.4^{a}	-13.6^{b}	-13.4^{b}	-11.7^{x}	-11.9^{x}	-11.7^{x}
	b*	43.1^{a}	41.5^{a}	43.1^{a}	42.2^{x}	41.5^{x}	41.9^{x}
Water activity		0.601^{a}	0.449^{b}	0.610^{a}	0.626^{x}	0.422^{z}	0.591^{y}
Moisture content (%)		2.0^{a}	0.3^{b}	0.2^{c}	0.9^{x}	0.5^{z}	0.8^{y}
Oxidative stability index (h)		1.3^{ab}	1.1^{b}	1.6^{a}	1.0^{x}	1.0^{x}	1.1^{x}
	Chlorophylls	7.73^{a}	1.29^{b}	0.91^{c}	2.58^{x}	$< LOQ^{y}$	$<LOQ^{y}$
Pigments (mg/kg)	Carotenoids	3.18^{a}	0.83^{b}	0.88^{b}	1.19^{x}	0.17^{y}	0.24^{y}
	Total pigments	10.91^{a}	2.12^{b}	1.79^{b}	3.77^{x}	0.17^{y}	0.24^{y}

The two samples were purchased in bulk and treated with raw clay (VOO-NC, VOO-OC) and activated clay (VOO-NA, VOO-OA), respectively. Positive sensory attributes and the main perceived defects are expressed as median values of intensities. n.p. not perceived. Values in the same row with different superscript letters represent significant differences between treatments for the same sample at $p < 0.05$ by Tukey's test (n = 3).

olive oil, often being the key criterion for preference. The typical green color of virgin olive oil comes from chlorophyll; while the yellow one is the result of the presence of carotenoids. The comparison of color data between samples before and after treatement with mineral clay is reported in Table 1. The ANOVA test showed a statistically significant influence of the clarification process on some colorimetric parameters, in particular for the freshly produced oil. The treatement significantly increased the lightness (*L*) (VOO-NT moved from 63.6 to 90.1 and 89.1 for oil treated with raw and activated clay, respectively) and decreased the greenness (-a*) (the a* value for VOO-NT was -8.4 and after treatement with raw and activated clay, it lowered to -13.6 and -13.4, respectively). On the other hand, yellowness (b*) was not influenced and no statistically significant differences were observed between treated and untreated samples (Table 1).

The modification of the colorimetric parameters in the case of VOO-O is not highly remarkable in comparison to the case of VOO-N. This may be explained by the lowest quantities of pigments present in the product that are subjected to degradation during storage. The results, depicted in Table 1, demonstrated a marked decrease in chlorophylls and carotenoids after the treatement process with raw and activated mineral clay for both the oils, but the pigment concentrations in VOO-O before the treatment was substantially lower than VOO-N due to the long storage period. Generally, the treatment with mineral clay was more effective on chlorophylls than on carotenoids, in agreement with the colorimetric values. These observations were in agreement with Cerretani *et al.,* (2008), who reported that the luminosity values (L*) increased with the reduction in the pigment contents in the oils. In fact, it was noticed that the color of the oil,

mainly the green one, which is related to the amount of chlorophylls, decreased with the increase in lightness (L*) in the samples. According to Cerretani *et al.,* (2008), significant correlations between the content in pigments and the tristimulus color measurement were obtained, specifically the total carotenoids *vs* the values for a* (green area) and b* (yellow area) *vs* a*/b* (green/yellow balance).

The volatile substances identified and quantified in the headspace of the VOOs before and after the treatment with mineral clay, together with those for raw clay, activated clay, and used clay impregnated with olive oil were studied (Table 2).

Virgin olive oil has a highly distinctive flavor due to specific volatile organic compounds belonging to several chemical classes, namely aliphatic and aromatic hydrocarbons, aliphatic and triterpene alcohols, aldehydes, ketones, ethers, esters and furan and thiophene derivatives (Kiritsakis *et al*., 1998). The main objective of this research was to study the behavior of clay towards olive oil, mainly whether it can adsorb oxidation compounds and to determine the degree of permeability/impermeability of clay *in* VOOs.

TABLE 2. Volatile compounds, expressed in %, of freshly produced VOO-N and VOO-O before (T) and after clarification with raw clay (C) and activated clay (A); raw clay (Cr), activated clay (C_{rA}), raw clay with VOO-N (C_{NB}), raw clay with VOO-O (C_{RO}).

Samples	**VOO-N**			**VOO-O**			**Clay**			
Treatment	**T**	**C**	**A**	**T**	**C**	**A**	**C_r**	**C_{ra}**	**C_{NB}**	**C_{ro}**
Markers of LOX pathway (expressed in %)										
Hexanal	6.3^{a}	6.9^{a}	6.7^{a}	6.3^{x}	6.1^{x}	5.3^{x}	nd	nd	19.9^{1}	18.9^{1}
(E)-2-hexenal	47.7^{b}	55.0^{a}	57.2^{a}	4.1^{x}	4.7^{x}	5.0^{x}	nd	nd	0.1^{2}	0.5^{1}
(Z)-2-hexen-1-ol	10.0^{a}	10.3^{a}	10.3^{a}	nd	nd	nd	nd	nd	nd	nd
1-hexanol	nd	nd	nd	29.1^{y}	31.6^{x}	31.8^{x}	nd	nd	0.5^{1}	0.5^{1}
3-ethyl-1,5-octadiene*	1.2^{a}	1.1^{a}	1.1^{a}	1.0^{x}	0.8^{x}	0.8^{x}	nd	nd	nd	nd
3-ethyl-1,5-octadiene*	0.8^{a}	0.8^{a}	0.8^{a}	1.3^{x}	1.2^{x}	1.2^{x}	nd	nd	nd	nd
Markers of lipid oxidation (expressed in %)										
Heptanal	nd	nd	nd	nd	nd	nd	nd	nd	1.5^{1}	0.7^{2}
(Z)-2-heptenal	nd	nd	nd	nd	nd	nd	nd	nd	0.1	0.4^{1}
(E)-2-heptenal	1.5^{b}	3.2^{a}	2.2^{ab}	1.4^{x}	2.1^{x}	1.7^{x}	nd	nd	2.9^{2}	8.0^{1}
Octanal	0.4^{b}	1.1^{a}	0.5^{b}	nd	0.6^{y}	0.8^{x}	nd	nd	13.6^{1}	3.3^{2}
(Z)-2-octenal	nd	nd	nd	nd	nd	nd	nd	nd	1.6^{2}	5.6^{1}
(E)-2-octenal	nd	nd	nd	nd	0.5^{x}	nd	nd	nd	9.0^{2}	15.4^{1}
Nonanal	2.4^{b}	3.3^{a}	3.3^{a}	1.7^{z}	3.0^{x}	2.1^{y}	nd	nd	4.0^{1}	1.6^{2}
(Z)-2-nonenal	nd	nd	nd	nd	nd	nd	nd	nd	0.3^{1}	0.4^{1}
(E)-2-nonenal	nd	nd	nd	nd	nd	nd	nd	nd	2.2^{1}	1.7^{1}
(E,E)-2,4-nonadienal	nd	nd	nd	nd	nd	nd	nd	nd	0.4^{2}	0.8^{1}
(Z)-2-decenal	nd	nd	nd	nd	nd	nd	nd	nd	1.0^{1}	0.9^{1}
(E)-2-decenal	nd	1.3^{a}	nd	1.3^{xy}	2.2^{x}	1.8^{x}	nd	nd	8.7^{1}	7.8^{1}
(E,Z)-2,4-decadienal	nd	nd	nd	1.1^{x}	nd	nd	nd	nd	0.1^{1}	0.3^{1}
(Z)-2-undecenal	nd	nd	nd	nd	nd	nd	nd	nd	0.7^{1}	0.5^{1}
(E)-2-undecenal	nd	nd	nd	nd	nd	nd	nd	nd	4.0^{1}	2.7^{2}

Values in the same row with different superscript letters represent significant differences between treatments for the same sample at $p < 0.05$ by Tukey's test (n = 3). nd, not detected.

* Correct isomer not determined.

The major volatile compounds in virgin olive oil which contribute to the positive attributes of olive oil aroma (fruity and positive notes) include C_6-LOX aldehydes (hexanal, *(E)*-2-hexenal) and C_6-LOX esters (hexyl acetate and (*Z*)-3-hexenyl acetate). Thus, volatile compounds, which are responsible for most of the sensory properties in virgin olive oils, play a significant role in the evaluation of the overall oil quality and a decisive influence on its acceptability.

Freshly produced VOO-N was characterized by a high percentage of *(E)*-2-hexenal (47.7%) in its profile. On the contrary, VOO-O exhibited a very low content of this compound (4.7%). This evidence confirmed the general judgment that *(E)*-2-hexenal is a biomarker of freshness of olive oil (Issaoui *et al*., 2015). (*Z*)-2-hexen-1-ol was also present in a considerable amount in VOO-N. However, 1-hexanol was the predominate compound in the volatile fraction of VOO-O (29.1%) and it was not detected in VOO-N. This molecule was the main C_6-LOX compound found in the headspace of VOO-O before and after the treatment with mineral clay. On the other hand, this sample, characterized by a sensory rancidity more perceived than in VOO-N, presented a higher content in (*E*)-2-decenal, an unsaturated aldehyde with a low value for odor threshold. It can be noticed that *(E)*-2-hexenal was practically not absorbed by clay. Hence, this compound was present in a percentage of 55.0% in VOO-N after treatment with raw clay and in a percentage of about 0.1% in raw clay soaked in VOO-N (C_{NB}) (Table 2). Clay showed a very low permeability to the virgin olive oils' aroma compounds which belonged to the LOX pathway. On the other hand, based on the obtained results, all the compounds resulting from lipid oxidation were swept by the clay to a different degree. It is worth noting that hexanal was the highest absorbed volatile by clay, with a percentage near 20%. This phenomenon may be explained by the fact that hexanal has two sources of production, enzymatic via lipoxygeanse and oxidation and the oil may have remained in the clay after treatment, being subjected to a rapid lipid oxidation before analysis (Table 2). However, the data did not show a clear effect of the treatment with mineral clay on the volatile compounds responsible for the aroma of samples.

As previously mentioned, the colour perception represents one of the most important factors in the consumers' purchase choice, which is sometimes more relevant than other factors, such as package, price, producer, brand, etc (Dekhili *et al*., 2011). Its importance is probably due to the fact that color is the only sensory attribute that a consumer can generally evaluate during the purchase phase. The color of VOO, which is not significantly related to its quality, may produce expectations. Several investigations have demonstrated how different shadings of VOO color could affect consumer acceptability (Gutiérrez González, 1987; Tous and Romero, 1992; Gambaro *et al*., 2014). These results are linked with the familiarity of consumers with VOOs produced in different countries and should be taken into account that several agronomic and technological parameters may influence the content in pigments of VOOs and therefore their color. Considering data related to consumer preferences expressed in the hedonic session (Table 3, Figures 1 and 2), the highest color acceptability was obtained for the freshly produced oil treated with raw mineral clay that evidenced a degree of clarity higher than 90% with a light green shade. However, for both samples, freshly produced and stored for one year, the treatment with raw and activated mineral clay enhanced the color acceptability compared to the untreated oils but only the raw clay reached a very high degree of clarity (Table 3). Figures 1 and 2 show that the more appreciated samples for appearance characteristic (color-liking) were the oils treated with raw mineral clay (VOO-NC and VOO-OC) with 93.9 and 68.7% of consumers, respectively, with a 7-9 score. Consumers also seem to prefer also the light green of VOO-NC over the deep one of VOO-NT; this result is in agreement with another study that showed that the deep green color of VOOs can negatively affect people (Recchia *et al*., 2012); whereas its clarity is positively linked with the color liking (McEwan *et al*., 1994).

4. CONCLUSIONS

This preliminary study, performed on two different kinds of unfiltered virgin olive oils, one freshly produced and one stored for one year, demonstrated that the treatment with raw and activated mineral clay, performed under mild conditions (36-38 °C under stirring), did not lead to remarkable changes in the sensory characteristics or the volatile profile nor a modification of hydrolytic or oxidative parameters. On the other hand, its ability to remove moisture and pigments, in particular chlorophylls was proven. This last effect was

TABLE 3. Color description, degree of clarity and rated color acceptability for the freshly produced VOO (VOO-N) and the one-year-stored VOO (VOO-O).

Samples		Color Description	Degree of clarity	Color acceptability
VOO-N	NT	Green	Medium (≈ 60)	5.75^{c}±0.07
	NC	Light green	Very high (> 90)	8.09^{a}±0.87
	NA	Light green	High (>70)	6.39^{b}±0.61
VOO-O	NT	Pale yellow	High (>70)	5.96^{c}±0.14
	NC	Pale yellow	Very high (> 90)	6.78^{b}±0.30
	NA	Pale yellow	High (>70)	6.36^{b}±0.26

Each sample was purchased in bulk and treated with raw clay (VOO-NC, VOO-OC) and activated clay (VOO-NA, VOO-OA). Results of color acceptability are expressed as mean and standard deviation values and, in addition, different superscript letters represent significant differences between samples at $p < 0.05$ by Tukey's test (n = 3).

responsible for the increase in the luminosity value of the oil, a characteristic that seems to positively affect consumers' preference in terms of acceptability.

In general, the comparison of the data obtained after applying raw or activated mineral clay, evidenced a greater efficacy of the raw clay in lowering the humidity (with a concurrent decrease in water activity), enhancing the luminosity as well as the color.

Further studies which include the use of a set of olive oil samples with different physico-chemical and sensory characteristics should be conducted in order to confirm these preliminary results and to check the possible impact of this kind of treatment with mineral clay on some minor (e.g. sterols and phenolic molecules) and major compounds (e.g. glycerides) in the olive oil as well as the main markers of the refining process (e.g. steradiene content, *trans*-isomers of fatty acids, specific absorption in UV of diene and trienes conjugated chromophores). Moreover, specific tests will be performed on selected oil samples to compare the effects of treatment with mineral clay and the current method of filtration. Future results could evidence the feasibility of a potential industrial-scale application.

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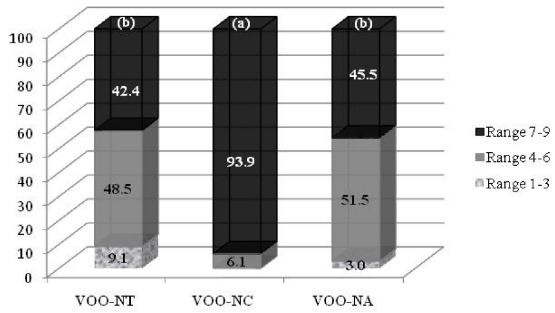

FIGURE 1. Percentages of color-liking for freshly produced VOO (untreated: VOO-NT, clarified with raw clay: VOO-NC and clarified with activated clay: VOO-NA). Acceptance test performed on 33 subjects. Values with different letters (a-b) represent significant differences between samples at $p < 0.05$ by Duncan's test with F value (15.83) > F critical (3.07).

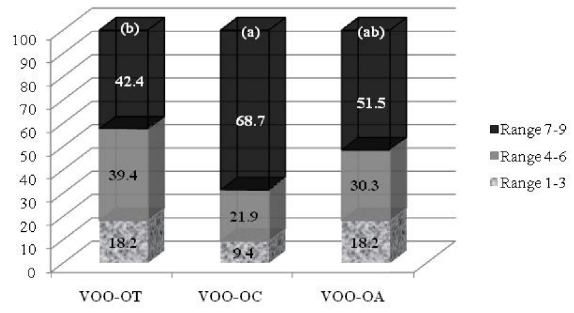

FIGURE 2. Percentages of color-liking for 1 year-stored VOO (untreated: VOO-OT, clarified with raw clay: VOO-OC and clarified with activated clay: VOO-OA). Acceptance test performed on 33 subjects. Values with different letters (a-b) represent significant differences between samples at $p < 0.05$ by Duncan's test with F value (1.49) > F critical (3.07).

REFERENCES

Adams R.P. 1995. Identification of Essential Oil Components by Gas Chromatography-Mass Spectroscopy. Allured Carol Stream, IL USA

Bendini A, Cerretani L, Carrasco-Pancorbo A, Gómez-Caravaca AM, Segura-Carretero A, Fernández-Gutiérrez A, Lercker G. 2007. Phenolic molecules in virgin olive oils: a survey of their sensoryproperties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules* **12**, 1679-1719. https://doi.org/10.3390/12081679

Boskou D. 2006. Olive Oil Composition. In: Olive Oil: Chemistry and Technology. D. Boskou (Ed.) p. 41-7. 2ndedition AOCS Press, Champaign, IL, USA.

CodexAlimentarius Commission (2003). Codex Standard for Olive Oils and Olive Pomaceoils. Codex stan 33-1981. Adoptedin 1981. Revisedin 1989. 2015, 2017. Amendedin 2009, 2013.

Cerretani L, Motilva MJ, Romero MP, Bendini A, Lercker G. 2008. Pigment profile and chromatic parameters of monovarietal virgin olive oils from different Italian cultivars. *Eur. Food Technol*. **226**, 1251-1258. https://doi.org/10.1007/s00217-007-0651-7

Conte L, Bendini A, Valli E, Lucci P, Moret S, Maquet A, Lacoste F, Brereton P, García-González D. L, Moreda W, Gallina Toschi T. 2019. Olive Oil Quality and Authenticity: A review of current EU legislation, standards, relevant methods of analyses, their drawbacks and recommendations for the future. *Trends Food Sci. Technol*. In press. https://doi.org/10.1016/j.tifs.2019.02.025

Davies NW. 1990. Gas chromatographic retention indexes of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20M phases. *J. Chromatogr.* A. **503**, 1.

Dekhili S, Sirieix L, Cohen E. 2011. How consumers choose olive oil: The importance of origin cues. *Food Qual. Prefer*. **22**, 757-762. https://doi.org/10.1016/j.foodqual.2011.06.005

Escolar D, Haro MR, Ayuso J. The Color Space of Foods: Virgin Olive Oil. *J. Agric. Food Chem.* 2007, **55**, 6, 2085–2093. https://doi.org/10.1021/jf062899v

European Union Commission, Regulation EEC 2568/91. 1991. Characteristics of olive and olive-pomace oils and on their analytical methods. Official Journal of the European Communities, L 248.

European Union Commission, Regulation CE 1989/2003. 2003. Amending Regulation EEC 2568/91. Official Journal of the European Communities, L 295.

European Community, Commission Regulation (2011) 61/2011. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, January 24, L23, 1-14.

FAO. 2003. Handling and Preservation of Fruits and Vegetables by Combined Methods for Rural Areas. Chapter 3, Agricultural Services Bulletin 149. ISBN 92-5-104861-4.

Gámbaro A, Raggio L, Ellis A.C, Amarillo M. 2014. Virgin olive oil color and perceived quality among consumers in emerging olive-growing countries. *Grasas Aceites* **65** (2), 1-7. https://doi.org/10.3989/gya.122213

Gutiérrez González R. 1987. Parámetros de calidad en el aceite de oliva. I. En su utilización en crudo. Pon. 2a. III Simp. Nal. Aceite de Oliva. Jaén.

Gracia A, Brenes M, Martínez F, Alba J, García P, Garrido A. 2001. High performance liquid chromatography evaluation of phenols in virgin olive oil during extraction at laboratory and industrial scale. *J. Am. Oil Chem. Soc*. **78**, 625-629. https://doi.org/10.1007/s11746-001-0316-x

Gila AM, Beltran G, Bejaoui MA, Jimenez A. 2017. How clarification systems can affect virgin olive oil composition and quality at industrial scale. *Eur. J. Lipid Sci. Technol*. **119**, 1-8. https://doi.org/10.1002/ejlt.201600479

Gómez-Caravaca AM, Cerratani L, Bendini A, Segura-Carretero A, Fernández-Gutiérrez A, Lercker G. 2007. Effect of filtration systems on the phenolic content in virgin olive oil by HPLC-DAD-MSD. *Am. J. Food Technol*. **2**, 671-678. https://doi.org/10.3923/ajft.2007.671.678

International Olive Council, Sensory analysis of olive oil-method for the organoleptic assessment of virgin olive oil, COI/T.20/Doc. No. 15/Rev. 10, 2018.

International Olive Council IOC/T.15/Doc No 3/Rev 8 (February 2015). Trade standard applying to olive oils and olive pomace oils.

Isengard HD. 2001. Water content, one of the most important properties of food. *Food Control* **12**, 395-400. https://doi.org/10.1016/S0956-7135(01)00043-3

Issaoui M, Gharbi I, Flamini G, Cioni PL, Bendini A, Gallina Toschi T, Hammami M. 2015. Aroma compounds and sensory

characteristics as biomarkers of quality of differently processed Tunisian virgin olive oils. *Int. J. Food Sci. Technol.* **50** (8), 1764-1770. https://doi.org/10.1111/ijfs.12830

Issaoui M, Bendini A, Souid S, Flamini, G, Barbieri S, Gallina Toschi T, Hammami M. 2019. Flavored olive oils: Focus on their acceptability and thermal stability. *Grasas Aceites* **70** (1), 293-303. https://doi.org/10.3989/gya.0224181

Jennings W, Shibamoto T. 1980. Qualitative analysis of flavor and fragrance volatiles by glass capillary chromatography. New York: Academic Press.

Kalua CM, Allen S, Bedgood Jr DR, Bishop AG, Prenzler PD, Robards K. 2007. Olive oil volatile compounds, flavour development and quality: a critical review. *Food Chem*. **100**, 273-286. https://doi.org/10.1016/j.foodchem.2005.09.059

Lazzerini C, Domenici V. 2017. Pigments in Extra-Virgin Olive Oils Produced in Tuscany (Italy) in Different Years. *Foods* **6**, 25. https://doi.org/10.3390/foods6040025

López-Feria S, Cárdenas S, García-Mesa JA, Valcárcel M. 2007. Quantification of the intensity of virgin olive oil sensory attributes by direct coupling headspace mass spectrometry and multivariate calibration techniques. *J. Chromatogr*. A. **1147**, 144-152. https://doi.org/10.1016/j.chroma.2007.02.107

Lozano-Sanchez J, Cerretani L, Bendini A, Segura-Carretero A, Fernandez-Gutierrez A. 2010. Filtration process of extra virgin olive oil: Effect on minor components, oxidative stability and sensorial and physico-chemical characteristics. *Trends Food Sci. Technol*. **21**, 201-211. https://doi.org/10.1016/j.tifs.2009.12.004

Lozano-Sánchez J, Cerretani L, Bendini A, Gallina-Toschi T, Segura-Carretero A, Fernández Gutiérrez A. 2012. New filtration systems for extra-virgin olive oil: Effect on antioxidant compounds, oxidative stability, and physicochemical and sensory properties. *J. Agric. Food Chem*. **60** (14), 3754-3762. https://doi.org/10.1021/jf205353b

Masella P, Parenti A, Spugnoli P, Baldi F, Mattei A. 2011. A predictive classification model for the management of virgin olive oil filtration at industrialscale. *Sep. Sci. Technol*. **46**, 1709-1715. https://doi.org/10.1080/01496395.2011.578606

McEwan JA. 1994. Consumer attitudes and olive oil acceptance: the potential consumer. *Grasas Aceites* **45**, 9-15. https://doi.org/10.3989/gya.1994.v45.i1-2.963

Maheswaran D. 1994. Country of origin as a stereotype: Effects of consumerexpertise and attribute strength on product evaluations. *J. Consumer Res*. **21**, 354-365. https://doi.org/10.1086/209403

Massada Y. 1976. Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry. New York: Wiley and Sons.

Masella P, Parenti A, Spugnoli P, Calamai L. 2009. Influence of vertical centrifugation on extra virgin olive oil quality. *J. Am. Oil Chem. Soc*. **86**, 1137-1140. https://doi.org/10.1007/s11746-009-1445-9

Mathlouthi M. 2001. Water content, water activity, water structure and the stability of foodstuffs. *Food Control*. **12**, 409-417. https://doi.org/10.1016/S0956-7135(01)00032-9

Minguez-Mosquera MI, Rejano L, Gandul B, Sanchez AH, Garrido J. 1991. Color-pigment correlation in virgin olive oil. *J. Am. Oil Chem. Soc*. **68**, 332-336.

Mosbahi M, Tlili A, Khlifi M, Jeddoui Y. 2008. Activation acido-basique des argiles de la formation El Haria du Bassin Meknassy - Mezzouna et essais de clarification des huiles neutres de soja. Congrès national de 3èmes journée de l'Association Tunisienne de Géologie Appliquée (ATGA). Mars 2008, Sousse- Tunisie.

Mosbahi M, Tlili A, Khlifi M, Jamoussi F. 2017. Basic activation of lower eocene clay from Mekmassy-Mezzouna basin (centezwestern Tunisia), synthesis of zeolite and clarification of soybean oils. *Appl. Clay Sci.* **138**, 1-11. https://doi.org/10.1016/j.clay.2016.12.011

Pryam DR, Girardot NF. 1952. Advanced taste method. *J. Food Eng*. **24**, 58-61.

Pagliarini E, Stramba P, Semeria L. 1994. Study of the subjective affective meaning and motivational aspects towards extra virgin olive oil. *Grasas Aceites* **45**, 65-67. https://doi.org/10.3989/gya.1994.v45.i1-2.973

Uceda M, Jimenez A, Beltran G. 2006. Olive oil extraction and quality. *Grasas Aceites* **57**, 25-31. https://doi.org/10.3989/gya.2006.v57.i1.19

Recchia A, Monteleone E, Tuorila H. 2012. Responses to extra virgin olive oils in consumers with varying commitment to oils. *Food Qual. Prefer*. **24**, 153-161. https://doi.org/10.1016/j.foodqual.2011.10.009

Stefanoudaki E, Williams M, Harwood J. 2010. Changes in virgin olive oil characteristics during different storage conditions. *Eur. J. Lipid Sci. Technol*. **112** (8), 906-914. https://doi.org/10.1002/ejlt.201000066

Stenhagen E, Abrahamsson S, Mc Lafferty FW. 1974. Registry of Mass Spectral Data. New York: Wiley and Sons.

Swigar AA, Maestri DM. 2006. The effects of genotype and extraction methods on chemical composition of virgin olive oils from Treslasierra Valley (Cordoba, Argentina). *Food Chem*. **96**, 507-511. https://doi.org/10.1016/j.foodchem.2005.03.003

Tous Martí J, Romero Aroca A. 1992. Caracterización del color de los aceites de olive vírgenes de cultivares catalanes. *Grasas Aceites* **43** (6)**,** 347-351. https://doi.org/10.3989/gya.1992.v43.i6.1131

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Fatty acid composition and antioxidant activity of *Angelica glauca* and *Chenopodium album* seed extracts from Kashmir

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SUMMARY: *Angelica glauca* Edgew. and *Chenopodium album* Linn. are medicinally important plants with aromatic, nutritious and flavor-enhancing properties. In this study the fatty acid composition of petroleum ether seed extracts (PE) of processed plants were analyzed by gas chromatography-mass spectrometry (GC-MS). The PE consisted mainly of unsaturated fatty acids, such as petroselinic acid 74.26% and oleic acid 7.37% for *A. glauca* and linoleic acid 53.05% and oleic acid 20.74% for *C. album*. The de-fatted seed extracts were screened for their antioxidant activities using 2,2-di-phenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), hydrogen peroxide (H_2O_2) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay. Almost all the assays resulted in defatted seed extracts showing powerful radical scavenging activity. These findings suggest that the processed plants could be used as ingredients (as a source of natural antioxidants and unsaturated fatty acids) for the formulation of functional foods.

KEYWORDS: *Angelica glauca; Antioxidant activity; Chenopodium album; Fatty acid composition; Linoleic acid; Petroselinic acid*

RESUMEN: ***Composición en ácidos grasos y actividades antioxidantes de extractos de semillas de* Angelica glauca *y de* Chenopodium álbum *de Cachemira.*** *Angelica glauca* Edgew. y *Chenopodium album* Linn. son plantas medicinales importantes que tienen propiedades aromáticas y nutritivas y se usan como condimento. En este estudio, la composición en ácidos grasos de los extractos de éter de petróleo (EP) de semillas de la planta se analizó por cromatografía de gases-espectrometría de masas (GC-MS). El EP consiste principalmente en ácidos grasos insaturados, especialmente ácido petroselínico 74,26% y ácido oleico 7,37% para *A. glauca* y ácido linoleico 53,05% y ácido oleico 20,74% para *C. album*. Además, a los extractos de semillas desgrasados se estudió sus actividades antioxidantes utilizando 2,2-di-fenil-1-picrylhydrazyl (DPPH), nitroazul de tetrazolio (NBT), peróxido de hidrógeno (H_2O_2) y 2,2'-azino-bis (3-etilbenzotiazolina-6-ácido sulfónico) (ABTS) ensayos de eliminación de radicales. Casi todos los ensayos sugieren que los extractos de semillas desgrasados mostraron potentes actividades de eliminación de radicales. Por último, los resultados sugieren que las plantas estudiadas podrían usarse como ingrediente (como fuente de antioxidantes naturales y ácidos grasos insaturados) para la formulación de alimentos funcionales.

PALABRAS CLAVE: *Ácido linoleico; Ácido petroselínico; Actividad antioxidante; Angelica glauca; Chenopodium album; Composición en ácidos grasos*

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1. INTRODUCTION

Lipids are found in all parts of plants but are mostly dominant in seeds. They are nutritional sources of food (Chen and Chuang, 2002). Monounsaturated fatty acids, such as oleic acid, are highly stable against oxidation and when blended with other oils have been found to have benefitial protective effects (Lampi and Kamal-Eldin, 1998). The antioxidants in plants prevent lipid oxidation, and the deterioration of color, flavor and nutritional quality of various foods (Kozlowska *et al*., 2014). They may also be an alternative to toxic synthetic antioxidants (Koleva *et al*., 2012). The extraction and preservation of natural antioxidants to replace the synthetic ones is the main target of the food, pharmaceutical and cosmetic industries (Binic *et al*., 2013).

Angelica glauca Edgew is an ornamental, perennial herb which is native to the temperate northern region from Kashmir to Uttarakhand. It is locally known as "Chora". Traditionally it is used in medicines, aromatic spices and condiments. It is used for various drug formulations at the domestic and international levels. The roots of *A. glauca* contain valeric acid, angelic acid and angelisine resin and have various medicinal properties such as stimulant, cardioactive, carminative, digestive, sudorific and expectorant (Chopra *et al*., 1956). Essential oil from the whole plant possesses antioxidant, antimicrobial and phytotoxic activity (Irshad *et al*., 2011). *Chenopodium album* Linn. (Chenopodiaceae) locally known as "Bathu" is an erect, annual plant growing up to 150 cm tall in the temperate zone. Traditionally it is used to cure arthritis and rheumatism (Prajapati *et al*., 2003). It is a medicinally important plant as it possesses anticancer (Ankita and Chauhan, 2012), anti-inflammatory (Usman *et al*., 2010), hepatoprotective (Pal *et al*., 2011), spasmolytic and analgesic activity (Ahmad *et al*., 2012). Our laboratory is also currently working on the analysis of novel seed oils for fatty acid composition (Sharma *et al*., 2009). A scanning of the literature has revealed that the whole plant extracts of *A. glauca* and *C. album* possess antioxidant activity (Irshad *et al*., 2011; Lone *et al*., 2017). However, no work on seed extracts for fatty acid composition and antioxidant activity from Kashmir (India) have been reported. Moreover, these plants possess tremendous nutritional and medicinal properties, less input care cost and high natural abundance. Therefore, this study was carried on *A. glauca* and *C. album* to determine their fatty acid composition and antioxidant activity, in order to evaluate them as potential natural sources of antioxidants and fatty acids used for nutritional purposes in the food industry.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

The seeds of *A. glauca* and *C. album* used in this work were collected from local nurseries in the Sher-e-Kashmir University of Agricultural Sciences and Technology (SKAUST-K) in 2019. 2,2-di-phenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), hydrogen peroxide (H_2O_2) and nitroblue tetrazolium (NBT) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). All other chemicals were of analytical grade.

2.2. Successive soxhlet extraction

The dried seeds were crushed separately into powder with a grinder. The seed powder from each plant was mixed with sodium sulphate and kept in an oven for about 2 h at 60-70 °C to remove any remaining moisture. A 50 g sample (Raaman, 2006) from each plant was put in a soxhlet apparatus along with 180 mL of solvent in a 250 mL round-bottom flask. Extractions were then performed using the petroleum ether solvent (PE), chloroform (CF), ethyl acetate (EA), acetone (AT) and methanol (MT). The extraction time ranged from 4-6 hours. The extracted oil (PE) was passed through anhydrous sodium sulphate (Na_2SO_4). All seed extracts were kept at 4 °C until further analysis. The oil characteristics were determined according to the standard AOCS procedures (Link, 1973) and the data are presented in Table 1.

2.3. Fatty acid methyl esters (FAMEs) preparation

One gram oil (PE) was saponified with 0.5N alcoholic potassium hydroxide (KOH). The unsaponifiable matter was removed with diethyl ether and the aqueous layer was acidified with 6N hydrochloric acid (HCl) followed by extraction with diethyl ether to get mixed fatty acids (MFAs) which were further treated with excess absolute methanol with a few drops of sulphuric acid (H_2SO_4) as catalyst and the reaction was refluxed

TABLE 1. Saponification and iodine values of petroleum ether extracts of of *A. glauca* and *C. album* and yield (%, *w/w*) of different extracts.

Name of plant	Seed extracts (%, *w/w*)					S.V (PE)	I.V (PE)
	PE	CF	EA	AT	MT		
A. glauca	14.62± 0.08^{c}	7.61± 0.29^{e}	10.54± 0.35^{d}	15.61 ± 0.09^{b}	19.82 ± 0.70^{a}	155.18± 1.37	73.80± 0.93
C. album	15.01± 0.69^{b}	9.32± 0.40^{e}	11.91± 0.03^{d}	13.32 ± 0.20^{c}	22.73 ± 0.48^{a}	145.59 ± 0.97	118.04 ± 1.43

PE: petroleum ether extract; CF: chloroform extract; EA: ethyl acetate extract; AT: acetone extract: MT: methanol extract. Values are arranged as means ± S.D. (n=3)

Different letters in each row are statistically significant different at ($p< 0.05$) according to Duncan's test.

for 1-2 hours. After completion of the reaction, as monitored by thin layer chromatography (TLC), the resulting mixture was diluted with water and continuously extracted with diethyl ether. The combined extracts were washed with 5% aqueous sodium bicarbonate and dried over anhydrous sodium sulphate to yield FAMEs which were further purified with n-hexane and diethyl ether (98/2, *v/v*) by column chromatography.

2.4. Fourier-transform infrared spectroscopy (FTIR) analysis

Transmittance spectra were obtained using a Fourier-transform infrared spectroscopy (FTIR) (Frontier, Perkin-Elmer Ltd, UK) fitted with an Attenuated total reflectance (ATR) crystal of zinc selenide and the length was recorded in cm^{-1}. The solvent used in the FTIR analysis was carbon tetrachloride (CCl_4) because of its transparent nature in the main region of the IR spectrum. The samples were put on ATR crystal kept at 65 °C so as to fully cover the surface of the crystal. A small amount of sample (50-100 µL) was taken. The samples were measured in duplicate. The spectra were continuously measured over a range of 3500-500 cm^{-1} and data resolution of 4 cm^{-1} and air were taken as reference background material. After each scan the ATR crystal was removed, cleaned and dried with tissue paper and ethanol.

2.5. Gas chromatography mass spectrometry analysis

The fatty acid composition was determined by using Gas chromatography, Perkin Elmer (GC, Clarius 600) coupled to a mass spectrometer (Perkin Elmer Technologies, Inc., Wilmington, D.E). An Elite-5MS capillary column (0.25mm × 30mm) with a flame ionization detector was used. Helium was used as carrier gas at a flow rate of 0.5 mL/min. The injector, column and detector temperatures were 180 °C, 260 °C and 280 °C, respectively. The oven temperature was programmed as follows: 180 °C for 2 min, then raised to 200 °C at 2 °C/min, held at 200 °C for a further 10 min, then raised to 215 °C at 2 °C/min, and held for 10 min. The injector and detector temperatures were maintained at 260 and 280 °C, respectively. Individual fatty acids were recognized with typical mass spectra from The National Institute of Standards and Technologies (NIST) library of the GC-MS.

2.6. Antioxidant activity

2.6.1. Radical scavenging activity (DPPH assay)

The antioxidant activity of seed extracts was determined by using DPPH following the procedure of (Shimada *et al*., 1992) with some modifications. Briefly, 200 µL of each extract (25-200 µg/mL) with 3.8 mL DPPH solution were incubated in the dark at room temperature for 1 h. The absorbance of the mixture was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as a positive control. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (I\%)} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

Where, A control is the absorbance of the DPPH radical (without the test sample), and A sample is the absorbance of the DPPH radical with the different extract samples of various concentrations.

2.6.2. Nitroblue tetrazolium assay (NBT assay)

Superoxide anion scavenging activity was determined as described (Vyas and Kumar, 2005). The reaction was performed in 50 mM/L phosphate buffer (PH 7.8) containing concentrations of (25-200) µg/mL of the extract, 50 mM/Lnitroblue tetrazolium (NBT), 10 mmol/L D,L-methionine, and 0.025% (*v/v*) Triton X-100. The reaction was initiated by illuminating the reaction mixture, the absorbance of formazan was recorded at 560 nm, and the percentage scavenging activity was described as the inverse of the produced formazan. BHT was used as a positive control. The percentage scavenging of NBT radicals was calculated using the equation described in the DPPH assay. The results were compared with standard drug BHT.

2.6.3. Hydrogen peroxide (H_2O_2) scavenging activity

The ability of the extracts to scavenge H_2O_2 radicals was determined according to the method described by (Ruch *et al*., 1989). A hydrogen peroxide solution of 40 mM was prepared in phosphate buffer (pH 7.4). A spectrum was produced using a UV-Visible spectrophotometer with phosphate buffer solution as a blank. The extracts at different concentrations (25-200 µg/mL) in 3.4 mL phosphate buffer were added to 0.6 mL of H_2O_2 solution (0.6 mL, 43 mM). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with BHT, the reference compound.

The percentage scavenging of H_2O_2 radicals was calculated using the equation described in the DPPH assay. The results were compared with BHT.

2.6.4. Radical scavenging activity (ABTS assay)

The antioxidant activity of the seed extracts was determined by ABTS according to (Re *et al*., 1999). The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 5 mL of 14 mM ABTS solution and 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, and stored in the dark at room temperature for 16 h. Various concentrations (25-200 µg/mL) of the plant extracts were mixed with 1 mL of $ABTS^{\bullet+}$ solution and homogenized. Absorbance was then recorded at 734 nm. Ethanol blanks were run in each assay, and all measurements were made after at least 6 min. The reaction mixture of the standard group was prepared by mixing 950 µL of $ABTS^{\bullet+}$ solution and 50 µL of BHT. As for the antiradical activity, the ABTS scavenging ability was expressed as IC_{50} (µg/mL). The reaction mixture of the standard group was obtained by mixing 950 µL of $ABTS^{\bullet+}$ solution and 50 µL of BHT. The ABTS scavenging ability was expressed as IC_{50} (µg/mL). The percentage scavenging of ABTS radicals was calculated using the equation described in the DPPH assay. The results were compared with standard drug BHT.

2.7. Stastical analysis

The antioxidant parameters were expressed as the mean ± SD with three replicates each. The results of all the antioxidant activities were analyzed with one-way analysis of variance (ANOVA). Duncan's post hoc test was applied for comparisons of means and differences were considered significant at 95% statistical significance using IBM SPSS Statistics 20.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of the extracts

The percentage (%, *w/w*) of various de-fatted extracts of *A. glauca and C. album* is depicted in Table 1. The yield of almost all the extracts was found to be significantly good, but seed extraction through methanol (MT) was found to be predominantly high for *C. album* 22.73% and *A. glauca* 19.82% and through petroleum ether (PE) 15.01% and 14.62% for *C. album* and *A. glauca,* respectively. The order of extracted yield through other solvents was acetone (AT) > ethyl acetate (EA) > chloroform (CF). The saponification value (SV) is the indicator of the average molecular weight and hence chain length (Nehdi *et al*., 2012). The higher the saponification value, the smaller is the chain length of fatty acids in a triacylglycerol. The saponification value (PE) of *A. glauca* at 155.18 is higher than *C. album* at 145.59, mainly because of the dominance of low molecular weight fatty acids (Table 1). The iodine value (IV) gives an indication of the degree of

TABLE 2. Saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids of petroleum ether extracts of *A. glauca* and *C. album.*

Common and systematic names	Carbon numbers	Chemical formula	Area (%)	
			A. glauca	*C. album*
Decanoic acid	C10:0	$C_{10}H_{20}O_2$	-	1.67 ± 0.02
Myristic acid	C14:0	$C_{14}H_{28}O_2$	0.32 ± 0.01	1.15 ± 0.02
Palmitic acid	C16:0	$C_{16}H_{32}O_2$	0.61 ± 0.35	6.32 ± 0.21
Stearic acid	C18:0	$C_{18}H_{36}O_2$	0.79 ± 0.20	2.79 ± 1.37
Petroselinic acid	C18:1 Δ6	$C_{18}H_{34}O_2$	74.26 ± 0.33	-
Oleic acid	C18:1 Δ9	$C_{18}H_{34}O_2$	7.37 ± 0.55	20.74 ± 0.12
Linoleic acid	C18:2	$C_{18}H_{32}O_2$	0.65 ± 0.07	53.05 ± 0.05
Linolenic acid	C18:3	$C_{18}H_{32}O_2$	-	2.01 ± 0.04
Eicosanoic acid	C20:0	$C_{20}H_{40}O_2$	-	1.11 ± 0.16
Eicosenoic acid	C20:1	$C_{20}H_{38}O_2$	0.59 ± 0.08	0.83 ± 0.13
Docosanoic acid	C22:0	$C_{22}H_{44}O_2$	0.40 ± 0.01	-
Docosenoic acid	C22:1	$C_{22}H_{42}O_2$	-	0.94 ± 0.03
Unidentified acids			1.39 ± 0.15	1.80 ± 0.23
∑TSFA[a]			2.12 ± 0.36	13.04 ± 1.38
∑TUFA[b]			82.28 ± 0.26	77.57 ± 0.04

∑TSFA[a]: total unsaturated fatty acids; ∑TUFA[b]: total saturated fatty acids.
Values are arranged as mean ± S.D. (n=3).

unsaturation and could be used to determine the oxidative stability of oils. The iodine value of *A. glauca* is lower at 73.80; while as *C. album* was found to be 118.04 higher than that of its earlier study (Ahmad *et al*., 1986). This may be mainly due to presence of a high percentage of polyunsaturated acids (linoleic acid) Table 2.

3.2. Functional group analysis by FTIR

The FTIR spectrum is one of the most important and powerful tools for the determination of functional groups in various plant extracts. This technique works on the basis of functional groups and gives information in the form of peak values. In this work the ATR-FTIR analysis was used to observe mainly the ester peak in the FAMEs of *A. glauca* and *C. album* as compared to its respective petroleum ether (PE) seed extracts from which they are synthesized. As displayed in Figure 1, the FAME of *A. glauca* showed a sharp, strong transmittance band at 1742 cm^{-1} as compared to the PE extract. Similarly, the FAME of *C. album* displayed a sharp peak at 1741 cm^{-1} as compared to its respective PE extract. In general, apart from TLC, the FTIR analysis gives valuable information regarding the formation of FAMEs from PE extracts, which is compulsory for GC-MS analysis.

3.3. Fatty acid composition

The fatty acid composition is a good indicator of the quality and stability of the oil. From the GC graphs as given in (Figure 2) and mass spectra as given in (Figure 3), the total numbers of fatty acids identified for *A. glauca* and *C. album* were 8 and 10, respectively. The analyzed (PE) plant seed extracts were rich in unsaturated fatty acids (TUFA) with 82.28% for *A. glauca* and 77.57% for *C. album*. Petroselinic acid at 74.26% was found to be the dominant monounsaturated acid in *A. glauca* compared to *Petroselinium crispum* (Ngo-Duy *et al*., 2009), but higher than other species in the Apiaceae family (Knothe and Steidley, 2019). The petroselinic acid composition found in *A. glauca* is similar to that of *Coriandrum sativum,* which is the valuable raw material for the pharmaceutical and food

industries (Delbeke *et al*., 2016); while 7.37% oleic acid was found, which is comparatively similar to other species in its family (Knothe and Steidley, 2019). 0.61% palmitic acid and 0.40% docosanoic acid were found. Linoleic acid was found to be dominant in the seed oil of *C. album* at 53.05% and oleic acid was found at 20.74%, which is similar to the Ayani variety of *C. album* from Mantaro valley (Peru) (Peiretti and Tassone, 2013). It also contained 2.01% linolenic acid, 6.32% palmitic acid, 1.67% decanoic acid, 1.11% eicosanoic acid and 0.94% docosenoic acid. Other fatty acids were found in smaller amounts, such as myristic acid (0.32 and 1.15%), stearic acid (0.79 and 2.79%), and eicosenoic acid (0.59 and 0.83%) for *A. glauca* and *C. album,* respectively.

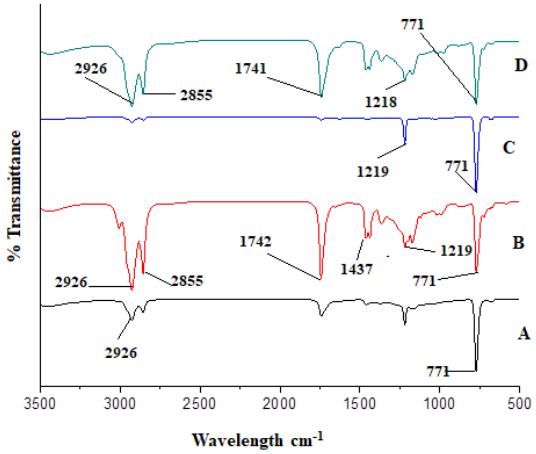

FIGURE 1. FTIR analyses of A: petroleum ether extract (PET) of *A. glauca*; B: FAME of *A. glauca*; C: petroleum ether extract (PET) of *C. album*; D: FAME of *C. album*.

3.4. Antioxidant activity

The antioxidant activities of the de-fatted seed extracts of *A. glauca* and *C. album* were determined using the DPPH radical scavenging, hydrogen peroxide, superoxide anion radical and NBT radical assay. The radical scavenging activity assay provides information about the antiradical activity of the extracts. The seed extracts were compared with standard antioxidant drug butylated hydroxyltoluene (BHT).

3.4.1. DPPH radical scavenging activity

DPPH radical scavenging assay is based on the fact that when the antioxidants present in de-fatted seed extracts react with DPPH radicals, they convert them into the yellow-colored compound di-phenyl hydrazine. The lower the absorbance of the reaction mixture, the higher is the free radical scavenging activity. As shown in Table 3, all the de-fatted seed extracts showed the good results for DPPH radical activity but the most enhanced effect was shown at 100 µg/mL and 200 µg/mL concentrations of extracts. The EA extracts of *A. glauca* at 25 µg/mL and 50µg/mL showed better results for radical scavenging compared to other extracts of the same plant ($p < 0.05$) at the same concentration with reference to BHT. All the extracts of *A. glauca* showed comparatively similar radical scavenging at 100 µg/mL and 200 µg/mL. However, at 100 µg/mL AT showed a dominant effect (81.26%) followed by EA (80.15%) and CF (78.81%); while MT showed the least inhibition (77.29%) compared to BHT (88.31%). At 200 µg/mL EA (92.73%) showed a dominant effect followed by AT (90.30%), MT (89.73%) and CF (85.84) with BHT (95.60%), which is similar to the inhibition shown by the essential oil of whole *A. glauca* plant in an earlier study with respect to BHT (Irshad *et al*., 2011). All the MT extracts of *C. album* at all concentrations showed good results compared to other extracts of the same plant. However, a dominant effect was shown by MT (79.49%), followed by AT (77.01%); while EA (65.26%) showed the least inhibition with BHT (88.31) at 100 µg/mL. At 200 µg/mL, the MT extract showed (87.73%) inhibition of radicals better than earlier reports of the whole plant (Lone *at al*., 2017), followed by EA (86.41%) and AT (84.55%) and CF (80.44%) showed the least inhibition compared to standard BHT (95.60%) at ($p < 0.05$) significance.

3.4.2. NBT radical scavenging assay

Superoxide scavenging activity was determined by the NBT assay and is depicted in Table 4. In general, the de-fatted seed extracts of *A. glauca* showed slightly better NBT radical scavenging compared to *C. album*. At 25µg/mL and 100 µg/mL the MT and EA extracts of *A. glauca* showed dominant effects followed by CF; while the least effect was shown by AT ($p < 0.05$) with respect to BHT. At concentrations of 50 µg/mL and 200 µg/mL MT and CF extracts showed dominant NBT scavenging activity followed by EA and AT. Earlier studies (Noh *et al*., 2014) found that the SOD activity of *A. gigas* (apiaceae) was 34%, which is lower than the one found in

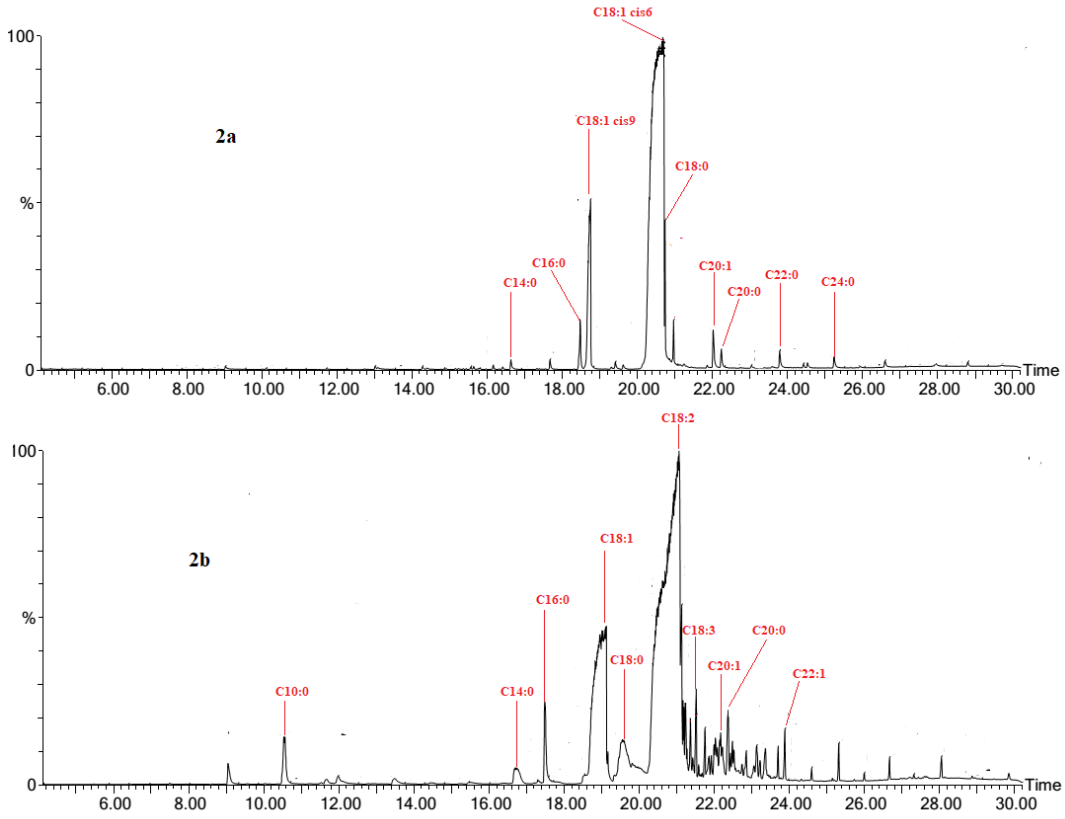

FIGURE 2. GC-MS chromatogram of the fatty acid methyl esters isolated from 2a:*A. glauca*; 2b:*C. album* seeds.

this study. This may be due to the difference in extraction method, different plant material used and contents of extracts taken. In the case of *C. album* at 25 µg/mL, MT and AT predominated; while at 50 µg/mL EA showed a dominant effect followed by MT and CF and the least inhibition of radicals was shown by AT extract compared to positive standard BHT. At 100 µg/mL, MT showed a dominant inhibition of radicals followed by EA; while the least effect was shown by AT and CF. However, at 200 µg/mL, EA and MT showed dominant scavenging followed by the CF and AT extracts of *C. album* ($p < 0.05$) with respect to BHT (Table 4). These results are comparatively better than those shown by *Chenopodium murale* extracts at similar concentrations (Khan *et al*., 2019).

3.4.3. H_2O_2 radical scavenging assay

The results for H_2O_2 radical scavenging activity of extracts is shown in Table 5. At 25 µg/mL and 50 µg/mL, the dominant radical scavenging was shown by the MT (31.05% and 55.79%) and AT (28.80 and 54.97%) seed extracts of *A. glauca* at ($p < 0.05$), respectively, with respect to BHT. AT displayed dominant (78.96 and 87.63%) inhibition followed by MT (75.49 and 83.12%) with BHT, which showed (79.06 and 92.44%) at 100 µg/mL and 200 µg/mL, respectively ($p < 0.05$), which is comparably similar to the earlier results shown by the Methanolic plant extract of *Withania somnifera* 84.49% and better than *Petroselinium crispum* 60.27% (Tupe *et al*., 2013). The CF extracts of *A. glauca* showed better inhibition and the least inhibition was observed in EA at 100 µg/mL and 200 µg/mL ($p < 0.05$) with respect to BHT. In *C. album* at 25 µg/mL and 50 µg/mL concentrations of extracts radical scavenging was shown in the following increasing order of magnitude: MT > EA > AT > CF. At 100 µg/mL and 200 µg/mL MT was shown to be dominant (72.42 and 87.67%) with BHT (79.06 and 92.44%) inhibition followed by CF and EA and AT showed the least inhibition ($p < 0.05$). Lone *et al.,* (2017) reported that the Methanol extract of *C. album* showed the highest H_2O_2 radical scavenging inhibition of 94% at 300 µg/mL with respect to BHT.

3.4.4. ABTS radical scavenging activity

Radical scavenging activity (ABTS) is shown in (Table 6). At 25 µg/mL and 100 µg/mL EA and AT extracts of *A. glauca* showed powerful radical inhibition effect while as CF and MT extracts of same plant showed normal inhibition ($p < 0.05$) with respect to BHT. At 50 µg/mL EA and MT extracts of *A. glauca* showed dominant effect followed by AT while CF shows least inhibition. At 200 µg/mL almost all seed extracts of *A. glauca* showed comparatively same results with the following increasing order of radical scavenging EA (91.24%) > MT (90.41) > AT (89.72) > CF (80.19) with BHT showed (93.12) ($p < 0.05$). Earlier reports on ABTS radical scavenging have found that the plant extracts of *Terminalia chebula*, *Salacia reticulate*, *Hemidesmus indicus*, *Aegle marmelos* and *Terminalia arjun* showed 100, 99.95, 99.43, 98.91 and 98.91 % inhibition respectively (Tupe *et al.*, 2013). This increase in % inhibition is mainly due to high concentration of plant extracts taken.

For *C. album* at 25 µg/mL and 200 µg/mL all seed extracts show good ABTS scavenging activity with the increasing order of their radical scavenging CF > EA > MT > AT ($p < 0.05$). At 50 µg/mL the radical scavenging results are almost similar with CF (61.64%), MT (60.17%), EA (59.99%) and AT (57.90%) with BHT (71.46%) and at 100 µg/mLEA, MT and CF showed dominant effect while AT showed least inhibition (Table 6). Adedapo *et al.,* (2011) in earlier studies on this plant found that the acetone leaf extract possesses 52-53% and 66% inhibition of ABTS radicals at 50 µg/mL and 100 µg/mL respectively, while they found methanol extracts have relatively better inhibition capacity.

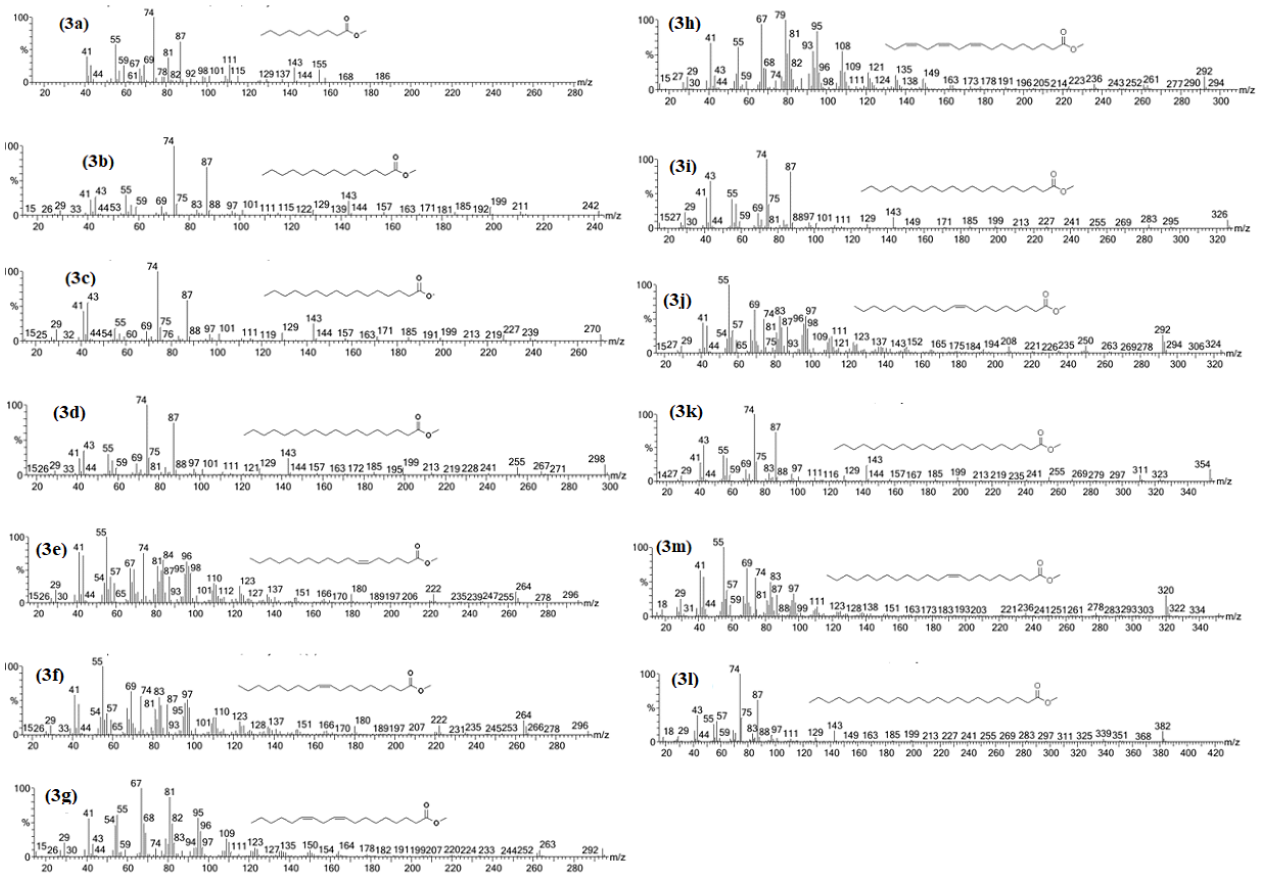

FIGURE 3. GC-MS spectra of 3a: Methyl decanoate; 3b: Methyl tetradecanoate; 3c: Methyl palmitate; 3d: Methyl stearate; 3e: Methyl petroselinate; 3f: Methyl oleate; 3g: Methyl linoleate; 3h: Methyl linolenate; 3i: Methyl eicosanoate; 3j: Methyl 9-eicosenoate; 3k: Methyl docosanoate; 3m: Methyl docosenoate, 3l: Methyl tetracosanoate.

TABLE 3. 2,2-di-phenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of defatted seed extracts of *A. glauca* and *C. album*.

Seed extracts	*A. glauca* Concentration (µg/mL)				*C. album* Concentration (µg/mL)			
	25	**50**	**100**	**200**	**25**	**50**	**100**	**200**
Chloroform	30.12 ± 0.02^{e}	61.62 ± 0.04^{e}	78.81 ± 0.12^{d}	85.84 ± 0.04^{e}	21.60 ± 0.22^{e}	44.21 ± 0.07^{e}	69.92 ± 0.09^{d}	80.44 ± 0.06^{e}
Ethyl acetate	36.58 ± 0.03^{b}	68.03 ± 0.07^{b}	80.15 ± 0.02^{c}	92.73 ± 0.07^{b}	28.55 ± 0.14^{c}	49.02 ± 0.16^{c}	65.26 ± 0.12^{c}	86.41 ± 0.08^{c}
Acetone	33.52 ± 0.01^{c}	64.23 ± 0.09^{d}	81.26 ± 0.16^{b}	90.30 ± 0.07^{c}	24.63 ± 0.17^{d}	47.13 ± 0.26^{d}	77.01 ± 0.09^{c}	84.55 ± 0.12^{d}
Methanol	31.55 ± 0.07^{d}	66.08 ± 0.17^{c}	77.29 ± 0.03^{e}	89.73 ± 0.07^{d}	30.69 ± 0.16^{b}	63.01 ± 0.10^{b}	79.49 ± 0.04^{b}	87.73 ± 0.09^{b}
BHT	39.33 ± 0.03^{a}	74.80 ± 0.02^{a}	88.31 ± 0.05^{a}	95.60 ± 0.06^{a}	39.33 ± 0.03^{a}	74.80 ± 0.02^{a}	88.31 ± 0.05^{a}	95.60 ± 0.06^{a}

Data are expressed as means ± S.D (n=3).
The different letters in each column are statically significant different at (p< 0.05) by one way ANOVA and Duncan's test.BHT: Butylated hydroxytoluene.

TABLE 4. Nitroblue tetrazolium (NBT) radical scavenging activity of defatted seed extracts of *A. glauca* and *C. album*.

Seed extracts	*A. glauca* Concentration (µg/mL)				*C. album* Concentration (µg/mL)			
	25	**50**	**100**	**200**	**25**	**50**	**100**	**200**
Chloroform	22.70 ± 0.03^{d}	37.15 ± 0.04^{c}	51.31 ± 0.03^{d}	64.37 ± 0.03^{c}	16.67 ± 0.01^{d}	31.20 ± 0.05^{d}	43.76 ± 0.07^{c}	64.04 ± 0.03^{d}
Ethyl acetate	24.63 ± 0.03^{b}	36.49 ± 0.07^{d}	52.79 ± 0.02^{c}	62.64 ± 0.08^{d}	14.04 ± 0.13^{e}	34.81 ± 0.20^{b}	50.94 ± 0.15^{c}	68.29 ± 0.01^{b}
Acetone	21.96 ± 0.20^{e}	34.69 ± 0.16^{e}	46.14 ± 0.05^{e}	60.14 ± 0.04^{e}	19.79 ± 0.03^{c}	28.53 ± 0.02^{e}	45.24 ± 0.02^{d}	60.35 ± 0.04^{e}
Methanol	24.18 ± 0.02^{c}	38.67 ± 0.03^{b}	54.27 ± 0.01^{b}	68.64 ± 0.01^{b}	20.57 ± 0.09^{b}	32.27 ± 0.25^{c}	53.12 ± 0.02^{b}	66.79 ± 0.01^{c}
BHT	30.96 ± 0.02^{a}	51.18 ± 0.08^{a}	73.39 ± 0.08^{a}	82.21 ± 0.05^{a}	30.96 ± 0.02^{a}	51.18 ± 0.08^{a}	73.39 ± 0.08^{a}	82.21 ± 0.05^{a}

Data are expressed as means ± S.D (n=3).
The different letters in each column are statically significant different at (p< 0.05) by one way ANOVA and Duncan's test.
BHT: Butylated hydroxytoluene.

TABLE 5. Hydrogen peroxide (H_2O_2) radical scavenging activity of defatted seed extracts of *A. glauca* and *C. album*.

Seed extracts	*A. glauca* Concentration (µg/mL)				*C. album* Concentration (µg/mL)			
	25	**50**	**100**	**200**	**25**	**50**	**100**	**200**
Chloroform	20.16 ± 0.09^{e}	39.99 ± 0.09^{d}	69.37 ± 0.06^{d}	82.10 ± 0.01^{d}	14.86 ± 0.10^{e}	36.32 ± 0.11^{e}	65.18 ± 0.07^{c}	85.57 ± 0.01^{c}
Ethyl acetate	24.77 ± 0.03^{d}	38.38 ± 0.34^{e}	62.27 ± 0.08^{e}	76.08 ± 0.05^{e}	22.48 ± 0.09^{c}	48.99 ± 0.10^{c}	64.95 ± 0.10^{cd}	78.86 ± 0.06^{d}
Acetone	28.80 ± 0.11^{c}	54.97 ± 0.05^{c}	78.96 ± 0.04^{ab}	87.63 ± 0.01^{b}	20.94 ± 0.06^{d}	41.39 ± 0.05^{d}	61.81 ± 0.06^{c}	75.85 ± 0.04^{e}
Methanol	31.05 ± 0.09^{b}	55.79 ± 0.08^{b}	75.49 ± 0.07^{c}	83.12 ± 0.09^{c}	28.08 ± 0.08^{b}	58.12 ± 0.09^{b}	72.42 ± 0.05^{b}	87.67 ± 0.04^{b}
BHT	35.18 ± 0.10^{a}	68.68 ± 0.07^{a}	79.06 ± 0.06^{a}	92.44 ± 0.01^{a}	35.18 ± 0.10^{a}	68.68 ± 0.07^{a}	79.06 ± 0.06^{a}	92.44 ± 0.01^{a}

Data are expressed as means ± S.D (n=3).
The different letters in each column are statically significant different at (p< 0.05) by one way ANOVA and Duncan's test.
BHT: Butylated hydroxytoluene.

TABLE 6. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of defatted seed extracts of *A. glauca* and *C. album*.

Seed extracts	*A. glauca* Concentration (µg/mL)				*C. album* Concentration (µg/mL)			
	25	50	100	200	25	50	100	200
Chloroform	28.55 ± 0.03^{d}	53.58 ± 0.05^{e}	73.38 ± 0.09^{e}	80.19 ± 0.01^{e}	32.15 ± 0.03^{b}	61.64 ± 0.05^{b}	76.71 ± 0.07^{d}	88.22 ± 0.03^{b}
Ethyl acetate	34.73 ± 0.10^{b}	59.42 ± 0.43^{b}	78.03 ± 0.02^{b}	91.24 ± 0.10^{b}	31.79 ± 0.01^{c}	59.99 ± 0.02^{cd}	78.51 ± 0.01^{b}	87.17 ± 0.04^{c}
Acetone	32.84 ± 0.06^{c}	56.48 ± 0.10^{d}	77.04 ± 0.05^{c}	89.72 ± 0.03^{d}	29.06 ± 0.06^{e}	57.90 ± 0.46^{e}	72.19 ± 0.07^{e}	84.77 ± 0.04^{e}
Methanol	27.83 ± 0.06^{e}	58.73 ± 0.12^{c}	73.62 ± 0.08^{d}	90.41 ± 0.10^{c}	30.53 ± 0.06^{d}	60.17 ± 0.14^{c}	77.28 ± 0.06^{c}	85.70 ± 0.11^{d}
BHT	44.24 ± 0.15^{a}	71.46 ± 0.02^{a}	83.57 ± 0.09^{a}	93.12 ± 0.01^{a}	44.24 ± 0.15^{a}	71.46 ± 0.02^{a}	83.57 ± 0.09^{a}	93.12 ± 0.01^{a}

Data are expressed as means ± S.D (n=3).

The different letters in each column are statically significant different at ($p< 0.05$) by one way ANOVA and Duncan's test.

BHT: Butylated hydroxytoluene.

4. CONCLUSIONS

The present study indicates that *A. glauca* and *C. album*are potent medicinal plants with their seeds being rich in unsaturated fatty acids especially petroselinic acid (an uncommon positional isomer of oleic acid) and linoleic acid respectively. They also contain significant oleic acid. Moreover, due to their powerful DPPH, NBT, H_2O_2 and ABTS radical scavenging activities which suggest that the seed extracts of worked plants could be used as non-exploited, natural source in future food and pharmaceutical industries. However, more work is needed to isolate target product. Also, green extraction methods, cost effective analysis and safety measurements should be taken in concentration to determine the commercial potential of these plants. Anyhow this encouraging work adds more knowledge to existing literature of these plants from Kashmir.

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REFERENCES

Adedapo A, Jimoh F, Afolayan A. 2011. Comparison of the nutritive value and biological activities of the acetone, methanol and water extracts of the leaves of *Bidens pilosa* and *Chenopodiumalbum*. *Acta Pol. Pharm*. **68**, 83-92.

Ahmad R, Ahmad I, Mannan A, Ahmad F, Osman SM. 1986. Studies on minor seed oils XI. *Fette, Seifen, Anstrichm.* **88**, 147-148. https://doi.org/10.1002/lipi.19860880408

Ahmad M, Mohiuddin OA, Jahan N, Anwar MUNIR, Habib S, Alam SM, Baig IA. 2012. Evaluation of spasmolytic and analgesic activity of ethanolic extract of *Chenopodium album* Linn. and its fractions. *J. Med. Plants Res.* **6**, 4691-4697.

Ankita J, Chauhan RS. 2012. Evaluation of anticancer activity of *Chinopodium album* leaves in BHK-21 cells. *Int. J. Univ. Pharm. Bio. Sci.* **1**, 92-102.

Binic I, Lazarevic V, Ljubenovic M, Mojsa J, Sokolovic D. 2013. Skin ageing: natural weapons and strategies. *Evidence-Based Comp. Alt. Med.* **1**, 827-248.

Chen SH, Chuang YJ. 2002. Analysis of fatty acids by column liquid chromatography. *Anal. Chim. Act.* **465**, 145-155. https://doi.org/10.1016/s0003-2670(02)00095-8

Chopra RN, Nayar SL, Chopra IC. 1956. Glossary of Indian Medicinal Plants, CSIR, New Delhi, pp.4-5.

Delbeke EI, Everaert J, Uitterhaegen E, Verweire S, Verlee A, Talou T, Soetaert W, Van Bogaert IN, Stevens CV. 2016. Petroselinic acid purification and its use for the fermentation of new sophorolipids. *Amb Exp.* **6**, 28. https://doi.org/10.1186/s13568-016-0199-7

Irshad M, Shahid M, Aziz S, Ghous T. 2011. Antioxidant, antimicrobial and phytotoxic activities of essential oil of *Angelica glauca*. *Asian J. Chem.* **23**, 1947.

Khan N, Ahmed M, Khan RA, Gul S. 2019. Antioxidant, cytotoxicity activities and phytochemical analysis of *Chenopodium murale* (Linn.). *Int. J. Bot.* **4**, 25-28.

Knothe G, Steidley KR. 2019. Composition of Some Apiaceae Seed Oils Includes Phytochemicals, and Mass Spectrometry of Fatty Acid 2-Methoxyethyl Esters. *Eur. J. Lipid Sci. Technol.* **121**, 1800386. https://doi.org/10.1002/ejlt.201800386

Koleva II, Van Beek TA, Linssen JP, Groot AD, Evstatieva LN. 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis: *An Int. J. Plant Chem. Biochem. Tech.* **13**, 8-17. https://doi.org/10.1002/pca.611

Kozlowska M, Zbikowska A, Gruczynska E, Zontała K, Połtorak A. 2014. Effects of spice extracts on lipid fraction oxidative stability of cookies investigated by DSC. *J. Therm. Anal. Calorim.* **118**, 1697-1705. https://doi.org/10.1007/s10973-014-4058-y

Lampi AM, Kamal-Eldin A. 1998. Effect of α-and γ-tocopherols on thermal polymerization of purified high-oleic sunflower triacylglycerols. *J. Am. Oil Chem. Soc.* **75**, 1699-1703. https://doi.org/10.1007/s11746-998-0319-x

Link WE. 1973. *Official and Tentative Methods of the American Oil Chemist's Society, third ed.* AOCS, Champaigh, 11, USA (Methods Da 15-48 and Da 16-48).

Lone BA, Chishti MZ, Bhat FA, Tak H, Bandh SA, Khan A. 2017. Evaluation of anthelmintic antimicrobial and antioxidant activity of *Chenopodium album*. *Trop. Anim. Health Prod.* **49**, 1597-1605. https://doi.org/10.1007/s11250-017-1364-y

Nehdi IA, Sbihi H, Tan CP, Zarrouk H, Khalil MI, Al-Resayes SI. 2012. Characteristics, composition and thermal stability of *Acacia senegal* (L.) Willd. seed oil. *Ind. Crops Prod.* **36**, 54-58. https://doi.org/10.1016/j.indcrop.2011.08.005

Ngo-Duy CC, Destaillats F, Keskitalo M, Arul J, Angers P. 2009. Triacylglycerols of Apiaceae seed oils: Composition and regiodistribution of fatty acids. *Eur. J. Lipid Sci. Technol.* **111**, 164-169. https://doi.org/10.1002/ejlt.200800178

Noh BY, Lee HJ, Do JR, Kim HK. 2014. Antioxidant and ACE inhibitory activity of cultivated and wild Angelica gigas Nakai extracts prepared using different extraction conditions. *Prev. Nut. Food Sci.* **19**, 274-280. https://doi.org/10.3746/pnf.2014.19.4.274

Pal A, Banerjee B, Banerjee T, Masih M, Pal K. 2011. Hepatoprotective activity of *Chenopodium album* Linn. plant against paracetamol induced hepatic injury in rats. *Int. J. Pharm. Pharm. Sci.* **3**, 55-57.

Peiretti PG, Gai F, Tassone S. 2013. Fatty acid profile and nutritive value of quinoa (*Chenopodium quinoa* Willd.) seeds and plants at different growth stages. *Anim. Feed Sci. Technol.* **183**, 56-61. https://doi.org/10.1016/j.anifeedsci.2013.04.012

Prajapati ND, Purohit SS, Sharma AK, Kumar TA. 2003. Hand Book of Medicinal plants: A Complete Source Book. Agrobios, India, p 134.

Raaman N. 2006. *Phytochemical techniques*. New India Publishing. Agency, New Delhi, India, P. 10.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical decolorization assay. *Free Rad. Bio. Med.* **26**, 1231-1237. https://doi.org/10.1016/s0891-5849(98)00315-3

Ruch RJ, Cheng SJ, Klaunig JE. 1989. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogen.* **10**, 1003-1008. https://doi.org/10.1093/carcin/10.6.1003

Sharma S, Gangal S, Rauf A. 2009. Lipase mediated hydrolysis of *Mimusops elengi* and *Parkinsonia aculeata* seed oils for the determination of positional distribution of fatty acids. *Ind. Crops Prod.* **30**, 325-328. https://doi.org/10.1016/j.indcrop.2009.04.004

Shimada K, Fujikawa K, Yahara K, Nakamura T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* **40**, 945-948. https://doi.org/10.1021/jf00018a005

Tupe RS, Kemse NG, Khaire AA. 2013. Evaluation of antioxidant potentials and total phenolic contents of selected Indian herbs powder extracts. *Int. Food Res. J.* **20**, 1053-1063.

Usman LA, Hamid AA, Muhammad NO, Olawore NO, Edewor TI, Saliu BK. 2010. Chemical constituents and anti-inflammatory activity of leaf essential oil of Nigerian grown *Chenopodium album L. EXCLI J.* **9**, 181-186.

Vyas D, Kumar S. 2005. Purification and partial characterization of a low temperature responsive Mn-SOD from tea (*Camellia sinensis* (L.) O. Kuntze). *Biochem. Biophy. Res. Commun.* **329**, 831-838.

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Quality attributes of roasted Arabica coffee oil extracted by pressing: composition, antioxidant activity, sun protection factor and other physical and chemical parameters

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SUMMARY: This research reports a comprehensive characterization of the composition profile and physical and chemical characteristics of roasted Arabica coffee oil obtained by mechanical pressing. The oil presented a peroxide value of 3.21 meq·kg^{-1} and an acid value of 7.3 mg KOH·g^{-1}. A higher proportion of unsaturated fatty acids (58%), predominantly linoleic (L) and palmitic (P) acids, was observed; PLL and PLP were estimated as the main triacylglycerols. The oil was characterized by high contents in diterpenes and tocopherols (3720 and 913 mg·100g^{-1}, respectively), the presence of caffeine and chlorogenic acids, as well as a high sun protection factor (9.7) and ABTS free radical-scavenging capacity (12.5 mg Trolox·mL^{-1}). Among the 35 volatile compounds studied, furfurythiol and pyrazines were the main components of the oil. These properties showed that roasted coffee oil has good potential for use in food and cosmetics.

KEYWORDS: *Coffee Arabica; Diterpenes; Tocopherols; Volatile compounds*

RESUMEN: ***Atributos de calidad del aceite de café Arábica tostado extraído por prensado: composición, actividad antioxidante, factor de protección solar y otros parámetros físicos y químicos.*** Esta investigación reporta una caracterización completa del perfil de composición y características físicas y químicas del aceite de café Arábica tostado obtenido por prensado mecánico. El aceite presentó un índice de peróxido de 3,21 meq·kg^{-1} y un índice de acidez de 7,3 mg de KOH·g^{-1}. Se observó una mayor proporción de ácidos grasos insaturados (58%), ácido linoleico, (L) y palmítico (P); PLL y PLP se estimaron como los principales triacilgliceroles. El aceite se caracterizó por un alto contenido de diterpenos y tocoferoles (3720 y 913 mg·100g^{-1}, respectivamente), la presencia de cafeína y ácidos clorogénicos, así como un alto factor de protección solar (9,7) y capacidad de captación de radicales libres ABTS (12,5 mg de Trolox·mL^{-1}). Entre los 35 compuestos volátiles estudiados, el furfuritiol y las pirazinas fueron los componentes principales del aceite. Estas propiedades mostraron que el aceite de café tostado tiene un buen potencial para su uso en alimentos y cosméticos.

PALABRAS CLAVE: *Café Arabica; Compuestos volátiles; Diterpenos; Tocoferoles*

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1. INTRODUCTION

Coffee is one of the most popular beverages worldwide. In the past 10 years, global coffee production has grown at an average annual rate of around 2.6% from 140.16 million 60-kg bags in 2010/11 to an estimated 168.71 million 60-kg bags in 2019/20. Brazil is the world's second-largest coffee consumer. In addition to being the main producer (57 million in 2019/20), in 2019 Brazil was also the world's largest exporter (37.7 million up to November), and soluble coffee represented around 10% of this total (Ico, 2019).

The mechanical pressing of coffee beans, green (raw) or roasted, is the most common industrial methods for oil extraction in Brazil (Oliveira *et al.,* 2005). Iy is eco-friendly, and does not require the use of any solvents. Roasted coffee oil is a co-product of the soluble coffee industry, and can be obtained by pressing the roasted beans before extraction of the soluble coffee. The roasted coffee oil is applied as a food flavoring, while green coffee oil is used in cosmetic formulations due to its antioxidant, emollient and UV protection properties (Calligaris *et al.,* 2009; Wagemaker *et al.,* 2011; Hurtado-Benavides *et al.,* 2016).

Lipids are among the most abundant coffee components, accounting for 3.2 to 11% of the total green beans and 8.6 to 17% of the roasted coffee. The increase in lipid content with the roasting process is due to losses in CO_2, water vapor and volatile compounds, and the degradation of carbohydrates, amino acids, and chlorogenic acids (Budryn *et al.,* 2012; Dias *et al.,* 2014; Pacetti *et al.,* 2015). Owing to their relatively high thermal stability, lipids protect aromatic compounds from degradation (Wagemaker *et al.,* 2011). The lipid fraction contains the majority of the volatile compounds responsible for the aroma (Calligaris *et al.,* 2009; Wagemaker *et al.,* 2011; Hurtado-Benavides *et al.,* 2016). It also contributes to coffee brew viscosity (Pacetti *et al.,* 2015). Triacylglycerols are the main components of coffee oil (about 75%), which also presents from 15 to 18% of the unsaponifiable matter (UM) (Speer and Kölling-Speer, 2006), composed of hydrocarbons, steroids, and tocopherols (Belitz *et al*., 2009). It has a high proportion of UM compared to other vegetable oils (0.2 - 1.5 %), such as soybean (from 0.6 to 1.2%), olive (from 0.4 to 1.1%), and sunflower (from 0.3 to 1.2 %) (Belitz *et al*., 2009).

Coffee oil composition varies with harvesting and post-harvest handling practices, bean origin and genetics (species and varieties), as well as roasting and extraction conditions (Pacetti *et al.,* 2015). The literature reports some data on coffee oil from the Arabica and Robusta species, although the majority of them are related to green coffee - extracted with solvents or more sophisticated methods (such as supercritical extraction). In general, researches focus on specific classes of compounds, such as fatty acids and volatile compounds (Oliveira *et al.,* 2005; Calligaris *et al.,* 2009; Budryn *et al.,* 2012; Getachew and Chun, 2016; Hurtado-Benavides *et al.,* 2016; Raba *et al.,* 2018). Less information is available on UM compounds –such as diterpenes and tocopherols– and on the presence of hydrosoluble components, which could be carried during pressing, such as caffeine and chlorogenic acids (González *et al.,* 2001; Oliveira *et al.,* 2014; Guercia *et al.,* 2016; Bitencourt *et al.,* 2018).

Regarding the physico-chemical characteristics, which are essential for technological use, some studies focus on quality indices such as the peroxide value (Turatti, 2001; Budryn *et al.,* 2012; Getachew and Chun, 2016) and thermal properties (Calligaris *et al.,* 2009; Budryn *et al.,* 2012; Raba *et al.,* 2018), generally correlating these parameters with the fatty acid profile.

Considering the interest and potential use of roasted coffee oil as a food ingredient as well as in the cosmetic area, where green coffee oil is more common nowadays, this study aimed to report a comprehensive characterization of the composition profile and properties of roasted Arabica coffee oil obtained by mechanical pressing.

2. MATERIALS AND METHODS

2.1. Materials

The coffee oil was supplied by Company Iguaçu Soluble Coffee (Cornélio Procópio, Brazil). Commercial dry Arabica coffee beans (4.5 to 5.0% w/w moisture) were medium roasted at 220 °C (air temperature) and 5 mbar for 10 to 12 min. The extraction was carried out at room temperature by cold pressing in an oil expeller SCOTTECH ERT 50 (Scott Tech USA, USA); the coffee reached a maximum of 60 °C during the process. The efficiency of the extraction was around 5 to 6% of oil (w/w). The oil was kept in a freezer at -22 °C until analysis.

2.1.1. Reagents and standards

The HPLC-grade solvents were *tert*-butyl methyl ether (Acros Organics, USA), acetonitrile (Mallinckrodt Baker, USA), and methanol (Merck, Germany). The following reagents and analytical grade materials were also used: potassium hydroxide (Quimex, Brazil), ethanol 98% (JTBaker, Mexico), sulfuric acid 95-97% (Merck, Germany), hydrochloric acid (Quimex, Brazil), sodium hydroxide (Sigma-Aldrich, USA), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, USA), acetic acid (Merck, Germany), ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, USA), potassium persulfate (Anidrol, Brazil), ethyl acetate (Sigma-Aldrich, USA), sodium thiosulphate (Synth, Brazil),Wijs solution (Anidrol, Brazil), potassium iodide (Synth, Brazil), carbon tetrachloride (Dinâmica, Brazil) and chloroform (Synth, Brazil).The water used to prepare standards and solutions was obtained by a purification system Elga Purelab Option-Q (Veolia Water Technologies, France). Nylon membranes were applied for filtration of solvents (Millipore, USA) and samples (0.22 µm) (Whatman, UK). Standards of 5-caffeoylquinic acid (5-CQA), caffeine, fatty acid methyl esters (FAME Mix C4-C24) and tocopherols (α, β, γ, and δ) (Sigma-Aldrich, USA), and cafestol and kahweol (Axxora, USA) were used. For the volatile profile, the following standards were used: 2-3-dimethylpyrazine, pyrazine, 4-methylthiazole, 2-isobutyl-3-methylpyrazine, 2,3-butanedione, 2,3-pentanedione, acetoin, benzyl alcohol, maltol, furaneol, furfuryl acetate, 3-methylbutanal, 2,5-dimethylpyrazine, pyridine, 2,6-dimethylpyrazine, 4,5-dimethylthiazole, 2-furfurylthiol, 2-acetylpyridine, vanillin, phenylethyl alcohol, 4-ethylguaiacol, 4-vinylguaiacol, cis-isoeugenol, isovaleric acid, methanethiol, dimethyldisulfite, acetic acid, propanoic acid, acetaldehyde, guaiacol, 2,3-diethyl-5-methylpyrazine, furfural, linalool, 2-isobutyl-3-methoxypyrazine and 2-acetyl-3,5-dimethylpyrazine (Sigma Aldrich, USA).

2.2. Physico-chemical analyses

The acid, iodine, and peroxide values were determined according to AOCS (2014). The peroxide value was determined using titrator TitroLine easy (Schott, Germany) with a 0.1 N sodium thiosulphate solution; results were expressed as meq of peroxide·kg^{-1}. The iodine value was determined by the Wijs method using a 0.1 N sodium thiosulphate solution; results were expressed as g of I_2·100 g^{-1}. The saponification value was determined by the fatty acid composition, and was expressed as mg KOH·g^{-1}. All analyses were performed in triplicate.

The moisture and volatile matter were determined in triplicate according to the AOCS (2014) and expressed as a percentage. The oil (5 g) was oven-dried with air circulation TE-394/1 (Tecnal, Brazil) at 130 °C for 2 h.

The antioxidant capacity was estimated based on the ABTS free radical scavenging capacity as described by Corso *et al*., (2016). The ABTS^{+} solution was produced by reacting 7 mmol·L^{-1} of a ABTS stock solution with 2.45 mmol·L^{-1} of potassium persulfate solution; the mixture stood in the dark at room temperature for 12-16 h prior to use. The ABTS^{+} solution was diluted with 5 mmol·L^{-1} phosphate buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. Ethyl acetate (1:12) was used for dilution. After the addition of 10 µL of the sample or standard Trolox in 4 mL of ABTS^{+} solution diluted, 6 min was taken for reaction, and 730 nm readings were performed on a UV-visible Libra S22 spectrophotometer (Biochrom, UK). Quantification was performed using the 5-point analytical curve (in triplicate) with Trolox. The analysis was performed in duplicate, and the results were expressed as mg Trolox·mL^{-1}.

2.3. Physical analysis

The refractive index was determined, in triplicate, in a refractometer RM 40 (Mettler Toledo, USA) at 20 ºC.

The density was determined by an electronic digital densimeter, model DMA-35 (Anton Paar, Austria) using 10 mL of coffee oil; the result was expressed as g·mL^{-1}. The viscosity was evaluated in a Viscometer DV-II (Brookfield, USA) and expressed in mPas. The analyses were performed at 25 ºC in triplicate.

In order to determine the sun protection factor (SPF) in vitro, the oil was dissolved in ethyl alcohol at the concentration of 0.2 µL·mL^{-1}. Three-fold readings were performed in the range of 290 to 320 nm (in 5 nm increments) in a UV-visible spectrophotometer Libra S22. The absorbance was multiplied by the erythemal effect of the radiation at each wavelength (Table 1), and the sum of the values was multiplied by a

correction factor (determined according to two sunscreens with known SPF), as described by Wagemaker *et al.,* (2011) (Eq. 1).

TABLE 1. Normalized product function used for sun protection factor calculation.

Wavelength (nm)	EEx I (normalized)[a]
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180

[a]EE (λ) = erythemal effect spectrum; I (λ) = solar intensity spectrum.

$$SPF = CF \cdot \sum_{290}^{320} EE(\lambda) \cdot I(\lambda) \cdot Abs(\lambda) \quad \text{(Eq. 1)}$$

Where:
CF = correction factor (= 10);
EE (λ) = erythemal effect spectrum;
I (λ) = solar intensity spectrum;
Abs (λ) = absorbance of the sunscreen product.

2.4. Chemical composition

2.4.1. Fatty acids and triacylglycerols

The hydrolysis and transesterification of the fatty acids were performed according to ISO method 5509, using 2 mol·L^{-1} NaOH in methanol and n-heptane, in triplicate. After separation, the phase containing n-heptane and fatty acid methyl esters was stored in an amber vial at -18 °C until analysis. Methyl esters of fatty acids were analyzed using CG Shimadzu 17A (Kyoto, Japan) equipped with a flame ionization detector and a CP SIL 88 capillary column (100m x 0.25 mm) (Agilent Technologies Inc., USA). The column temperature was programmed as follows: 65 °C (15 min); raised at 10 °C·min^{-1} until 165 °C and held for 2 min; raised at 4 °C·min^{-1} to 185 °C and held for 8 min; raised at 4 °C·min^{-1} to 235 °C and held for 5 min. The detector and injector were maintained at 260 ºC, using 1/100 Split. The gas flow rate was 1.2 mL.min^{-1} for the carrier gas (H_2) and 30 mL·min^{-1} for make-up gas (N_2). Identification of the fatty acids was based on comparison with standards and the results were expressed as relative percentages of the fatty acids identified.

Oil composition in triacylglycerols (TAG) was estimated by software available in the Plataforma Lames (2019) based on the fatty acid profile. This method results in a large number of TAGs, and in order to reduce the number of components, all structural isomers were divided into a set of components with the same number of carbon and double bonds. Each set of isomers was named according to the major TAG and groups with a total TAG content lower than 0.5% (w/w) were not considered, as suggested by Bitencourt *et al.,* (2018).

2.4.2. Diterpenes

Extraction was performed according to Dias *et al.,* (2014), in duplicate. Samples (0.2 g) were saponified with 2.0 mL of 2.5 mol·L^{-1} potassium hydroxide in ethanol (96% v / v) at 80 °C for 1 h. For the extraction of the unsaponifiable matter, 2.0 mL of distilled water and 2.0 mL of *tert*-butyl methylether were added. After stirring and centrifugation at room temperature (3 min at 3000 rpm), the organic phase was collected. The last step was repeated 3 times. Distilled water (2 mL) was added for cleaning, and the organic extract was collected and evaporated to dryness in a water bath (70 °C) and re-suspended in the mobile phase.

The analysis was performed as described by Mori *et al.,* (2016), using UPLC Waters Acquity (Waters, Milford, USA) equipped with an automatic sample injector, solvent quaternary pumping system, column oven, and DAD detector, controlled by the Empower 3 program. Detection was set at 230 nm (cafestol) and 290 nm (kahweol). Kinetex C18 column (150 mm x 4.6 mm, 2.6 µm) (Phenomenex, USA) and volume of injection of 1.4 µL were used. Isocratic elution with water: acetonitrile (45:55 v / v) at a flow rate of 1.2 mL·min^{-1} was performed. The analyses were made in duplicate.

Quantification was performed by external standardization using triplicate 6-point analytical curves ($r \geq 0.999$, $p < 0.001$), with a limit of quantification (LQ) of 3.2 mg·100 g^{-1} and 3.6 mg·100g^{-1} for kahweol and cafestol, respectively. The results were expressed as contents of kahweol and cafestol and as total diterpenes (mg·100 g^{-1}).

2.4.3. Tocopherols

The tocopherol profile was determined based on the AOCS Ce 8-89 methodology (AOCS, 2014). The oil was directly solubilized in hexane (1% w/v). A Lab Alliance LC305 HPLC (Scientific Systems, Inc., USA) with Radpump III pump and LC 305 fluorescence detector and a LiChrospher Si 60 column (125 mm x 4 mm, 5 µm) (Merck, Germany). Fluorescence excitation was set at 325 nm and emission at 480 nm. Isocratic elution was performed with hexane: ethyl acetate: glacial acetic acid (98: 1.3: 0.7% v / v / v), at a flow rate of 1.5 mL·min^{-1} and an injection volume of 250 µL.

Quantification was performed by external standardization using triplicate 6-point analytical curves for each compound (α, β, γ, and δ-tocopherol), with LQ of 0.1 mg·100 g^{-1}. The results were expressed as individual tocopherols and as total tocopherol (mg·100 g^{-1}).

2.4.4. Caffeine and chlorogenic acids

Extraction was performed as described by Carvalho *et al.*, (1990), in triplicate. Coffee oil (2 g), water (200 mL), and MgO (5 g) were boiled for 45 min. After cooling and filtration, 4 mL of a sulfuric acid solution (1:9 acid:water) and 20 mL of chloroform were added to the mixture in a separatory funnel. After stirring, the chloroform layer was transferred to another funnel; the step was repeated five times. A potassium hydroxide solution 1% (5 mL) was then added to the extract, and after stirring and phase separation, the extract was filtered and diluted with chloroform.

A chromatographic analysis was performed according to Corso *et al.*, (2016), using a Shimadzu HPLC (Kyoto, Japan) with two pumps (LC-10 AD), a Rheodyne injection valve with 20 µL loop, a UV/visible detector (SPD-10 A), CBM-101 interface and Program CLASS-CR10, version 1.2. A Spherisorb ODS1 column (250 × 4.6 mm, 5 µm) (Waters, Ireland) was used, and detection was set at 272 nm (caffeine) and 320 nm (chlorogenic acids). A gradient of 5% acetic acid (A) and acetonitrile (B) solution was used as follows: 0-10 min: 5% B; 10-25 min: 13% B; 25-35 min: 5% B, flow rate 0.5 mL min^{-1}. The injections were made in duplicate.

The quantification was performed by external standardization using duplicate 6-point analytical curves ($r \geq 0.999$, $p < 0.001$). The sum of the compounds was detected at 320 nm, using the 5-CQA as standard, and applied to estimate the total chlorogenic acid content (Corso *et al.*, 2016).

2.4.5. Volatile compounds

The analysis was performed by solid-phase micro-extraction followed by quantification in an Agilent 6890 N CG equipped with Agilent 5973 mass spectrometry detector and MSD Chemstation software (Agilent Technologies Inc., USA). Sample preparation and chromatographic conditions were applied according to Kalschne *et al.*, (2018).

The oil was weighed (1.0 g) in a 20 mL vial (Agilent, California, USA) immediately sealed with a silicone septum and kept in a water bath (70 ºC). After 10 min, the septum was punctured, and a DVB/CAR/PDMS fiber (Sigma Aldrich, USA) was exposed to the headspace for 30 min. After injection, the compounds were heat-desorbed from the fiber (desorption time 10 min) and transferred to an Innowax column (60 m x 0.32 mm x 0.25 µm) (Agilent, California, USA). Helium was used as carrier gas at 1.3 mL·min^{-1} flow rate and the injector temperature was 250 °C. The heating profile started at 40 °C, held 5 min, raised to 60 °C at 4 °C·min^{-1}, held at 60 °C for 5 min and up to 250 °C at 8 °C·min^{-1}, held for 3 min. The mass spectrometer operated at 280 °C interface temperature, ion source temperature of 230 °C, quadrupole temperature of 150 °C, scanning in a range of *m/z* of 35-400 amu.

The standards (1 mL) were placed in vials (20 mL), and injected into the GC-MS using the same extraction technique applied to volatile compounds. Quantification was performed by external standardization using duplicate 6-point analytical curves. Sensory attributes related to each volatile compound, based on those described in literature (Akiyama *et al.*, 2007, Belitz *et al.*, 2009. Dulsat-Serra *et al.*, 2016, Toledo *et al.*, 2016, and Kalschne *et al.*, 2018), were also reported (Table 5).

3. RESULTS AND DISCUSSION

Peroxide, acid, iodine and saponification values can be correlated with the stability and quality of oils. They indicate the oxidation degree, stability status, degree of unsaturation, and the relative amount of low fatty acids and high molecular weight (AOCS, 2014).

Coffee oil showed a peroxide value of 3.208 meq·kg^{-1} (Table 2). This was higher than that described by Sanches (2016) for roasted Arabica oil stored at a different time and under temperature conditions up to 2.38 meq·kg^{-1}, and Turatti (2001), up to 2.4 meq·kg^{-1}. However, it was still lower than the maximum value (15 meq·kg^{-1}) recommended for cold-pressed oil by the Brazilian regulation (Anvisa, 2005).

TABLE 2. Physico-chemical characterization of roasted Arabica coffee oil.

Parameters	Oil
Peroxide value (meq·kg^{-1})[a]	3.208 ± 0.001
Acid value (mg KOH·g^{-1})[b]	7.3 ± 0.2
Iodine value (g I_2 100 g^{-1})[b]	113.5 ± 0.3
Saponification value (mg KOH·g^{-1})[b]	195.26 ± 0.08
Moisture and volatile matter (%)[b]	0.85± 0.05
Refractive Index[b]	1.4798 ± 0.0000
Density (g·mL^{-1})[b]	0.938± 0.002
Viscosity (mPas a 25ºC)[b]	228.7 ± 0.5
ABTS (mg Trolox·mL^{-1})[a]	12.5 ± 0.1
Sun protection factor[b]	9.7 ± 1.2

[a]Means of duplicate ± standard deviation.
[b]Means of triplicate ± standard deviation.

An acid value of 7.3 mg KOH·g^{-1} was observed (Table 2), which was lower than that described by Turatti (2001) for roasted coffee oil (8.95 mg KOH g^{-1}) and by Amin *et al.,* (2019) for pumpkin seed oils (from 11.5 to 13.5 mg NaOH·g^{-1}). These values were higher than those defined for cold-pressed oils (maximum 4.0 mg KOH·g^{-1}) (Anvisa, 2005). However, no specific regulation can be found for oils that undergo a previous heat treatment such as roasted coffee oil. Furthermore, the literature describes that the roasting process can release acidic compounds, increasing acidity values (Sanches, 2016).

The coffee oil presented an iodine value of 113.50 and a saponification value of 195.26 mg KOH·g^{-1} (Table 2). The data were in the range of those reported by Sanches (2016) for roasted Arabica coffee oil: from 92.17 to 114.10 g I_2·100g^{-1} and from 192.98 to 233.44 mg KOH·g^{-1} for iodine and saponification values, respectively. Values in a similar range were reported by Amin *et al.*, (2019) for pumpkin seed oils: iodine value from 106.6 to 113.2 g I_2·100g^{-1} and saponification value from 115.7 to 236.0 mg KOH·g^{-1}.

The moisture and volatile matter of 0.85% (Table 2) were attributed to press extraction, since solvent-extracted oils do not contain water. Sanches (2016) reported lower moisture contents (up to 0.2%) for roasted coffee oil, although he pointed out that industrial limits varied between 0.30 and 2.00%.

The refractive index can be used as a physical parameter of oil quality. It increases with increasing fatty acid chain length and degree of unsaturation (AOCS, 2014). The coffee oil presented a refractive index of 1.4798 (Table 2), similar to that described by Amin *et al.*, (2019) for pumpkin seed oils (1.5).

Density and viscosity are important parameters for oil processing, since they are determinant for the correct design of the pumping, sedimentation, and filtration steps (Bonnet *et al.,* 2011). The coffee oil had a density of 0.938 g·mL^{-1} and a viscosity of 228.7 mPas at 25 °C (Table 2). Oliveira *et al.,* (2014), evaluating pressed green Arabica coffee oil, reported similar values for density and lower viscosity (from 95 to 127.9 mPas). Roasted coffee oil is denser than several other vegetable oils. Stanciu (2019) reported density values from 0.84 to 0.93 g·mL^{-1} for soybean, corn, sunflower, grape seed, and olive oils, among others.

Sun protection factor (SPF) and antioxidant capacity are important parameters to evaluate the potential of the oil as an ingredient in food and cosmetics. SPF indicates the relationship between the time of exposure to the sun without generating erythema (redness to the skin) with the use of the product compared to unprotected skin. Consequently, the higher the SPF, the longer the time the skin will be protected against UVB radiation (Wagemaker *et al.,* 2011). For the roasted coffee oil, a SPF of 9.7 and ABTS free radical-scavenging capacity of 12.5 mg Trolox mL^{-1} (Table 2) were observed. No data was found regarding the antioxidant capacity of coffee oil extracted by pressing. Wagemaker *et al.,* (2011) described a SPF of 1.50 for green Arabica coffee oil, traditionally used in cosmetics. Kaur and Saraf (2010) reported a wide range of SPF for several herbal oils used in cosmetics, from 0.248 (rose oil) to 7.549 (olive oil); besides olive oil, the highest values were found for coconut (7.119), peppermint (6.668), tulsi (6.571) and lemon grass (6.282) oils but they presented lower SPF than roasted coffee oil. Therefore, the efficient protection afforded by roasted coffee oil indicates its potential for use in cosmetic products.

Roasted coffee oil presented 57.5% of unsaturated fatty acids (Figure 1-a), indicating susceptibility to lipid oxidation, which highlights the importance of studying chemical parameters related to stability (Table 2). The literature described a wide range for the proportion of saturated, monounsaturated and polyunsaturated fatty acids in coffee oil from 29.45 to 47.3%, 42.72 to 59.17% and from 4.30 to 17.81%, respectively (Calligaris *et al.,* 2009; Getachew and Chun, 2016; Hurtado-Benavides *et al.,* 2016). These differences may be due to the coffee species used, as well as to the extraction method applied.

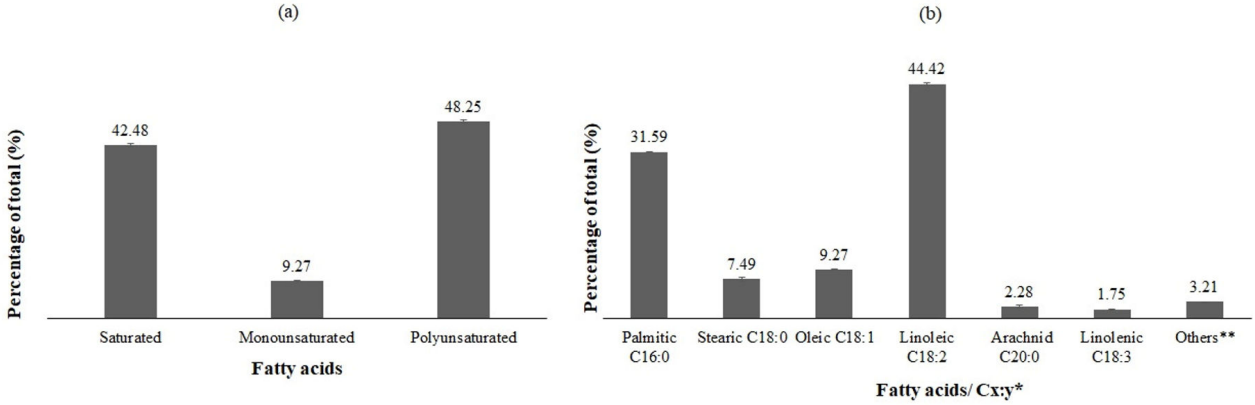
Regarding the fatty acid profile, the high proportion of polyunsaturated linoleic acid (44.42%) in roasted coffee oil (Figure 1) can be important for the health benefit of the compound ingestion, as it is an essential fatty acid (Spector, 1999). The presence (9.27%, Figure 1) of monounsaturated oleic acid –an omega-9 fatty acid – is also interesting because of its effect in reducing LDL cholesterol oxidation and as a precursor to the production of most other polyunsaturated fatty acids and hormones (Watkins and German, 2008). On the hand, palmitic acid, the main saturated fatty acid found in the roasted coffee oil (31.59%, Figure 1), which can increase low-density blood cholesterol levels, is interesting for extended use in several skin product formulations such as soaps and shaving creams and, along with linoleic, stearic and oleic fatty acids, is described as an excellent cosmetic material (Dangarembizi *et al*., 2015).

The fatty acid profile (Figure 1- b) was similar to that reported in other studies (Turatti, 2001; Oliveira *et al.,* 2005; Calligaris *et al.,* 2009; Raba *et al.,* 2018), since linoleic (L) and palmitic (P) are the main fatty acids, followed by oleic (O) and stearic (S). Some authors have reported higher palmitic acid contents, followed by linoleic acid (Rocha *et al.,* 2013; Hurtado-Benavides *et al.,* 2016). Cornelio-Santiago *et al.,* (2017) and Bitencourt *et al.,* (2018) described a predominance of linoleic, palmitic, oleic, and stearic acids in green coffee oil obtained by supercritical extraction.

Triacylglycerols are the main components of roasted coffee oil. It was estimated that the main TAGs in the roasted coffee oil were PLL (18.7%), PLP (13.3%), LLL (8.8%), PLO (7.8%), SLP (6.3 %) and OLL (5.5%) (Table 3).

González *et al.,* (2001) reported higher contents in PLL (20.1 to 31.5%) and PLP (15.8 to 28.9%) for Soxhlet-extracted roasted Arabica coffee oil. For green coffee oil, Cornelio-Santiago *et al.,* (2017) reported SLP (12.9%), PLL (12.3%), and PLP (11.6%) as the main TAGs, while Bitencourt *et al.,* (2018) highlighted the high PLP (22.9%) and PLL (22.6%) contents.

The profile of fatty acid and triacylglycerols observed for the studied roasted coffee oil (Figure 1 and Table 3) was similar to that described in the literature for oils obtained by different extraction methods and green coffee oil, showing the potential use of pressed roasted coffee oil.



Means of triplicate; error bars: standard deviation

*Cx:y where Cx = number of carbons and y = number of double bonds.

**Others: Myristic, Margaric, n-Heneicosanoic, Eicosadienoic, Behenic, Timnodonic, Adrenic and Clupanodonic acids presented in contents up to 1%.

FIGURE 1. Fatty acids of roasted Arabica coffee oil. (a) Percentage of saturated, monounsaturated and polyunsaturated fatty acids. (b) Fatty acid profile.

TABLE 3. Hypothetical triacylglycerol profile of roasted Arabica coffee oil.

Triacylglycerols[a]	Cx:y[b]	Percentage of total (%)[c]
PPP	48:0	3.16 ± 0.13
SPP	50:0	2.25 ± 0.04
POP	50:1	2.78 ± 0.06
PLP	50:2	13.30 ± 0.29
SOP	52:1	1.32 ± 0.03
SLP	52:2	6.31 ± 0.03
PLO	52:3	7.80 ± 0.06
PLL	52:4	18.69 ± 0.19
PLnL	52:5	1.46 ± 0.06
PLA	54:2	1.88 ± 0.03
SLO	54:3	1.85 ± 0.06
SLL	54:4	4.44 ± 0.11
OLO	54:4	1.14 ± 0.04
OLL	54:5	5.48 ± 0.13
LLL	54:6	8.75 ± 0.21
LLnL	54:7	1.03 ± 0.04
ALL	56:4	1.32 ± 0.05
Others[d]		8.25 ± 1.08

[a]Fatty acids: Arachnid (A), Adrenic (Ad), Behenic (Be), Clupanodonic (Cp), Linoleic (L), Linolenic (Ln), Oleic (O), Palmitic (P), Stearic (S), Timnodonic (Tm).[b]Cx: y where Cx = number of carbons and y = number of double bonds. [d]PLnP (50: 3), SPS (52: 0), PAP (52: 0), POO (52: 2), SLS (54: 2), PTmL (54: 7), BeLP (56:2), ALO (56: 3), PAdL (56: 6), PCpL (56: 7) presented in contents up to 1%.[c]Means of triplicate ± standard deviation.

Diterpenes, the major components of UM, correspond to 86 to 88% of the UM for Arabica coffee (Pacetti *et al*., 2015), and their contents remained stable during the roasting process (Dias *et al*., 2014). Kahweol and cafestol are the main diterpenes in coffee and produced only by plants of the *Coffea* genus (Dias *et al*., 2014). They are of interest due to their anticarcinogenic, antioxidant, anti-inflammatory, and hepatoprotective activities, and also to their skin hydration and sun protection effects (Kim *et al.,* 2009; Muriel and Arauz, 2010) although cafestol is also related to an increase in serum cholesterol levels (Speer and Kölling-Speer, 2006). The coffee oil presented a total diterpenes content of 3720 mg·100g^{-1}, with 1980 and 1740 mg 100 g^{-1} of kahweol and cafestol, respectively (Table 4). Oliveira *et al.,* (2014) and Bitencourt *et al.,* (2018) reported higher efficiency of supercritical extraction of green coffee oil diterpenes compared to pressing. It was also observed that the kahweol content (Table 4) was comparable to that reported by Bitencourt *et al*., (2018) (up to 1500 mg·100 g^{-1}) for green coffee oil using supercritical extraction.

TABLE 4. Unsaponifiable matter and hydrosoluble compounds of roasted Arabica coffee oil.

	Compounds	Content (mg·100g^{-1})
Diterpenes[a]	Kahweol	1980± 50
	Cafestol	1740 ± 60
	Total	3720
Tocopherols[b]	α	30.350 ± 0.250
	β	881.123 ± 17.080
	δ	2.226 ± 0.004
	Total	913
Hydrosoluble[a]	Caffeine	350± 10
	Chlorogenic acids	10.71 ± 0.03

[a]Means of duplicate of extraction ± standard deviation.
[b]Means of triplicate ± standard deviation.

In the UM, the presence of tocopherols also stands out, both for vitamin activity and antioxidant effect, which contributes to the stabilization of cell membranes which protect other bioactive compounds. The main component was β-tocopherol (97% of the total), followed by α and δ isomers; γ-tocopherol was absent (below the LD of 0.07 mg·100g^{-1}) (Table 4). Thus, a high total tocopherol content of 913 mg·100g^{-1}was observed, corresponding to 271 mg of vitamin E (expressed as α-tocopherol)·100g^{-1} or 298 IU of vitamin E·100g^{-1}.

In the literature, no consensus is found on the tocopherol profile of roasted Arabica coffee oil. González *et al.,* (2001) reported the β-isomer (from 9.4 to 16.1 mg·100g^{-1}) as the major one, followed by γ and α-tocopherol (5.9 to 9.5 and 2.1 to 3.4 mg·100g^{-1}) and the absence of δ-tocopherol. Ribeiro (2015) reported a higher γ-tocopherol content (182 mg·100g^{-1}), followed by β, δ, and α isomers (94, 25, and 1 mg·100g^{-1}, respectively). It should be noted that, besides the difference in the isomer profile, those authors reported lower total tocopherol contents than those obtained in this study, probably due to the high temperature used in Soxhlet extraction. For pressed green coffee oil, contents of 13.3 and 34.7 mg·100g^{-1} of α and β tocopherol, respectively, were reported (Bitencourt *et al.,* 2018). Therefore, the efficient extraction of the UM compounds of the roasted coffee by pressing can stand out.

As previously discussed, as roasted coffee was pressed, it was also possible to extract some hydrosoluble compounds of known antioxidant effects such as caffeine and chlorogenic acids. The coffee oil presented 350 mg·100g^{-1} of caffeine and 10.71 mg·100g^{-1} of total chlorogenic acids (Table 4). The higher caffeine extraction was attributed to its lower molecular weight (194.194 g·mol^{-1}), and higher water solubility (22 g·L^{-1}) (Pubchem, 2018) compared to chlorogenic acids.

Similar caffeine contents, from 320 to 340 mg·100g^{-1}, were reported by Sanches (2016) for roasted Arabica coffee oil obtained by pressing. Oliveira *et al.,* (2014) highlighted a higher efficiency of the supercritical extraction process with caffeine contents from 260 to 1650 mg· 100g^{-1} in green Arabica coffee oil.

No data on chlorogenic acid content in roasted coffee oil were found. For green coffee oil, Bitencourt *et al.,* (2018) reported 8.8 mg GAE·100g^{-1} using pressing extraction, and Oliveira *et al.,* (2014) reported a wider range of values (0 to 262 mg GAE·100g^{-1}) depending on supercritical extraction conditions. It should be considered, however, that the Folin-Ciocalteu estimation is not specific for phenolic compounds, and the response may also be due to other reducing compounds.

The presence of these bioactive compounds (Table 4) may also be associated with the antioxidant capacity and SPF characteristics observed for roasted coffee oil (Table 2).

Thirty-five volatile compounds of different classes (carboxylic acids, ketones, furans, thiols, pyrazines, phenols, pyridines, aldehydes, terpenes, alcohols, sulfur compounds, and thiazoles) were quantified in the oil (Table 5), several of them being described as typical of roasted coffee aroma (Akiyama *et al.,* 2007; López-Galilea *et al.,* 2006).

For roasted Arabica coffee oil, Getachew and Chun (2016) described the presence of aldehydes, ketones, furans, pyrroles, pyrazines, pyridines, and phenolic compounds (24 volatile compounds), and Hurtado-Benavides *et al.,* (2016) reported a greater number of compounds (41 volatiles), mainly furans and pyrazines, in products obtained by supercritical extraction. In pressed oil, Oliveira *et al.,* (2005) identified 32 volatile compounds, including hydrocarbons, pyrazines, furans, and ketones.

The volatile compounds found in higher contents belong to carboxylic acids, ketones, furans, and thiol classes. We highlight the acetic acid, with a negative impact on the aroma profile, and maltol, 2-furfurylthiol, furfuryl acetate, and 2,6-dimethylpyrazine, which presented potential positive aroma characteristics. The pyrazine class contained the highest number of volatile compounds, and the lowest number of compounds was observed in the classes of terpenes, alcohols, sulfur compounds, and thiazoles (Table 5). The volatile compounds in the roasted coffee oil can be formed by the thermal degradation of carbohydrates, amino acids, ascorbic acid, lipids, esters, and the auto-oxidation of aldehydes and ketones during the roasting process (Buffo and Cardelli-Freire, 2004).

Carboxylic acids account for a high proportion of roasted coffee's volatile fraction (Kalschne*et al.,* 2018). Volatile acids present characteristic odors, and acetic acid is present in high contents in the roasted coffee oil (1287.63 ng·g^{-1}) (Table 5), is related to vinegar odor (Belitz *et al.,* 2009).

Ketones are also abundant in roasted coffee (Toledo *et al.,* 2016), presenting aromas such as fruit, butter, mushroom, mold, caramel, and tea (López-Galilea *et al.,* 2006; Akiyama *et al.,* 2007). Maltol, present in higher contents (835.94 ng·g^{-1}) (Table 5), presents a caramel odor (Belitz *et al.,* 2009).

Furans are described as the main chemical class found in Arabica coffee, followed by pyrazines, pyridines, and pyrroles (Toledo *et al.,* 2016). They can give an aroma of roasted malt, sweet, grass, fruits, burnt, burnt sugar, and others (López-Galilea *et al.,* 2006; Akiyama *et al.,* 2007, Nascimento *et al.,* 2007; Belitz *et al.,* 2009). The furan found in higher contents in the roasted coffee oil was furfuryl acetate (539.88 ng·g^{-1}) (Table 5), which has a floral and fruity odor (Nascimento *et al.,* 2007).

Although presented in lower contents, thiols and pyrazines have a significant impact on the characteristic aroma and flavor of coffee brews. Thiols are related to aromas of roasted, fresh coffee, roasted meat, and nuts, among others (Dulsat-Serra *et al.,* 2016). A high content in 2-furfurylthiol in the oil (727.16 ng·g^{-1}) (Table 5) was observed, which is a key aromatic compound in roasted coffee products (Toledo *et al.,* 2016, Nascimento *et al.,* 2007; Belitz *et al.,* 2009). Pyrazines are described as presenting aromas of nut, earth, roasted and grass (Czerny and Grosch, 2000; Akiyama *et al.,* 2007; Toledo *et al.,* 2016).

TABLE 5. Profile of volatile compounds of roasted Arabica coffee oil.

Sensory Group[a]	Volatile Compound / Class	Compound content (ng·g^{-1})[b]	Class content (ng·g^{-1})
	Carboxylic acids		
Fermented	Isovaleric acid	68.82	
Chemist / Pungent	Acetic acid	1287.63	1422.01
Vegetable / Herbaceous	Propanoic acid	65.56	
	Ketones		
Sweet, Burned	2,3-Butanedione	1.58	
Sweet, Burned	2,3-Pentanedione	0.12	869.74
Sweet, Burned	Acetoin	32.10	
Sweet, Burned	Maltol	835.94	
	Furans		
Floral	Furfuryl acetate	539.88	
Vegetable	Furfural	199.90	741.87
Sweet, Burned	Furaneol	2.09	
	Thiols		
Burned (Coffee)	2-Furfurylthiol (Fufurylmercaptane)	727.16	727.17
Putrid	Methanethiol	0.01	
	Pyrazines		
Seasoning	2,3-Diethyl-5-methylpyrazine	1.05	
Nuts	2,5-Dimethylpyrazine	114.29	
Nuts	2,3-Dimethylpyrazine	39.59	
-	2-Isobutyl-3-methylpyrazine	0.27	560.29
-	2-Isobutyl-3-methoxypyrazine	0.31	
-	2-Acetyl-3,5-dimethylpyrazine	6.00	
Burned (Coffee)	2,6-Dimethylpyrazine	361.41	
Moldy / Earth	Pyrazine	37.37	
	Phenols		
Chemical	Guaiacol	113.54	
Sweet / Vanilla	Vanillin	3.31	
Smoked	4-Ethylguaiacol	89.14	491.95
Smoked	4-Vinylguaiacol	285.15	
Smoked	Cis-isoeugenol	0.81	
	Pyridines		
Burned (Coffee)	Pyridine	386.92	388.84
Burned (Coffee)	2-Acetylpyridine	1.92	
	Aldehydes		
Pungent	Acetaldehyde (ethanal)	87.88	113.83
Frutal	3-Methylbutanal	25.95	
	Terpenes		
Vegetable	Linalool	4.02	4.02
	Alcohols		
Sweet, Burned	Benzyl alcohol	1.52	3.71
Smoked	Phenylethyl alcohol	2.19	
	Sulfur compounds		
Putrid	Dimethyldisulfite	3.27	3.27
	Thiazoles		
Burned (Coffee)	4,5-Dimethylthiazole	0.48	1.11
Moldy / Earth	4-Methylthiazole	0.63	

[a]Sensory attributes related to each component are cited based on those described by Akiyama *et al.*,(2007), Belitz *et al*., (2009), Dulsat-Serra *et al*., (2016), Toledo *et al*., (2016), and Kalschne *et al*., (2018). [b]Means of duplicate.

The main pyrazine identified in the roasted coffee oil was 2,6-dimethylpyrazine (361.41 $ng \cdot g^{-1}$), which presents a characteristic aroma of burnt coffee and roasted cocoa/nut (Nascimento *et al.,* 2007).

4. CONCLUSIONS

Roasted coffee oil proved to be a high quality product due to its low peroxide and acid values, significant contents in tocopherols and diterpenes, in addition to the presence of caffeine and chlorogenic acids, resulting in high antioxidant capacity. The roasted oil presented a high sun protection factor effect (compared to green coffee oil and others herbal oils), and the profile of fatty acids and triacylglycerols was similar to that described in the literature for green coffee oil. In the complex profile of volatiles, thirty-five compounds of different classes were identified, with pyrazines and furfurylthiol as the predominant ones. These properties show that roasted coffee oil has good potential for use in food and cosmetics.

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REFERENCES

Akiyama M, Murakami K, Ikeda M, Iwatsuki K, Wada A, Tokuno K, Onishi M, Iwabuchi H. 2007. Analysis of the headspace volatiles of freshly brewed arabica coffee using solid-phase microextraction. *J. Food Sci.* **72**, 388-396. https://doi.org/10.1111/j.1750-3841.2007.00447.x

Amin MZ, Islam T, Mostofa F, Uddin MJ, Rahman MM, Satter MA. 2019. Comparative assessment of the physicochemical and biochemical properties of native and hybrid varieties of pumpkin seed and seed oil (*Cucurbita maxima* Linn.). *Heliyon* **5**, 1-6. https://doi.org/10.1016/j.heliyon.2019.e02994

Anvisa. 2005. Agência Nacional de Vigilância Sanitária (ANVISA). RDC nº 270, de 22 de setembro de 2005. Regulamento técnico para óleos vegetais, gorduras vegetais e creme vegetal. *Diário Oficial [da] República Federativa do Brasil,* Brasília, DF, n. 184, Seção 1. p.372.

AOCS. 2014. *Official methods and recommended practices of the American Oil Chemist's Society*. Official Method CE 8-89. Champaign: AOCS Press. Vol 1 and 2.

Belitz HD, Grosch W, Schieberle P. 2009. *Food Chemistry*. 4th, Springer, Berlin.

Bitencourt RG, Ferreira NJ, Oliveira AL, Cabral FA, Meirelles AJA. 2018. High pressure phase equilibrium of the crude green coffee oil - CO2 - ethanol system and the oil bioactive compounds. *J. Supercrit. Fluids* **133**, 49-57. https://doi.org/10.1016/j.supflu.2017.09.017

Bonnet JP, Devesvre L, Artaud J, Moulin P. 2011. Dynamic viscosity of olive oil as a function of composition and temperature: A first approach. *Eur. J. Lipid Sci. Technol*. **113**, 1019-1025. https://doi.org/10.1002/ejlt.201000363

Budryn G, Nebesny E, Zyzelewicz D, Oracz J, Miśkiewicz K, Rosicka-Kaczmarek J. 2012. Influence of roasting conditions on fatty acids and oxidative changes of Robusta coffee oil. *Eur. J. Lipid Sci. Technol*. **114**, 1052-1061. https://doi.org/10.1002/ejlt.201100324

Buffo RA, Cardelli-Freire C. 2004. Coffee flavour: An overview. *Flavour Fragr. J*. **19**, 99-104. https://doi.org/10.1002/ffj.1325

Calligaris S, Munari M, Arrighetti G, Barba, L, 2009. Insights into the physicochemical properties of coffee oil. *Eur. J. Lipid Sci. Technol*. **111**, 1270-1277. https://doi.org/10.1002/ejlt.200900042

Carvalho CRL, Mantovani DMB, Carvalho PRN, Moraes RMM. 1990. *Análises químicas de alimentos*. Manual Técnico. Campinas: ITAL, 121p.

Cornelio-Santiago HP, Gonçalves CB, Oliveira NA, Oliveira AL. 2017. Supercritical CO2 extraction of oil from green coffee beans: Solubility, triacylglycerol composition, thermophysical properties and thermodynamic modelling. *J. Supercrit. Fluids* **128**, 386-394. https://doi.org/10.1016/j.supflu.2017.05.030

Corso MP, Vignoli JA, Benassi MT. 2016. Development of an instant coffee enriched with chlorogenic acids. *J. Food Sci. Technol*. **53**, 1380-1388. https://doi.org/10.1007/s13197-015-2163-y

Czerny M, Grosch W. 2000. Potent odorants of raw Arabica coffee. Their changes during roasting. *J. Agric. Food Chem*. **48**, 868-872. https://doi.org/10.1021/jf990609n

Dangarembizi R, Chivandi E, Dawood S, Erlwanger KH, Gundidza M, Magwa ML, Muredzi P, Samie A. 2015. The fatty acid

composition and physicochemical properties of theunderutilised *Cassia abbreviata* seed oil. *Pak J. Pharm. Sci*. **28**, 1005-1008.

Dias RCE, Faria-Machado AF, Mercadante AZ, Bragagnolo N, Benassi MT. 2014. Roasting process affects the profile of diterpenes in coffee. *Eur. Food Res. Technol*. **239**, 961-970. https://doi.org/10.1007/s00217-014-2293-x

Dulsat-Serra N, Quintanilla-Casas B, Vichi S. 2016. Volatile thiols in coffee: A review on their formation, degradation, assessment and influence on coffee sensory quality. *Food Res. Int*. **89**, 982-988. https://doi.org/10.1016/j.foodres.2016.02.008

Getachew AT, Chun BS. 2016. Optimization of coffee oil flavor encapsulation using response surface methodology. *LWT - Food Sci. Technol*. **70**, 126-134. https://doi.org/10.1016/j.lwt.2016.02.025

González AG, Pablos F, Martín MJ, León-Camacho M, Valdenebro MS. 2001. HPLC analysis of tocopherols and triglycerides in coffee and their use as authentication parameters. *Food Chem*. **73**, 93-101. https://doi.org/10.1016/S0308-8146(00)00282-X

Guercia E, Berti F, Navarini L, Demitri N, Forzato C. 2016. Isolation and characterization of major diterpenes from *C. canephora* roasted coffee oil. *Tetrahedron: Asymmetry* **27**, 649-656. https://doi.org/10.1016/j.tetasy.2016.06.008

Hurtado-Benavides A, Dorado DA, Sánchez-Camargo ADP. 2016. Study of the fatty acid profile and the aroma composition of oil obtained from roasted Colombian coffee beans by supercritical fluid extraction. *J. Supercrit. Fluids* **113**, 44-52. https://doi.org/10.1016/j.supflu.2016.03.008

Ico. 2019. *International Coffee Organization*. Coffee Market Report - December 2019. Available in: <http://www.ico.org/documents/cy2019-20/cmr-1219-p.pdf> Access on 15 January 2020.

ISO. 1978. International Organization for Standardization. ISO *5509: Animal and Vegetable Fats and Oils: Preparation of Methyl Esters of Fatty Acids*. London: International Organization for Standardization.

Kalschne DL, Viegas MC, Conti AJ, Corso MP, Benassi MT. 2018. Steam pressure treatment of defective *Coffea canephora* beans improves the volatile profile and sensory acceptance of roasted coffee blends. *Food Res. Int*. **105**, 393-402. https://doi.org/10.1016/j.foodres.2017.11.017

Kaur CD, Saraf S. 2010. *In vitro* sun protection factor determination of herbal oils used in cosmetics. *Pharmacognosy Res*. **2**, 22-25. https://doi.org/10.4103/0974-8490.60586

Kim HG, Hwang YP, Jeong HG. 2009. Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells. *Toxicol. Lett*. **187**, 28-34. https://doi.org/10.1016/j.toxlet.2009.01.022

López-Galilea I, Fournier N, Cid C, Guichard E. 2006. Changes in headspace volatile concentrations of coffee brews caused by the roasting process and the brewing procedure. *J. Agric. Food Chem*. **54**, 8560-8566. https://doi.org/10.1021/jf061178t

Mori ALB, Kalschne DL, Ferrão MAG, Fonseca AFA, Ferrão RG, Benassi MT. 2016. Diterpenes in Coffea canephora. *J. Food Compos. Anal*. **52**, 52-57. https://doi.org/10.1016/j.jfca.2016.08.004

Muriel P, Arauz J. 2010. Coffee and Liver Disease. *Fitoterapia* **81**, 297-305. https://doi.org/10.1016/j.jceh.2016.02.003

Nascimento EA, Aquino FJT, Nascimento PM, Chang R, Morais SAL. 2007. Volatiles compounds and potent odorants of conilon coffee in different degrees of roasting. *Rev. Ciência Eng*. **16**, 23-30.

PubChem. 2018. *National Center for Biotechnology Information (NCBI)*. Available in: <https://pubchem.ncbi.nlm.nih.gov/> Access on 13 May. 2019.

Oliveira AL, Cruz PM, Eberlin MN, Cabral FA. 2005. Brazilian roasted coffee oil obtained by mechanical expelling: compositional analysis by GC-MS. *Ciência e Tecnol. Aliment*. **25**, 677-682. https://doi.org/10.1590/S0101-20612005000400009

Oliveira PMA, Almeida RH, Oliveira NA, Bostyn S, Gonçalves CB, Oliveira AL. 2014. Enrichment of diterpenes in green coffee oil using supercritical fluid extraction - Characterization and comparison with green coffee oil from pressing. *J. Supercrit. Fluids* **95**, 137-145. https://doi.org/10.1016/j.supflu.2014.08.016

Pacetti D, Lucci P, Frega NG. 2015. Unsaponifiable matter of coffee, in Preedy, VR, 1st Edition. *Coffee in Health and Disease Prevention*. London, England: Academic Press,119- 127.

Plataforma Lames. 2019. Available in: <http://projetos.extras.ufg.br/plames/>Access on 15 January. 2020.

Raba DN, Chambre DR, Copolovici DM, Moldovan C, Copolovici LO. 2018. The influence of high-temperature heating on composition and thermo-oxidative stability of the oil extracted from Arabica coffee beans. *PLoS One* **13**, 1-13. https://doi.org/10.1371/journal.pone.0200314

Ribeiro, JM. 2015. Estudo da composição química e das atividades antioxidante e antibacteriana dos óleos extraídos dos grãos de café (*Coffea arabica*) cru e torrado. Dissertação, 72f. Mestrado em Química Orgânica. Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, 2015. Available in: <http://acervo.ufvjm.edu.br/jspui/bitstream/1/817/1/juliana_martins_ribeiro.pdf> Access on 15 January 2020.

Rocha CC, Reis C, Chaves ARM. 2013. Caracterização qualitativa de ácidos graxos como componentes dos óleos de grão de café verde, café torrado e borra de café, in: *VIII Simpósio de pesquisa dos cafés do Brasil*. Salvador-BA, 1-5.

Sanches MZ. 2016. *Avaliação das características físico-químicas e sensoriais de óleo de café torrado (Coffea arabica) durante armazenamento em diferentes temperaturas*. Dissertação, 68 f. Mestrado em Tecnologia de Alimentos. Universidade Tecnológica Federal do Paraná, Londrina, 2016. Available in:<http://repositorio.utfpr.edu.br/jspui/bitstream/1/2236/1/LD_PPGTAL_M_Sanches%2c%20Marcelo%20Zuchi_2016.pdf>Access on 15 January. 2020

Spector AA. 1999. Essentiality of fatty acids. *Lipids* **34**, 1-3. https://doi.org/10.1007/bf02562220

Speer K, Kölling-Speer I. 2006. The lipid fraction of the coffee bean. *Brazilian J. Plant Physiol*. **18**, 201-216. https://doi.org/10.1590/S1677-04202006000100014

Stanciu I. 2019. A new mathematical model for the viscosity of vegetable oils based on freely sliding molecules. *Grasas Aceites* **70** (3), e318. https://doi.org/10.3989/gya.0824182

Toledo PRAB, Pezza L, Pezza HR, Toci AT. 2016. Relationship between the different aspects related to coffee quality and their volatile compounds. *Compr. Rev. Food Sci. Food Saf*. **15**, 705-719. https://doi.org/10.1111/1541-4337.12205

Turatti JM. 2001. Extração e caracterização de óleo de café, in: *II Simpósio de pesquisa dos cafés do Brasil*. 1-15.

Wagemaker TAL, Carvalho CRL, Maia NB, Baggio SR, Guerreiro Filho O. 2011. Sun protection factor, content and composition of lipid fraction of green coffee beans. *Ind. Crops Prod*. **33**, 469-473. https://doi.org/10.1016/j.indcrop.2010.10.026

Watkins SM, German JB. 2008. Unsaturated fatty acids, in Akoh CC, Min DB (Ed.) *Food Lipids - Chemistry, Nutrition, and Biotechnology*. 3ed, CRC Press, Boca Raton, 514-530.

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Impact of plum processing on the quality and oxidative stability of cold-pressed kernel oil

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SUMMARY: Plum kernels of the "Čačanska rodna" variety, by-products from plum brandy production, were collected before and after fermentation and distillation, and used for cold-pressed oil production. Fatty acid and tocopherol contents were determined by capillary GC and HPLC, while the oxidation stability of the resulting cold-pressed oils was tested by the Rancimat method. The results showed that oleic fatty acid was dominant in the oil samples with a content of 56.6 to 61.8%, regardless of the plum kernels' origin. The fermentation and distillation processes had a pronounced effect on the tocopherol content and oxidative stability of the resulting kernel oils. Tocopherol contents were 61.8 mg·100g^{-1}, 87.4 mg·100g^{-1}, 79.6 mg·100g^{-1} of oil, while the induction periods were 38.7, 44.4 and 33.6 hours for samples before fermentation, after fermentation and distillation, respectively. Based on the results, it could be concluded that the fermentation process increased the content of tocopherols in kernel oil whereas the high temperature during the distillation process adversely affected the tocopherol content and oxidative stability of the obtained kernel oil.

KEYWORDS: *By-product; Cold-pressing; Fatty acids; Oxidative stability; Plum kernels; Tocopherols*

RESUMEN: ***Impacto del procesamiento de ciruelas en la calidad y la estabilidad oxidativa del aceite del hueso prensado en frío.*** Los huesos de ciruelas de la variedad "Čačanska rodna", subproductos de la producción de brandy de ciruela, se procesaron antes y después de la fermentación y la destilación, y se utilizaron para la producción de aceite prensado en frío. El contenido de ácidos grasos y tocoferoles se determinó mediante GC capilar y HPLC, mientras que la estabilidad oxidativa de los aceites prensados en frío resultantes se determinó mediante el método Rancimat. Los resultados obtenidos mostraron que el ácido oleico era predominante en los aceites, con un contenido de 56,6 a 61,8%, independientemente del origen de los huesos de las ciruelas. Los procesos de fermentación y destilación tuvieron un efecto pronunciado sobre el contenido de tocoferoles y sobre la estabilidad oxidativa de los aceites de hueso resultantes. El contenido de tocoferoles fue de 61,8 mg·100g^{-1}, 87,4 mg·100g^{-1}, 79,6 mg·100g^{-1} de aceite, mientras que los períodos de inducción fueron de 38,7, 44,4 y 33,6 horas para las muestras antes de la fermentación, después de la fermentación y la destilación, respectivamente. En base a los resultados obtenidos, se podría concluir que el proceso de fermentación aumentó el contenido de tocoferoles en el aceite de hueso, mientras que la alta temperatura durante el proceso de destilación afectó negativamente al contenido de tocoferoles y la estabilidad oxidativa del aceite de hueso obtenido.

PALABRAS CLAVE: *Ácidos grasos; Estabilidad oxidativa; Huesos de ciruela; Prensado en frío; Subproducto; Tocoferoles*

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1. INTRODUCTION

Plum is the leading fruit species grown in Serbia and, in some ways, a symbol of Serbia. It has been grown in this region for centuries, thanks to several advantages: it reproduces relatively easily, starts bearing fruit fast and thrives in hilly and mountainous regions, where it represents 70% of the total fruit species. Serbia is the third plum producer in the world, with 500,000 tons annually, ranking below China with six million tons and India, with 600,000 tons per year (Mratinić, 2013). In Serbia, most of the plum yield, 90%, is processed into plum brandy, with the remaining fruit processed into jam, compote, preserves and a significant portion dried. Plum processing yields pomace, skins and pits as by-products. Pomace and skins are a valuable source of phenolic compounds, while pits are usually used as fuel or disposed of as waste. Pits represent 9 to 11% of the weight of plum fruits, while the kernels represent 17 to 20% of the weight of pits, depending on the plum variety. Bearing in mind that the average plum yield in Serbia is about 7 $t \cdot ha^{-1}$, according to the records of the National Office for Statistics, it can be concluded that about 700 $kg \cdot ha^{-1}$ of plum pits are produced as by-products.

According to the FAO data for 2000-2009, plum orchards in Serbia covered 191,021 ha, which leads to the conclusion that 1,300 tons of plum pits, i.e. 230 tons of kernels, remain as by-products. The kernel is high in oil content; the content varies and depends on both phenotypic and genotypic factors, but according to the literature, it can exceed 40% (Kamel and Kakuda, 1992; Uluata and Nurhayat, 2017). Such a high oil content makes plum kernel an excellent raw material for the production of cold-pressed oil, with all nutritionally valuable oil components preserved, since, as described by Codex Standard, *"Cold-pressed oils are obtained, without altering the oil, by mechanical procedures only, e.g. expelling or pressing, without the application of heat. They may have been purified by washing with water, settling, filtering and centrifuging only. (CX-STAN 210 - 1999, Vol. 8).* In addition to its favorable fat content, which is dominated by monounsaturated oleic acid and polyunsaturated linoleic acid, omega-6 fatty acid, plum kernel oil has a relatively high content in tocopherols with γ-tocopherol as its major component, which contributes to the excellent oxidative stability of this oil (Kamel and Kakuda, 1992; Picuric-Jovanovic *et al*., 1997; Özcan *et al*., 2014; Górnaś *et al*., 2017a).

There is insufficient data in the literature on cold-pressed plum kernel oil, or on the impact of raw material processing on the quality of the obtained oil. Since most of the plum in Serbia is processed into plum brandy ("šljivovica" *eng. slivovitz*), many changes (thermal, oxidative and hydrolytic) occurring during fermentation and distillation can affect the nutritional quality of the cold-pressed oil. The objective of this study was to examine the quality and oxidative stability of cold-pressed plum kernel oil derived from different stages of plum brandy production.

2. MATERIALS AND METHODS

2.1. Material

Kernels of the plum variety "Čačanska rodna" were used as starting material for the production of cold-pressed oil. Kernels from fresh plums before fermentation (PKBF) were used, as well as those extracted from different stages of the slivovitz production process, in particular, kernels separated after fermentation (PKAF) and kernels separated after distillation (PKAD). Plums were allowed to ferment for 13 days at 18-20 °C, while batch distillation was performed in a traditional alembic pot of 250 L for 3 hours by direct heating. In the beginning, distillation was induced by strong heating, which was continued for a short period after the condensed distillate began to trickle. Depending on the alcohol-to-water ratio, the mixture boils between 78.5 and 100 °C.

Once the pits were cracked open in a nutcracker with rotating cylinders, the released kernels were dried on paper, in a thin layer, at ambient temperature (22-24 °C) for 72 h, with occasional manual stirring. The kernels were dried to 6% humidity to facilitate cold-pressing.

2.2. Cold-pressing

The kernels (approximately 300 g per sample) were cold-pressed in a small-scale screw oil press (Gorenje, 2 $kg \cdot h^{-1}$ capacity, power 650 W). The temperature of the obtained oil was monitored by an IC thermometer and kept below 45 °C.

After cold-pressing, the oils were kept at room temperature (22-24 °C) for 24 hours for natural sedimentation and then decanted. The oil samples were stored in dark glass bottles in a refrigerator at 4 °C until analysis (within 2 weeks).

2.3. Determination of peroxide and acid value

2.3.1. Peroxide value

The peroxide value (PV), expressed in mmol·kg^{-1}, was determined according to SRPS EN ISO 660:2015. The determination was carried out in triplicate.

2.3.2. Acid value

The acid value (AV), expressed in mg KOH·g^{-1}, was determined according to SRPS EN ISO 3960:2016. The determination weas carried out in triplicate.

2.4. Determination of fatty acid composition

Fatty acid methyl esters (FAME's) were prepared using the standard method according to ISO E. 5509 (2000). FAME's were determined according to the gas chromatography method described by SRPS EN ISO 12966-4 (2016) by capillary gas chromatography on Agilent Technologies 6890 (USA) GC device equipped with split/splitless injector, flame ionization detector (FID) and capillary column Supelco SP-2560 (length 100 m, i.d. 0.25 mm, film thickness 0.20 µm, Supelco, Bellefonte, USA). Injector and detector temperatures were 250 and 260 °C, respectively. Helium was used as the carrier gas at a flow rate of 5 mL·min^{-1}. The injected volume was 1 µL and the injector split ratio was set at 20:1. The column temperature was programmed from the initial 50 °C (held 5 min) to 240 °C (held 20 min), with a temperature rate of 4 °C·min^{-1}. The chromatographic peaks in the samples were identified by comparing the relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, USA). Total fatty acids were calculated in mg·g^{-1} of lipids and individual fatty acids were expressed in relative quantities as mass % of total fatty acids.

2.4.1. Indexes of lipid nutritional quality (INQ)

The nutritional quality of the lipid fraction of samples was determined by examining the fatty acid profile and taking into consideration two indexes: atherogenicity (AI) and thrombogenicity (TI) (Ulbricht and Southgate, 1991).

$$(\mathrm{AI}) = [(4 \times \mathrm{C14{:}0}) + \mathrm{C16{:}0} + \mathrm{C18{:}0}]/\Sigma\mathrm{MUFA} + \Sigma\mathrm{PUFA}$$

$$(\mathrm{TI}) = (\mathrm{C14{:}0} + \mathrm{C16{:}0} + \mathrm{C18{:}0})/$$
$$(0.5\mathrm{MUFA} + 0.5\mathrm{PUFA} - \mathrm{n6} + 3\mathrm{PUFAn3} +$$
$$\mathrm{PUFA} - \mathrm{n3}/\mathrm{PUFA} - \mathrm{n6})$$

Where
MUFA - monounsaturated fatty acid
PUFA - polyunsaturated fatty acid

2.5. Determination of tocopherols

The determination of tocopherols was carried out using HPLC (Waters M600E, USA) on a reverse-phase Nucleosil 50-5 C18 column (Machery-Nagel, Germany) with fluorescence detection according to a method based on the procedure of Carpenter (1979) with some modifications. The following procedures were applied: 20 mL of 96% v/v of ethanol, 0.12 g of pyrogallol, and 3 mL KOH solution were added to 0.5 mL of oil, after which the solution was heated for 30 min at 60 °C with reflux and stirring.

Once the saponification process was completed, the content was cooled, transferred to a volumetric flask (50 mL) and topped with ethanol. An aliquot of 5 mL was then transferred to a separation funnel and 5 mL of cold deionized water and 5 mL of hexane were added. The mixture was vortexed for 3 min and 4 mL of the solution were dried under a nitrogen blanket. The dry matter was then dissolved in 4 mL of methanol. The sample was filtered using a membrane syringe filter and injected into the HPLC system. The mobile phase was 95% v/v methanol at a flow rate of 1.2 mL·min^{-1}. Detection was performed by a fluorescence detector (Shimadzu RF-535, Japan) operated with the excitation wavelength at λ=290 nm and the emission wavelength at λ=330 nm. The relative retention time and maximum values of absorption at the given relative retention time were used for the identification of tocopherols in the oil samples.

Commercial tocopherol standards were suitably diluted and used for method validation (solution series from 0.001 to 0.5 µg·mL^{-1}), and for quantification purposes (solution series from 0.05 to 10.0 µg·mL^{-1}). Total time of the chromatographic analysis was 20 minutes. The signal was processed using "Clarify" software.

Furthermore, the tocopherol content was calculated based on a comparative analysis of peaks of standards and samples. According to Table 1, the method is sufficiently sensitive and specific for determining the acceptable levels of tocopherols.

2.6. Oxidative stability determination

2.6.1. Rancimat test

The oxidative stability of the samples was determined on a Rancimat apparatus model 743 (Metrohm, Herisau, Switzerland) according to ISO 6886:2016. Oil samples (2.5 g) were transferred to reaction vessels. The samples were heated at 100 °C, with an airflow of 20 L·min^{-1}. The volatile components released during oxidation were collected in a cell with water, and the increase in electrical conductivity of the water was constantly measured and recorded over a period of time. In this way, the induction period, IP (h), was determined, which shows the oil's resistance to oxidation.

2.7. Statistical analysis

All determinations were carried out in triplicate and values were expressed as a mean of three measurements ± standard deviation. Statistical analysis was performed using the StatSoft Statistica 10 software (StatSoft, Inc. STATISTICA, ver. 10, data analysis software system). To test the mean differences, a variance analysis (one-way ANOVA) was performed, followed by the Tukey's HSD test at the significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Peroxide and acid values

The basic chemical quality of the oil was examined by determining the peroxide and acid values (Table 2). These two parameters are very important as they indicate the condition and quality of the oil.

TABLE 2. Peroxide and acid values

Sample	PV (mmol· kg^{-1})	AV (mgKOH· g^{-1})
PKBF	0.0±0.0^{a}	0.2±0.0^{a}
PKAF	0.9±0.1^{b}	3.4±0.3^{b}
PKAD	4.3±0.4^{c}	3.7±0.3^{b}

*The results are presented as mean value ± SD (n=3); different letters in columns indicate that there is significant difference at $p < 0.05$, (according to Tukey's HSD test).
PV - Peroxide Value, AV - Acid value. PKBF - Kernels from fresh plums before fermentation, PKAF - Kernels separated after fermentation, PKAD - kernels separated after distillation.

The peroxide value of edible oils is an important indicator of quality and the values obtained were far below the upper limit values prescribed by the Codex Alimentarius Commission (El-Adawy and Taha, 2001). Oil samples from fresh and fermented kernels were found to have peroxide values of 0.0 mmol·kg^{-1} and 0.9 mmol·kg^{-1}, respectively, showing that the oil had not been oxidized, i. e. that there had not been any changes in the contents in primary oxidation products during fermentation.

However, even though the peroxide value of oil from kernels that had been distilled was within the limits acceptable to the Codex Alimentarius Commission (El-Adawy and Taha, 2001), a significantly higher value was found, 4.3 mmol·kg^{-1}, indicating that the higher temperatures during distillation had affected oxidative processes and the kernel oil quality.

As for acid value, fresh plum kernel oil showed the lowest value, 0.2 mg KOH·g^{-1}, while the highest value was found in the oil from the kernels of distilled pits, 3.7 mg KOH·g^{-1}. The oil produced from fermented kernels had an acid value of 3.4 mg KOH·g^{-1}. The obtained values were expected since fresh plum kernels had not been processed in any way, and the oil had been pressed immediately after pit cracking;

TABLE 1. Analytical performance of the method based on HPLC with fluorescence detection

Tocopherols	Linearity	Calibration range (n=6) (µg·mL^{-1})	LOD (µg·mL^{-1})	LOQ (µg·mL^{-1})	Recovery (%) at level of 1 µg·mL^{-1}	RSD (%)
α	0.9995	0.05 - 10.0	0.007	0.03	98.1	1.90
β+γ	0.9998	0.05 - 10.0	0.006	0.02	97.7	1.98
δ	0.9999	0.05 - 10.0	0.006	0.02	98.4	1.09

*LOD - Limit of Detection; LOQ - Limit of Quantitation; RSD - Relative Standard Deviation

conversely, triacylglycerols were hydrolyzed in the presence of water, heat and certain microflora during fermentation and distillation. Dulf *et al*., (2016) also concluded that there was an increase in free fatty acids during solid-state fermentation of plum pomace (from the juice industry) and brandy distillery wastes, due to hydrolysis and the presence of lipid-degrading enzymes.

3.2. Fatty acid composition

When considering the biologically active components of edible oils, fatty acid composition and content are of particular significance. In addition to providing the highest amount of energy, certain fatty acids are essential for the normal functioning of the body; they can even have a protective effect from various health conditions. Table 3 presents the composition of fatty acids in the examined oil samples derived from the kernels of damson plums var. "Čačanska rodna" from plum kernels before fermentation (PKBF), plum kernels after fermentation (PKAF) and plum kernels after distillation (PKAD).

Table 3. Fatty acids (FA) and indexes of atherogenicity (AI) and thrombogenicity (TI)

FA (g·100g^{-1})	PKBF	PKAF	PKAD
C16:0	5.3±0.6^{b}	5.6±0.9^{b}	5.4±0.9^{c}
C16:1	1.4±0.6^{a}	1.5±0.4^{a}	1.5±0.5^{a}
C18:0	1.6±0.4^{a}	1.7±0.3^{a}	1.6±0.5^{a}
C18:1 *cis*	61.8±1.1^{d}	61.6±1.4^{d}	56.5±2.0^{f}
C18:1 *trans*	-	-	3.1±0.2^{b}
C18:2 *cis*	29.9±1.3^{c}	29.6±0.9^{c}	29.1±1.1^{e}
C18:2 *trans*	-	-	2.8±0.6^{b}
ΣSFA	6.9±1.1^{b}	7.3±1.2^{b}	7.0±1.4^{d}
ΣMUFA	63.2±1.7^{d}	63.1±1.8^{d}	59.6±2.7^{f}
ΣPUFA	29.9±1.3^{c}	29.6±0.9^{c}	31.9±1.7^{e}
O/L	2.1	2.1	1.9
AI	0.07	0.08	0.08
TI	0.15	0.16	0.16

Oleic acid was found to be dominant in all samples, with 55.7-63.2%. It was followed by linoleic (27.0-29.9%), palmitic (5.3-5.6%) and stearic acids (1.6-1.7%). The obtained data are in line with the results reported by other authors (Hassanein, 1999; Özcan *et al.,* 2014; Uluata and Nurhayat, 2017; Górnaś *et al.*, 2017a). In terms of fatty acid content, especially when it comes to the dominant monounsaturated oleic acid, plum kernel oil resembles olive oil and as such has a significant nutritional value. In addition, the linoleic fatty acid content is also very important, since this is an essential ω-6 fatty acid, necessary for the formation of cell membranes, vitamin D metabolism and synthesis of various hormones (Simopoulos, 2002). Also, the oleic/linoleic acid ratio (O/L ratio) is a significant indicator of the quality of kernels and oils of the genus *Prunus*. The higher the O/L ratio, the better the quality and oxidative stability of the kernels and oil. In the examined samples, this ratio was in the range of 1.9-2.1. By comparison, the O/L ratio ranged from 2.20-4.97 in 20 almond selections examined by Čolić *et al*., (2017).

Palmitoleic acid was also detected in oils from kernels that had undergone fermentation and distillation, representing 1.5 and 1.4%, respectively. Even though the three examined oils showed very similar fatty acid compositions, there was a difference in presence and content in *trans* fatty acids in oils originating from kernels that had passed through distillation. Since the distillation was carried out in an alembic pot directly heated by a wood-fuelled fire, we assume that the lower layers of the fruit mash (containing the majority of pits) were occasionally overheated in the absence of intense stirring, which may have caused the isomerization of double bonds and the appearance of trans fatty acids. Such a high content in *trans* fatty acids (5.9%) is not desirable and it decreases the nutritional value of the oil derived from distilled kernels.

The range of different fatty acid types in the examined kernel oils was as follows: saturated (6.9-7.3%), monounsaturated (59.6-63.2%) and polyunsaturated (29.6-31.9%). MUFA-containing oils are more stable and less susceptible to oxidation than those containing PUFAs. Also, several studies have shown that MUFAs increased HDL cholesterol and decreased both total and LDL cholesterol, lowered blood pressure, improving circulation by reducing clogging and hardening of arteries (Mariotti and Peri, 2014).

The atherogenicity and thrombogenicity indexes are indicators of cardiovascular disease risk increase and are related to fatty acid composition. Given the predominant monounsaturated oleic fatty acids in plum kernel oil, these indices have low values in the examined samples (AI 0.07-0.08 and TI 0.15-0.16), indicating that the oil lowers the risk of cardiovascular disease and is, therefore, healthier.

3.3. Tocopherol content and composition

Tocopherols are essential unsaponifiable components of vegetable oils. The total content of these natural antioxidants, as well as the presence of some of their homologues, depend on multiple factors (raw material variety, type of oil, climate conditions, oil production process, tocopherol measurement methods, etc). There are four tocopherol homologues: α-, β-, γ- and δ-tocopherol; α-tocopherol increases the biological value of vegetable oils while γ- and δ-tocopherols increase oxidative stability. β-tocopherol is seldom found in vegetable oils (Pongracz *et al.*, 1995; Kamal-Eldin, 2006). The content and composition of tocopherols in the examined oils are shown in Table 4.

TABLE 4. Content and composition of tocopherols (mg·100g^{-1})

Sample	α-tocoferol	β+γ-tocoferol	δ-tocoferol	Total tocopherols
PKBF	7.0±0.2^{a}	48.5±0.8^{a}	6.3±0.4^{a}	61.8±1.4^{a}
PKAF	18.6±0.6^{c}	57.7±1.3^{b}	11.1±0.7^{c}	87.4±2.6^{c}
PKAD	15.0±1.1^{b}	56.4±0.9^{b}	8.2±1.1^{b}	79.6±3.1^{b}

*The results are presented as mean value ± SD (n=3); different letters in columns indicate that there is significant difference at $p < 0.05$, (according to Tukey's HSD test).
PKBF - Kernels from fresh plums before fermentation, PKAF - Kernels separated after fermentation, PKAD - kernels separated after distillation.

As can be seen in Table 4, the lowest total tocopherol content was in the oil from PKBF, 61.8 mg·100 g^{-1} while the total tocopherol content was significantly higher in the oil after fermentation (87.4 mg·100g^{-1}) and distillation (79.6 mg·100g^{-1}). The increase in tocopherol content can be explained by the effect of alcohol during the fermentation process, which accelerates the separation of individual components, affecting primarily the porosity of the kernel. However, during distillation, although this process may continue, high temperatures cause degradation of the tocopherols and decrease their content (Bjelica *et al.*, 2019). The β+γ-tocopherol were dominant in all oil samples, with 48.5 mg·100g^{-1} in PKBF, while in oil samples from PKAF and PKAD, this tocopherol homologues were represented by 57.7 and 56.4 mg·100g^{-1}, respectively. According to the literature, the remaining representatives of the *Prunus* genus also have γ-tocopherol as the dominant tocopherol in their kernel oils, with its content ranging up to 1333 mg·kg^{-1}, which was found in sour cherry kernel oil (Matthaus and Özcan, 2009; Manzoor*et al.*, 2012; Górnaś *et al.*, 2016b; Górnaś *et al.*, 2017b).

The content of δ-tocopherol, the homologue that greatly increases oxidative stability, is particularly interesting. The highest content in this homologue was found in the oil from kernels after fermentation, 11.1 mg·100g^{-1}. The content of α-tocopherol ranged from 7.0 mg·100g^{-1} in oil from PKBF to 18.6 mg·100g^{-1} in PKAF and 15.0 mg·100g^{-1} in PKAD oil. The results are comparable to the results for α- and γ-tocopherol content found by Górnaś *et al.*, (2015) in their analysis of the composition of tocochromanols in the kernels of 28 different plum varieties. However, the δ-tocopherol content in the samples analyzed by Górnaś *et al.*, (2015) was far lower, ranging from 0.71 to 4.04 mg·100g^{-1}. In further investigations, Górnaś *et al.*, (2016a) found the content of total tocochromanols for 28 different plum varieties to be quite high, in a wide range of 70.7-208.7 mg·100g^{-1} oil.

Even though the plum kernel oil can be compared to olive oil in terms of its fatty acid composition and content, this is not the case when it comes to tocopherol content. α-tocopherol is dominant in olive oil, ranging up to 370 mg·kg^{-1} in content (Psomiadou *et al.*, 2000). In terms of tocopherol content and composition, this oil can be compared to maize, soy and rapeseed oils (Moreau, 2002).

3.4. Oxidative stability

When determining the quality of cold-pressed oils, another important parameter is oxidative stability, i.e. the preservation of oil from oxidation over a certain period of time. Oxidative stability was tested using the Rancimat test based on IP in hours. The results are shown in Table 5.

The results of the IP determination show a very good oxidative stability of the examined oils. The highest IP was found in the PKBF sample, 44.4 h, with the oxidative stability decreasing after fermentation and distillation, so the IP in the PKAF and PKAD samples was found to be 38.7 h and 33.6 h, respectively. Comparing the obtained results using the Tukey test ($p < 0.05$) shows that there is a statistically significant difference in the induction period between oil samples from kernels before and after treatments (fermentation, distillation).

TABLE 5. Oxidative stability

Sample	IP (h) 100°C, 20L·h^{-1}
PKBF	38.7±1.9^{b}
PKAF	44.4±1.7^{c}
PKAD	33.6±1.2^{a}

*The results are presented as mean value ± SD (n=3); different letters in the column indicate that there is significant difference at $p < 0.05$, (according to Tukey's HSD test).
IP - induction period. PKBF - Kernels from fresh plums before fermentation, PKAF - Kernels separated after fermentation, PKAD - kernels separated after distillation.

There is insufficient data on the IP of cold-pressed plum kernel oil in literature. Uluata and Nurhayat (2017) listed an IP of 15.1 h for plum kernel oil, without specifying whether this oil was cold-pressed; the Rancimat test was performed at a slightly higher temperature (110 °C). The same authors listed IP values for sour cherry kernel oil, another plant from the *Prunus* genus, of only 1.5 h (Uluata and Nurhayat, 2017). Uluata (2016) compared IPs (Rancimat test at 110 °C) of apricot kernel oil obtained by two methods, cold pressing and solvent extraction. The solvent-extracted oil showed a longer induction period (20.1 h) than the cold-pressed (15.1 h). Comparing oxidative stability of cold-pressed plum kernel oil and apricot kernel oil during thermal and photooxidation, Kiralan *et al*., (2018) found plum kernel oil to be more stable than apricot kernel oil.

Such high oxidative stability of the tested samples is probably due to the predominant MUFA and total tocopherols, but other bioactive components not covered by this research could have certainly contributed.

4. CONCLUSIONS

Plum kernels are a good material for cold-pressed oil production, bearing in mind the total oil content. The oils from kernels obtained before and following fermentation and distillation vary in terms of fatty acid composition, tocopherol content and oxidative stability. *Trans* fatty acids were detected in the oil from kernels obtained after distillation. This was the oil sample with the lowest tocopherol content and the lowest oxidative stability; therefore, plum kernels obtained after distillation of the mash are not recommended for the production of cold-pressed oil.

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REFERENCES

Bjelica M, Vujasinović V, Rabrenović B, Dimić, S. 2019. Some chemical characteristics and oxidative stability of cold pressed grape seed oils obtained from different winery waste. *Eur. J. Lipid Sci. Technol.* **121,** 1800416. https://doi.org/10.1002/ejlt.201800416

Čolić S, Fotirić Akšić M, Lazarević K, Zec G, Gašić U, Dabić Zagorac D, Natić, M. 2017. Fatty acid and phenolic profiles of almond grown in Serbia. *Food Chem.* **234**, 455-463. https://doi.org/10.1016/j.foodchem.2017.05.00 6

Dulf FV, Vodnar DC, Socaciu, C. 2016. Effects of solid-state fermentation with two filamentous fungi on the total phenolic contents, flavonoids, antioxidant activities and lipid fractions of plum fruit (*Prunus domestica* L.) by-products. *Food Chem.* **209**, 27-36. https://doi.org/10.1016/j.foodchem.2016.04.016

El-Adawy TA, Taha KM. 2001. Characteristic and composition of different seed oils and flours. *Food Chem*. **74**, 47-54. https://doi.org/10.1021/jf001117+

Górnaś P, Rudzińska M, Soliven A. 2017a. Industrial by-products of plum *Prunus domestica* L. and *Prunus cerasifera* Ehrh. as potential biodiesel feedstock: Impact of variety. *Ind. Crops Prod.* **100,** 77-84. https://doi.org/10.1016/j.indcrop.2017.02.014

Górnaś P, Radziejewska-Kubzdela E, Mišina I, *Biegańska-Marecik* R, Grygier, A, *Rudzińska* M. 2017b. Tocopherols, tocotrienols and carotenoids in kernel oils recovered from 15 apricot (*Prunus armeniaca* L.) genotypes. *J. Am. Oil Chem. Soc.* **94,** 693-699. https://doi.org/10.1007/s11746-017-2978-y

Górnaś P, *Rudzińska* M, Raczyk M, Mišina I, Soliven A, Lacis, G, Segliņa D. 2016a. Impact of species and variety on concentrations of minor lipophilic bioactive compounds in oils recovered from plum kernels. *J. Agric. Food Chem.* **64**, 898-905. https://doi.org/10.1021/acs.jafc.5b05330

Górnaś P, Rudzińska M, Raczyk M, Mišina I, Soliven A, Segliņa D. 2016b. Composition of bioactive compounds in kernel oils recovered

from sour cherry (*Prunus cerasus* L.) by-products: Impact of the cultivar on potential applications. *Ind. Crops Prod.* **82,** 44-50. https://doi.org/10.1016/j.indcrop.2015.12.010

Górnaś P, Grāvīte I, Mišina I, Lacis G, Radenkovs V, Olšteine A, Segliņa D, Kaufmane E, Rubauskis E. 2015. Composition of tocochromanols in the kernels recovered from plum pits: the impact of the varieties and species on the potential utility value for industrial application. *Eur. Food Res. Technol*. **24**, 513-520. https://doi.org/10.1007/s00217- 015-2480-4

Hassanein M. 1999. Studies on non-traditional oil: Detailed studies on different lipid profiles of some *Rosaceae* kernel oils. *Grasas Aceites* **5,** 379-384. https://doi.org/10.3989/gya.1999.v50.i5.682

ISO. 2000. International Standards Official Methods 5509:2000. Animal and Vegetable Fats and Oils-Preparation of methyl esters of fatty acids. International Organization for Standardization, Geneve, Switzerland.

ISO. 2016. International Standards Official Methods 6886:2016 - Animal and Vegetable Fats and Oils —Determination of Oxidative Stability (Accelerated Oxidation Test). International Organization for Standardization, Geneve, Switzerland.

Kamal-Eldin A. 2006. Effect of fatty acids and tocopherols on the oxidative stability of vegetable oils. *Eur. J. Lipid Sci. Technol.* **58**, 1051-1061. https://doi.org/10.1002/ejlt.200600090

Kamel BS, Kakuda Y. 1992. Characterization of the seed oil and meal from apricot, cherry, nectarine, peach and plum. *J. Am. Oil Chem. Soc.* **69**, 492-494. https://doi.org/10.1007/BF0 2540957

Kiralan M, Kayahan M, Kiralan SS, Ramadan M F. 2018. Effect of thermal and photooxidation on the stability of cold-pressed plum and apricot kernel oils. *Eur. Food Res. Technol*. **244**, 31-42. https://doi.org/10.1007/s00217-0 17-2932-0

Manzoor M, Anwar F, Ashraf M, Alkharfy KM. 2012. Physico-chemical characteristics of seed oils extracted from different apricot (*Prunus armeniaca* L.) varieties from Pakistan. *Grasas Aceites* **63**, 193-201. https://doi.org/10.3989/gya.095011

Mariotti M, Peri C. 2014. The composition and nutritional properties of extra-virgin olive oil, in Peri C (Ed.) *The extra-virgin olive oil handbook*. John Wiley & Sons, Oxford, UK.

Matthaus B, Özcan MM. 2009. Fatty acids and tocopherol contents of some *Prunus* spp. kernel oils. *J. Food Lipids* **16**, 187-199. https://doi.org/10.1111/j.1745-4522.2009.01140.x

Moreau RA, Whitaker BD, Hicks KB. 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog. Lipid Res.* **41**, 457-500. https://doi.org/10.1016/S0163-7827(02)00006-1

Mratinić E. 2013. *Šljiva*, Partenon, Beograd. *(in Serbian)*

Özcan MM, Ünver A, Arslan D. 2014. A research on evaluation of some fruit kernels and/or seeds as a raw material of vegetable oil industry. *Qual. Accur. Saf. Crop. Foods* **7**, 187-191. https://doi.org/10.3920/QAS2013.03 19

Picuric-Jovanovic K, Vrbaski Z, Milovanovic M. 1997. Aqueous enzymatic extraction of plum kernel oil. *Fett/Lipid* **99**, 433-435. https://doi.org/10.1002/lipi.19970991205

Pongracz G, Weiser H, Matzinger D. 1995. Tocopherol-Antioxidantien der Natur. *Fett/Lipid* **97,** 90-104. https://doi.org/10.1002/lipi.19950970303

Psomiadou E, Tsimidou M, Boscou D. 2000. Alpha-tocopherol content of Greek virgin olive oils. *J. Agric. Food Chem.* **48**, 1770-5. https://doi.org/10.1021/jf990993o

Simopoulos AP. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **56,** 365-379. https://doi.org/10.1016/S0753-3322(02)00253-6

SRPS EN ISO 660:2015 - Animal and vegetable fats and oils - Determination of acid value and acidity. Institute for Standardization of Serbia.

SRPS EN ISO 3960:2016 - Animal and vegetable fats and oils - Determination of peroxide value

SRPS EN ISO 12966-4:2016 - Gas chromatography of fatty acid methyl esters - Part 4: Determination by capillary gas chromatography. Institute for Standardization of Serbia.

Ulbricht TLV, Southgate DAT. 1991. Coronary heart disease: Seven dietary factors. *Lancet* **338**, 985-992. https://doi.org/10.1016/0140-6736(91)91846-M

Uluata S, Özdemir N. 2017. Evaluation of chemical characterization, antioxidant activity and oxidative stability of some waste seed oil. *Turkish J. Agric. Food Sci. Technol.* **5**, 48-53. https://doi.org/10.24925/turjaf.v5i1.48-53.909

Uluata S. 2016. Effect of extraction method on biochemical properties and oxidative stability of apricot seed oil. *Academic Food J.* **14,** 333-340.

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Nutritional, functional and microbiological characteristics of Jordanian fermented green Nabali Baladi olives

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SUMMARY: The quality characteristics of green olives produced by the traditional spontaneous fermentation method in Jordan have never been studied. We investigated the nutritional, functional, and microbiological characteristics of Jordanian fermented green Nabali Baladi olives (GNBFO). Proximate composition, fatty acids, and total polyphenols were determined by standard protocols. Cultivable microflora was monitored over 3 months of fermentation. Isolated microorganisms were identified by molecular sequencing and *in vitro* probiotic traits were tested. GNBFO contained fiber (3g⁄100g), total polyphenols (306mg⁄100g), oil (19.3g ⁄100g), and oleic acid (70%). Yeast strains (*Candida diddensiae* and *Candida naeodendra*) were predominant and showed acid (pH=2.5) and bile salt (0.1% and 0.3%) resistant and high adhesion ability (ca∼10^7CFU/ml) to intestinal cell lines; they were positive to catalase and negative to lipase and none possessed antimicrobial activity against selected pathogens. Lactic acid bacteria were not detected. In conclusion, the GNBFO have promising functional characteristics as they contain valuable nutrients, antioxidants, and yeast strains with potential probiotic traits.

KEYWORDS: *Oleic Acid; Olive; Polyphenols; Probiotics; Spontaneous Fermentation; Yeast*

RESUMEN: ***Características nutricionales, funcionales y microbiológicas de las aceitunas verdes jordanas fermentadas Nabali Baladi.*** Hasta la fecha, no se han estudiado las características de calidad de las aceitunas verdes producidas por el método tradicional de fermentación espontánea en Jordania. En este trabajo, investigamos las características nutricionales, funcionales y microbiológicas de las aceitunas Nabali Baladi verdes fermentadas jordanas (GNBFO). La composición proximal, los ácidos grasos y los polifenoles totales se determinaron mediante protocolos estándar. La microflora cultivable se controló durante 3 meses de fermentación. Se identificaron microorganismos aislados por secuenciación molecular y se probaron los rasgos probióticos in vitro. GNBFO contenía fibra (3g/100g), polifenoles totales (306mg/100g), aceite (19,3g/100g) y ácido oleico (70%). Las cepas de levaduras (*Candida diddensiae* y *Candida naeodendra*) fueron predominantes y mostraron acidez (pH=2.5) y sales biliares (0,1% y 0,3%) resistentes y alta capacidad de adhesión (ca ∼ 10^7CFU/ml) a las líneas celulares intestinales; fueron positivos para catalasa y negativos para lipasa y ninguno poseía actividad antimicrobiana contra patógenos seleccionados. No se detectaron bacterias del ácido láctico. En conclusión, GNBFO tiene características funcionales prometedoras, ya que contienen valiosos nutrientes, antioxidantes y cepas de levaduras con posibles rasgos probióticos.

PALABRAS CLAVE: *Ácido Oleico; Fermentación Espontánea; Levadura; Oliva; Polifenoles; Probióticos*

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1. INTRODUCTION

The olive (*Olea europaea* L.) is an ancient cultivated human plant food. It originates in the Middle East and the Mediterranean region and is central to the local diet (Al-Ismail *et al*., 2011; Ghanbari *et al.,* 2012). Despite the global spread of olive cultivation, most of its production comes from the Mediterranean basin, (Ghanbari *et al.,* 2012). In Jordan, almost 80% of the olives are used for oil production and 20% for fermentation to produce table olives (MOA, 2016). In 2016, table olive consumption in Jordan reached 28 thousand tons (MOA, 2016). Consumption of olives is related to higher-quality diets, including higher intakes of dietary fiber, proteins, a number of vitamins, mineral elements, phenolic compounds, organic acids, pigments, and phytosterols, monounsaturated oleic acid, and lower intakes of saturated fat (López *et al.,* 2007; Ghanbari *et al.,* 2012). These components are known to possess multiple biological, medicinal, and therapeutic effects such as antioxidant, antihyperlipidemic, antimicrobial, anti-inflammation, antihypertensive, anticarcinogenic, and antithrombotic (Lanza *et al.,* 2010). In essence, the intake of olives and their oil is associated with several health benefits, particularly reduced risks of cardiovascular disease, insulin resistance, and cancer (Saibandith *et al.,* 2017). Nevertheless, the salt content in table olives greatly varies depending on the preparation process (López *et al.,* 2008), a matter that may represent a health concern. This is especially important in populations which consume high amounts of olives and subsequently salt, because of its association with hypertension (Pino *et al.,* 2018).

The olive's functional nutrients, bioactive, and health-promoting components have been shown to vary greatly with olive genotype, maturity, product quality, postharvest handling and storage conditions, agricultural practices, and methods of processing and analysis (Bleve *et al*., 2014). Worldwide, about two thousand olive varieties have been identified (Ghanbari *et al.,* 2012); several of these varieties inhabit Jordan, mainly Nabali Baladi, Muhasan, Rassei, Shami and Nasohi (Al-Ismail *et al.,* 2011). The Nabali olive is the major olive genotype grown in Jordan and is widely used in traditional fermentation and oil production as it is naturally adapted to the dry conditions in Jordan and provides a high oil yield (Humeid *et al.,* 1991). In fact, the available literature on the nutritional and health benefits of olives and olive oil is basically derived from results of studies investigating olive varieties other than those grown in Jordan (Lanza *et al.,* 2010; Aponte *et al.,* 2010; Issaoui *et al.,* 2011).

Furthermore, a number of technological methods to produce table olives are available (Issaoui *et al.,* 2011) and yield olives with varied chemical and microbiological characteristics (Abriouel *et al.,* 2011). These methods of olive fermentation greatly influence the competitive activities of the natural microflora that lead to spontaneous fermentation (Abriouel *et al.,* 2011). Several Lactic acid bacteria (LAB) and yeast species are the main natural microbes engaged in olive fermentation (Bleve *et al*., 2014). In Jordan and eastern Mediterranean countries, the traditional fermentation method of green olives depends on spontaneous fermentation in high brine concentration after debittering the green olives by soaking in water for 3 days (NCARE, 2000). This method results in fermented olives with a unique sour taste that differs remarkably from Greek, Italian or Spanish methods. To the best of our knowledge, the nutritional, functional and microbiological characteristics of the fermented Nabali olive have not yet been elucidated. Hence, this study was performed to identify some of such characteristics of table olives produced by the traditional fermentation method in Jordan.

2. MATERIALS AND METHODS

2.1. Olives fermentation

Green Nabali Baladi olives (15 kg) were purchased from a local farm in Jerash, Jordan, in two successive seasons (November 2017 and November 2018) and processed according to the traditional method of green olive fermentation in Jordan (NCARE, 2000). Olives of each season were crushed under mechanical pressure to make a scratch in the flesh, soaked in water for three days, placed in two glass jars and the brine solution (10% wt/vol) was added. The brined olives were kept in a dark, dry place at room temperature (17-20 °C) to allow natural fermentation to take place (NCARE, 2000). The fermentation lasted 3 months.

2.2. Microbiological analysis

A 30 g sample of olive flesh was taken from each jar after 30, 60 and 90 days of fermentation. Samples were washed with sterile saline (0.9% NaCl wt/vol) solution (30 ml) and homogenized by a Stomacher lab blender (Bag Mixer® 400W, Interscience, France). A 10g sample of the homogenized olive paste was diluted with 90 ml peptone water, and then 10-fold serial dilution was carried out. Lactic acid bacteria (LAB) and yeasts were quantified as follows: samples of 100 µl of each dilution were applied to agar plates that contained Man, Rogosa and Sharpe Agar (MRS, Oxoid, England), followed by anaerobic incubation at 30 °C for 48-72 hours (Bleve *et al.,* 2014) and Potato Dextrose Agar (Oxoid, England) supplemented with chloramphenicol and chlortetracycline (Dar Al Dawa, Amman, Jordan), followed by incubation at 25 °C for 2-4 days (FDA, 2001). Gram staining and viable counts were performed to identify the isolated microorganisms. The pH of the brine solution was measured (Hanna Instrument, USA). Analysis of brine concentration was also performed by using Mohr's titration method (AOAC, 1995). The experiments were performed in triplícate.

2.3. Analysis of proximate composition

A proximate composition analysis was performed on the fermented olives according to the reference Weende method (AOAC 1995). For moisture content determination, samples of olive pulp were dried at 105 °C using the air oven (Memmert, Karlklob-West Germany) until a constant weight was reached. The dried olive pulp was used to determine the fat content following the solvent (ether 40 -70 °C) extraction procedure for 6 hours using the Soxhlet apparatus. The ether was evaporated and the residual oil was then weighed (AOAC, 1995). Crude protein in the pulp of the dried olives was measured using the micro Kjeldahl method (AOAC, 1995). The pre-weighed samples of olives were digested in 10 ml sulfuric acid in the presence of catalyst tablets containing K_2SO_4, $CuSO_4$, and selenium. The reduced ammonia was liberated by boiling with 50% sodium hydroxide and distilled (Rapid Distillation Unit, Lanconco Corporation, Kansas City, USA) into a boric acid solution to form ammonia borate that was titrated directly using a standard sulfuric acid solution. The resultant nitrogen content was multiplied by the factor 6.25 to determine the crude protein content.

Crude ash was analyzed by the dry ashing method (AOAC, 1995). The pre-weighed samples were ignited in a muffle furnace (Naber Model D2804, Bremen) at 550 °C for 6 hours. The inorganic ash residue was then weighed. The crude fiber of dried and defatted olive pulp was determined according to the Van Soest method (AOAC, 1995). In pre-weighed filter bags, a sample of 1 g of dried and defatted olives was weighed. The sample-containing bag was sealed and placed in the bag suspender trays of the fiber analyzer (ANKOM, USA). The sample was digested using a standard sulfuric acid solution (1.25%) for 30 minutes, and then in a sodium hydroxide solution (1.25%) for 30 minutes. The bags were then dried in an air oven at 105 °C and cooled before weighing. The sample-containing bag was ashed in pre-weighed crucibles for 2 hours at 550 °C, cooled in a desiccator, and then weighed. The crude fiber content was calculated by subtracting the weight before and after ashing (AOAC, 1995). The carbohydrate content of olive pulp was estimated as the resulting difference from subtracting the content of each of moisture, protein, fat, ash, and fiber from 100% (Bleve et al., 2014).

2.4. Analysis of fatty acid composition

The extraction of fat from fermented olives was performed according to Issaoui *et al*., (2011). The olive pulp was well ground to form a paste from which fat was extracted using a methanol/water/chloroform (1/1/1; v/v/v) solvent mixture. The fat-containing mixture was centrifuged, the solvent layer was evaporated at 60 °C, and the EC Regulation no. 2568/91 method was used to prepare the fatty acid methyl esters. In brief, a pre-weighed sample of fat extract (50 mg) was well mixed with hexane (2 ml, GC grade) and a freshly-prepared solution (200 µl) made up of 2 M-potassium hydroxide in anhydrous methanol was added and thoroughly mixed to form a clear solution, and then acetic acid (200 µl) was added with continuous mixing. Soon after esterification was complete, the capillary GC column (Restek, Rtx-225, USA, cross bond 50%-cyanopropyl-methyl 50%-phenylmethyl polysilo-xane, 60 m, 0.25 mm/D, 0.25 µm df) was used to determine the fatty acid methyl esters. The latter was identified using the chromatogram of the corresponding fatty acid methyl ester standards (Supelco Inc, Bellefonte, USA) as described elsewhere (Al-Ismail *et al.,* 2011).

2.5 Analysis of total polyphenols

The Folin-Ciocalteu assay was used to quantify the total polyphenols in olive pulp. The method is essentially based on the ability of phenols to reduce phosphomolybdic acid in aqueous alkali media. Methanol solvent was used to extract (3 times) olive pulp (5 g) and (0.5 ml) of this extract was well mixed with (0.5 ml) Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Buchs, Switzerland). A volume of (3 ml) of Na_2CO_3 saturated solution was added to the batter and brought up to (10 ml) with distilled wáter, and then allowed to stand for 30 minutes at room temperature. The final solution was centrifuged (10 minutes, 3000 g) and the supernatant absorbance was read (765 nm) by the spectrophotometer (LABOMED, Los Angeles, USA). The olive pulp's total polyphenols were expressed as mg of gallic acid/100g of fresh fruits (Bleve *et al.,* 2014).

2.6. Identification of yeast isolates

The isolated yeast from the fermented olives after 90 days of fermentation was named as Y1 and Y2 for the two yeast strains isolated in the 2017 season, and Y3 and Y4 for the two strains isolated in the 2018 season. Y1, Y2, Y3, and Y4 and the reference strain of *Candida albicans* ATCC 10231 were grown overnight in the YPG medium (Yeast extract 1%: oxoid, England, peptone 2%: oxoid, England, glucose 2%) at 30 °C. The manufacturer's instructions of Wizard Genomic DNA purification (Promega, Madison, WI, USA) were strictly followed in the extraction of the experimental genomic DNA. Sequencing the 5′ end of the 26S rDNA encompassing the D1 and D2 expansion domains using the primers NL1 (5′-GCATATCAATAAGGGGA GGAAAAG-3′) and a reversed primer NL4 (5′-GGT CCG TGT TTC AAG ACGG-3′) were then performed for the identification of experimental genomic DNA. Amplification was performed for 36 PCR cycles with annealing at 52 °C, extension at 72 °C for 2 minutes, and denaturation at 94 °C for 1 minute. The products of PCR were purified and sequenced for species identification (Macrogen, Korea). The National Center for Biotechnology Information BLAST online program was used to determine the DNA sequence similarity (BLAST, 2019) and as given by Wang et al., (2008).

2.7. *In Vitro* probiotic characterization of yeast

2.7.1. Tolerance of yeast to low pH and bile salt

An overnight culture of yeast isolates was tested for viability as affected by low pH and bile salt. Fresh yeast culture (100 µl; ca $1x10^8$ CFU/ml) of each yeast isolate was inoculated in sterile test tubes containing either: 1 ml of acidified YPG medium (5 M HCl, pH 2.5), 1 ml of YPG containing 0.1% bile salts, 1 ml of YPG containing 0.3% bile salts, or 1 ml of YPG alone. A viable count of yeast isolates in acidified YPG medium was measured at 0, 1, 2 and 3 hours of incubation at 37 °C by taking samples of 100 µl, serially diluted and plated onto YPG agar plates. Yeast isolates' survival in bile salts was measured after incubation for 24 hours at 37 °C. A yeast culture (one loopful of 2 mm in diameter) was streaked onto YPG agar to check viability. Yeast isolates inoculated in YPG medium alone was used as the positive control. The test was performed in duplicate (van der Aa Kuhle *et al.,* 2005).

2.7.2. Adhesion of yeast strains to intestinal cell lines

The colonocyte-like cell lines Caco-2 were used to determine the adhesion ability of the yeast isolates. Caco-2 cell line was kindly supplied by Prof. Y. Bustanji of the University of Jordan. The culture and maintenance of the cell lines were carried out following standard procedures using DMEM medium High Glucose (EuroClone, Italy) supplemented with fetal bovine serum (10%) and with a mixture of antibiotics (10 U/ml penicillin, 10 µg/ml streptomycin). Intestinal cells were seeded in 24-well plates and cultivated until a confluent differentiated state was reached (monolayers). Yeasts were grown in YPG media for 48 hours at 30 °C, then 1 ml of yeast culture was centrifuged and the pellet was washed twice with PBS solution. The yeast pellet was suspended in the DMEM media without antibiotics to reach an inoculum size of ca~10^8 CFU/ml. Cellular monolayers were also carefully washed with PBS solution, and yeast suspensions were added. Adhesion experiments were carried out for 90 minutes at 37 °C, 5% CO_2 and, afterward, wells were gently washed to release unattached yeasts before proceeding with the lysis of cellular monolayers using a 0.25%

Trypsin- EDTA solution (Sigma, USA). Dilutions of samples, before and after adhesion, were made in PBS solution and yeast counts were performed on YPG agar plates. The adhesion was calculated as: % CFU adhered yeasts/ CFU added yeasts. Experiments were carried out in two replicated plates and in each plate, two wells were used per sample (Zivkovic *et al*., 2015).

2.7.3. Antimicrobial activity of yeasts

A few common food-borne pathogens were selected, *Escherichia coli* (ATCC 8739), *Salmonella typhimurium* (02:8432), *Salmonella enteridis* (CRIES1016), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus aureus* (ATCC 6538P); they were cultured on Trypticase Soy Soft (TSS) agar (20 mL, with 8 g/L agar; Oxoid, England). The capacity of each yeast strain to inhibit the previous bacterial pathogens was determined using the TSS agar medium. One loopful (2 mm in diameter) of yeast was streaked as a line (2-3 cm) onto the soft-agar surface; the plates were then incubated at 27 °C for 24 hours and screened for inhibition zones around the bacterial colonies (Silva *et al.,* 2011).

2.8. Technical characteristics of yeast isolates

The lipolytic activity of yeast isolates was qualitatively tested with 5% (v/v) olive oil, emulsified by vigorous shaking. The plates were inoculated by streaking once across and incubated at 25 °C for 10 days. The plates were then flooded with a saturated copper sulphate solution and allowed to stand for 10 minutes. Where growth occurred, the appearance of a bluish color was taken as being indicative of positive lipolytic activity. The blue zones were intensified if the plates were kept in the refrigerator after pouring off the developer (Kurtzman *et al.,* 2011). Catalase activity was determined by adding drops of H_2O_2 (3%) to the cultured colonies; the release of gas was indicative of a positive result (Silva *et al.,* 2011).

2.9. Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences programme version 20 (SPSS®), Chicago, Il, USA). Values are given as the mean ± SD. Each datum represents six replicates for each season; i.e. three sample replicates for each of the two jars each season were measured. Appropriate data were tested using the independent sample t-test for significant differences. The statistical significance level was fixed at $p < 0.05$ (Issaoui *et al.,* 2011)

3. RESULTS

3.1. Microbiological Characteristics of fermented olives

After 3 months of olive fermentation, yeast was the predominantly isolated microorganism from the olive samples. As shown in Figure 1, the yeast count reached its highest level after 2 months of fermentation (ca~ $3x10^4$ CFU/ gram olive in the 2017 season). A significant (p=0.004) reduction in yeast count was detected by the end of the third month compared to the yeast count during the second month. The pH of the brine measured at zero time fermentation was 5.2. pH values and were significantly (p=0.001) reduced after 60 days of fermentation (pH= 4.45 in season 2017, pH=4.26 in season 2018) compared to values after 30 days of fermentation (Figure 1). At the start of fermentation, the brine concentration was 100 g/L. It markedly (p=0.001) decreased after 30 days of fermentation (90 g/L in season 2017 and 91 g/L in season 2018). A further, but stable drop in this variable, was noticed from 60 days till the end of fermentation (89 and 86 g/L for in the two seasons, respectively). In the 2018 season, lower yeast counts were detected after 3 months of fermentation compared to the yeast count in the 2017 season; the yeast count reached ca~ $5x10^2$ CFU/g olives on the 30^{th} day, $8.8x10^3$ CFU/g olives on the 60^{th} day and 7.8 x 10^3 CFU/g olives on the 90^{th} day of fermentation. By the end of 90 days of fermentation, the LAB was not detected at any level. Figure 1 clarifies the dynamics between yeast count and pH values during the olive fermentation process.

3.2. Proximate composition and total polyphenols in fermented olives

The analysis of proximate composition of fermented green Nabali Baladi olives revealed a high content in oil (19% wet weight). Table 1 shows the detailed proximate composition analysis results for fermented olive samples taken from 2 separate jars in two different seasons. The results of the two seasons' analysis were comparable. Crude fiber content was 3 g/100g edible part of olives. A low amount of protein was

detected in fermented olives (0.9 g/100g edible part). The total polyphenol content in green Nabali Baladi olives ranged from 290-310 mg gallic acid equivalent/100g of fermented green olive pulp (Table 1).

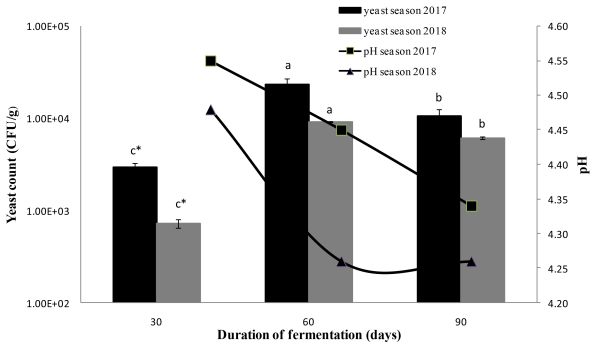

FIGURE 1. Viable count of yeasts (CFU/g) and brine pH changes during green Nabali Baladi olive fermentation.
Lactic acid bacteria were not detected during 90 days of the experiment. Values are given as mean ± SD (Based on three samples replicaes for each of the two jars each season; i.e. six replicates for each season). Independent sample t-test was used. Bars with different superscripts are significantly different ($p<0.05$). * Significantly different ($p<0.05$) in the same time duration.

3.3. Fatty acid composition of fermented olives

Table 2 presents the fatty acid composition of green Nabali Baladi fermented olives. Oleic acid was the major fatty acid (about 70% of total fatty acids). Other fatty acids such as palmitic acid, linoleic acid and stearic acid account for about 25% of the total fatty acids. Total saturated fatty acids compose 17% of the fatty acid, whereas total polyunsaturated fatty acids were only 9%.

3.4. Identification of yeast isolates

The identification of yeast isolates (Y1, Y2, Y3, and Y4) was performed by the sequencing of the 26S rDNA encompassing the D1/D2 expansion domains. BLAST identified the two DNA sequences as *Candida diddensiae*, *Candida naeodendra* strain MB14804, *Candida diddensiae,* and *Candida diddensiae* as 100%, 99%, 99%, and 99%, respectively (Table 3).

3.5. *In Vitro* probiotic characterization of yeast

3.5.1. Tolerance to low pH and bile salt and adhesion to intestinal cell lines

Table 3 shows the results of Y1, Y2, Y3 and Y4 *in vitro* probiotic characteristics testing. Y1 showed higher tolerance to HCl than Y2. On another hand, isolates Y3 and Y4 isolated in the second season had comparable tolerance to acidic media. Collectively, ca~ 10^7 CFU/ ml of yeasts were able to survive acidic conditions after 3 hr of incubation. In addition, all yeast isolates showed comparable tolerance to bile salt at different concentrations (0.1 and 0.3%). Y1, Y2, Y3, and Y4 also showed high adhesion ability to Caco-2 cell line, ca~ 10^7 CFU of yeast isolates were able to attach to intestinal cell lines after 90 min of incubation. Y3 and Y4 had higher adhesion ability to the Caco-2 cell line than Y1 and Y2, but it was considered low adhesion ability (Table 3).

TABLE 1. Proximate composition analysis of fermented green Nabali Baladi olives per 100 g of edible part [1-4]

Nutrient	g/ 100 g of edible part	
	Season 2017	Season 2018
Moisture	67.9±0.3[a]	67.5±0.6[a]
Fat	19.27±0.4[a] (60.0±0.3)	19.83±0.2[a] (61.0±0.2)
Carbohydrate	4.9±0.6[a] (15.26± 1.2)	4.3±0.4[a] (13.2±0.3)
Crude Protein	0.9±0.05[a] (2.8±0.3)	0.9±0.02[a] (2.8±0.3)
Crude Fiber	3.0±0.1[a] (9.5±0.7)	3.13±0.06[a] (10.2±0.1)
Ash	4.1±0.1[a] (12.5±1.9)	4.2±0.1[a] (12.9±1.3)
Total Polyphenols*	306.3±42.3[a] (954.2±130.8)	295.4±14.6[a] (908.9±45.5)

1. Edible part of fermented olive is 75.27 g/ 100 g of whole olives
2. Values between parenthesis are calculated based on dry weight
3. Values are given as mean ± SD (Based on three samples replicates for each of the two jars each season; i.e. six replicates for each season). Independent sample t-test was used.
4. Values with different superscripts within the same row are significantly different ($p<0.05$)
*Total polyphenols was expressed as mg of gallic acid/100 g edible olive

3.5.2. Antimicrobial activity of yeasts

The yeast isolates (Y1, Y2, Y3, and Y4) did not show any inhibition zone surrounding their colonies in the antimicrobial tests, which indicated that the isolates did not have antimicrobial activity against the selected pathogenic bacteria.

3.6. Technical characteristics of yeast isolates

Yeast isolates were tested for lipase and catalase production. They were found negative for lipase production and positive for catalase (Table 3).

TABLE 2. Fatty acid composition of oil extracted from fermented olives[1, 2]

Fatty acid	g/ 100 g of total fatty acid	
	Season 2017	Season 2018
C 16:0	14.3±0.06 [a]	14.3±0.06 [a]
C 16:1	1.5±0.02 [a]	1.6±0.02 [a]
C 18:0	2.6±0.08 [a]	2.6±0.08 [a]
C 18:1	70.6±0.3 [a]	69.8±0.3 [a]
C 18:2	8.2±0.1[a]	8.2±0.1[a]
C 18:3	0.73±0.02 [a]	0.74±0.02 [a]
C 20:0	0.4±0.01 [a]	0.4±0.01 [a]
C 20:1	0.23±0.01[a]	0.22±0.01[a]
C22:0	0.1±0.01[a]	0.1±0.01 [a]
Σ SFA	17.4	17.3
Σ MUFA	72.33	71.57
Σ PUFA	8.94	8.89

1. Values are given as mean ± SD (Based on three sample replicates for each of the two jars each season; i.e. six replicates for each season). Independent sample t-test was used.
2. Values with different superscripts within the same row are significantly different ($p<0.05$)

4. DISCUSSION

The Nabali Baladi olive is one of the major local olive varieties in Jordan; it is widely used for fermentation and oil production (Humeid *et al.,* 1991). A limited number of studies investigated the qualitative characteristics of fresh Nabali Baladi olives with an emphasis on oil production purposes. To our knowledge, this is the first study investigating the nutritional, functional and microbiological characteristics of fermented green Nabali Baladi olives in Jordan.

The results demonstrated that the oil content of fermented olives ranged from 19-20% (wet weight) of flesh (60% based on dry weight) after 3 months of fermentation in two different seasons (2017 and 2018). It has been reported that the oil content of ripened Nabali Baladi olives without fermentation was 60% of flesh dry weight in Jordan (Al-Ismail *et al.,* 2011) and 55% (dry weight) in Palestine (Ebiad and Abu-Qaoud, 2014). These results are comparable to those of the present study. However, the oil content of fermented green olives is widely variable depending on the olive variety. In Tunisia, one of the major olive producer in the region, the oil content of green table olives ranged from 29% (dry weight) in the *Sayali* olive variety (Sakouhi *et al.,* 2008) to 35% (dry weight) in the *Meski* olive variety (Issaoui *et al.,* 2011). Moreover, Turkish olive varieties such as the *Domat* cultivar had low oil content amounting to 23% based on dry weight (Savas and Uylaser, 2013), while *Gemlike* and *Edincik* varieties had high oil content reaching 38 and 50%, based on dry weight, respectively (Borcakli *et al.,* 1993). High oil content (68% of dry pulp) was found in the Italian *Intosso d'Abruzzo* cultivar table olives (Lanza *et al.,* 2010). Accordingly, Nabali Baladi olives can be considered a rich source of oil before and after fermentation compared to other olive varieties in the region.

In this study, it was demonstrated that 100 g of edible fermented green Nabali Baladi olives contained 0.9 g protein, 4.6 g carbohydrate and 3 g of crude fiber. Similar to our results, the Italian green *Intosso d'Abruzzo* fermented olives were found to contain 1 g protein, 2.6 g fiber and 2.8 g carbohydrates in 100 g fermented olives (Lanza *et al.,* 2010). However, it has been documented that the olive fresh fruit's average composition includes water (50%), protein (1.6%), oil (22%), carbohydrate (19.1%) and cellulose (5.8%) (Ghanbari *et al.,* 2012).

Indeed, the analysis of fatty acid profile in Nabali Baladi olive oil revealed that oleic acid is the main fatty acid followed by palmitic acid and linoleic acid (70, 14.3 and 8.3%, respectively). It was found that olive processing and fermentation did not cause any significant changes in the fatty acid profile; instead, the fatty acid profile was dependent on olive varieties (López-López *et al.,* 2015). Previous studies analyzed the fatty acid profile of olive oil extracted from Nabali Baladi olives cultivated in different regions of Jordan and Palestine and the results were highly variable (Humeid *et al.,* 1991; Al-Ismail *et al.,* 2011; Ebiad and Abu-Qaoud, 2014). It was found that oleic acid accounted for 67% of the oil extracted from Nabali Baladi olives collected from Bani Kenaneh in north Jordan, followed by linoleic acid (14%) and palmitic acid (12.5%) (Al-Ismail *et al.,* 2011). Oleic acid represented (66%) of total fatty acids in the oil extracted from Nabali Baladi olives in Palestine, followed by palmitic acid (15%) and linoleic acid (12.8%) (Ebiad and Abu-Qaoud 2014). On another hand, oleic acid represented a higher percentage (73-76%) and lower linoleic acid (8.5-9.7%) and palmitic acid (8.8-11.8%) in oil extracted from Nabali Baladi olives collected from Amman, Jordan (Humeid *et al.,* 1991). As in olive oil, fermented green Nabali Baladi olives are a valuable source of

TABLE 3. Yeast identification, HCl (pH 2.5) and bile salt tolerance, adhesive ability and technical characteristics for the yeast isolates from fermented green Nabali Baladi olives

	Identification by sequencing	Identity (%)	Viability of yeast				Adhesion ability CFU/ml		Technical characteristics	
			HCl (pH2.5) CFU/ml		Bile salt (%)		Time (min)		Lipase	Catalase
			0 h	3 h	0.1	0.3	0	90		
Y1	*Candida diddensiae*	100	$1.07x10^{8}$±$2x10^{7}$	$5.9x10^{7}$±$1.5x10^{6}$	+*	+	$1.6x10^{8}$±$3.1x10^{7}$	$3.5x10^{7}$±$1.1x10^{6}$	-	+
Y2	*Candida naeodendra* strain MB14804	99	$3.25x10^{7}$±$4.1x10^{6}$	$9.49x10^{6}$±$6.1x10^{5}$	+	+	$3.9x10^{8}$±$1.1x10^{7}$	$6.32x10^{7}$±$2.2x10^{6}$	-	+
Y3	*Candida diddensiae*	99	$5.35x10^{7}$±$5x10^{6}$	$2.43x10^{7}$±$3.6x10^{6}$	+	+	$1.35x10^{8}$±$5.2x10^{7}$	$4.34x10^{7}$±$1.6x10^{6}$	-	+
Y4	*Candida diddensiae*	99	$6.25x10^{7}$±$2.5x10^{6}$	$3.2x10^{7}$±$2x10^{6}$	+	+	$2.5x10^{8}$±$6.5x10^{7}$	$7.68x10^{7}$±$9.2x10^{5}$	-	+

*+ No inhibitions were observed compared to the control

Values are given as mean ± SD (Based on three sample replicates for each of the two jars each season i.e. six replicates for each season).

monounsaturated fatty acids (oleic acid), which is considered one of the health benefits of olives and olive oil (Ghanbari *et al.,* 2012).

Fermented olives have a wide range of functional properties besides high oil and oleic acid contents. Olive fruits are considered a rich source of polyphenols and other bioactive compounds (Ghanbari *et al.,* 2012). A clinical study demonstrated that olive intake increases polyphenols and total antioxidant potential in plasma, thus indicating that olive polyphenols have good bioavailability (Kountouri *et al*., 2007). In this study, the total polyphenol content in green Nabali Baladi olives was found to be moderate (300 mg gallic acid equivalent/100 g). Previous studies showed highly variable total polyphenol contents in fermented olives. The Italian green *Intosso d'Abruzzo* fermented olive total polyphenol content was 167 mg caffeic acid equivalent /100 g (Lanza *et al.,* 2010). In Tunisia, green *Meski* fermented olives contained 1170 mg hydroxytyrosol equivalent/100 g (Issaoui *et al.,* 2011). However, the total polyphenol content in fermented green olives has been reported to be dependent on olive variety, time of harvest and treatment method (Othman *et al.,* 2009; Bouaziz *et al*., 2010). As is well known, the debittering step in olive fermentation reduces the polyphenol content, mainly oleuropein, by either dilution or chemical hydrolysis (Issaoui *et al.,* 2011). The total polyphenol content in the green *Chetoui* olive variety was decreased by 58% after spontaneous fermentation for 67 days (Othman *et al.,* 2009). Polyphenol content was increased from 346 to 576 mg gallic acid equivalent/100 g in green *Chetoui* olives during 1 month of tree maturation (Bouaziz *et al*., 2010). Moreover, it was found that olive varieties with small drupe size have a higher quantity of oleuropein (Morello *et al.,* 2005). Green Nabali Baladi olives have a large fruit size and high oil content (Al-Ismail *et al.,* 2011), thus they are expected to have low total polyphenol content compared to other olive varieties.

Upon fermentation, olives became a rich source of beneficial microorganisms including LAB and yeasts (Aponte *et al.,* 2010; Hurtado *et al.,* 2012; Argyri *et al.,* 2013). Different isolates of LAB and yeasts were found to possess probiotic properties *in vitro* (Van der Aa Kuhle *et al.,* 2005, Zivkovic *et al.,* 2015). Traditional fermentation methods vary among countries; the Spanish-style green olives are characterized by treatment with NaOH before the addition of brine (brine concentration usually ranges between 6-8% w/v) and starter culture may be added (Abriouel *et al.,* 2011). Green olive fermentation according to the Sicilian method is characterized by repeated washing of the olives with water as a debittering mechanism and brining in a 6-10% salt solution (Aponte *et al.,* 2010). The Turkish traditional method uses either scratched or cracked green olives which are first placed in water to reduce bitterness and then transferred to the fermentation tanks where the brine-to-salt ratio is increased progressively and reaches 5-6% (Cillidag, 2013). Two famous traditional olive fermentation methods are reported in Tunisia. The first is a simple process and consists of a modest addition of a balanced amount of water and salt to the olive fruits that must be harvested unripe and kept in jars made of clay or glass (Issaoui *et al*., 2011). The second process is the oldest and consists of adding oil and salt to the olive fruits which must be kept in a fabric bag with a large opening. In the latter method, olives ripen faster and lose their color rapidly due to direct contact with air and temperature (Issaoui *et al*., 2011).

In Jordan, the traditional fermentation method is characterized by debittering olive fruit through soaking in water for three days followed by spontaneous fermentation in brine (> 10% w/v of raw salt) under low-temperature conditions (NCARE, 2000). Vegetable fermentation is a natural and spontaneous process that is achieved through the activity of indigenous microbiota, particularly LAB and yeast on the raw material (Bautista-Gallego *et al.,* 2011). It is well documented that LAB is the leading micro-organism in different vegetable fermentations including olives (Aponte *et al.,* 2010, Hurtado *et al.,* 2012). However, olive fermentation could be affected by different factors that would encourage specific microorganisms to dominate the fermentation process such as brine concentration, olive variety, previous alkali treatment and temperature during the fermentation process. Most green olive fermentations are dominated by LAB species (Hurtado *et al.,* 2012). Previous studies revealed that the Spanish-style treated olive fermentation is due to LAB, whereas black olives were fermented primarily by yeasts, and LAB represents a small proportion of the total microflora (Aponte *et al.,* 2010).

Indeed, our results showed that green Nabali Baladi olive fermentation was dominated by yeast throughout the 90 days of fermentation. The identification of isolated yeasts by sequencing of the D1/D2 region of the 26S rDNA region revealed that *Candida diddensiae* and *Candida naeodendra* were the predominant yeasts in the green Nabali Baladi fermented olives. High brine concentrations and low pH values at the start of fermentation would favor yeast growth and inhibit lactic acid bacteria. When brine concentration was maintained above 80g/L, yeast growth dominated the black olive fermentation for 90 days (Bleve *et al.,* 2014). In this study, the brine concentration started at 100 g/L and ended at 86 g/L after 90 days of fermentation. Furthermore, the fermentation of green cracked olives in brine concentrations (90-110 g/L) was dominated by yeast for 6 months of fermentation with little or no lactic acid bacteria growth (Abriouel *et al*., 2011).

Nevertheless, different yeast species were isolated from fermented green and black olives. Green Sicilian olive fermentation was dominated by *Candida parapsilosis*, *Pichia guilliermondii,* and *Pichia kluyveri* (Aponte *et al.,* 2010). The exploration of the biological diversity of yeasts in the mass fermentation activities of green table olives in Spain revealed that *Candida diddensiae*, *Saccharomyces cerevisiae,* and *Pichia membranifaciens* were the most abundant yeast species directly isolated from brined Aloreña olives; whereas for Gordal and Manzanilla cultivars, they were *Candida tropicalis*, *Pichia galeiformis* and *Wickerhamomyces anomalus*. In the case of Gordal and Manzanilla green olives which were prepared according to the Spanish method, the prevailing yeasts were *Debaryomyces etchellsii, C. tropicalis, P. galeiformis,* and *Kluyveromyces lactis* (Bautista-Gallego *et al.,* 2011). *Candida naeodendra* is a species of the *Candida diddensiae* group that has been frequently identified in olive fermentation processes (Bautista-Gallego *et al.,* 2011). Moreover, different strains of *Candida diddensiae* were shown to produce a wide range of lipase, esterase or β- glucosidase activities that qualify them to be used as a potential starter in olive fermentation (Ciafardini and Zullo, 2015).

On the other hand, the probiotic characteristics of the *Candida* species have been scarcely studied. However, strains of yeasts isolated from fermented olives can have some beneficial criteria that may improve the technical and functional properties of fermented olives (Hatoum *et al.,* 2012). Accordingly, *Candida diddensiae* and *Candida naeodendra* were tested for their acid and bile tolerance and adhesion to intestinal cell lines *in vitro*. The results presented in Table 3 demonstrate that the two isolates can tolerate gastric conditions and potentially survive in the gastrointestinal tract. Neither isolate showed antimicrobial activities against a group of pathogenic bacteria. It is possible that yeast isolates have positive immunomodulatory activity as documented previously (Smith *et al.,* 2014). In essence, the isolated yeasts were positive for catalase production. This character is important for olive preservation due to its high content of unsaturated fatty acids; in fact, it is, therefore, possible to effectively prevent oxidation and peroxide formation (Silva *et al.,* 2011).

5. CONCLUSIONS

Fermented green Nabali Baldi olives are a rich source of oleic acid, polyphenols, and dietary fiber. The fermentation of green Nabali Baladi olives according to the traditional fermentation method in Jordan is dominated by yeast strains, primarily *Candida diddensiae,* with no detection of LAB.This yeast strain can tolerate HCL and

bile salt and has a medium ability of adhesion to intestinal cell lines. Thus, *C. diddensiae* isolated from fermented green Nabali Baladi olives may represent a good probiotic candidate. In this respect, further *in vitro* and *in vivo* studies are required.

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REFERENCES

Association of the Official Analytical Chemists (AOAC). 1995. Official Methods of Analysis. Arlington, Virginia, USA.

Abriouel H, Benomar N, Lucas R ,Galvez A. 2011. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green tableolives. *Int. J. Food Microbiol.* **144**, 487-96. https://doi.org/10.1016/j.ijfoodmicro.2010.11.006

Al-Ismail KM, Ahmad R, Al-Dabbas M, Ajo RY, Rababah T. 2011. Some physiochemical properties of olive and olive oil of three jordanian olive varieties. *Riv. Ital. Sostanze Grasse* **88**, 191-198.

Aponte M, Ventorino V, Blaiotta G, Volpe G, Farina V, Avellone G, Lanza CM, Moschetti G. 2010. Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. *Food Microbiol.* **27**, 162-70. https://doi.org/10.1016/j.fm.2009.09.010

Argyri AA, Zoumpopoulou G, Karatzas KA, Tsakalidou E, Nychas GJ, Panagou EZ, Tassou CC. 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol.* **33**, 282-291. https://doi.org/10.1016/j.fm.2012.10.005

Bautista-Gallego J, Rodriguez-Gomez F, Barrio E, Querol A, Garrido-Fernandez A, Arroyo-Lopez FN. 2011. Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. *Int. J. Food Microbiol.* **147**, 89-96. https://doi.org/10.1016/j.ijfoodmicro.2011.03.013

BLAST: Basic Local Alignment Search Tool. Last retrieved, September 2019. https://blast.ncbi.nlm.nih.gov/Blast.cgi

Bleve G, Tufariello M, Durante M, Perbellini E, Ramires FA, Grieco F, Cappello MS, De Domenico S, Mita G, Tasioula-Margari M, Logrieco AF. 2014. Physico-chemical and microbiological characterization of spontaneous fermentation of Cellina di Nardò and Leccino table olives. *Front. Microbiol.* **5**, 570. https://doi.org/10.3389/fmicb.2014.00570

Borcakli M, Özay G, Alperden I, Özsan E, Erdek Y. 1993. Changes in chemical and microbiological composition of two varieties of olive during fermentation. *Grasas Aceites* **44**, 253-258. https://doi.org/10.3989/gya.1993.v44.i4-5.1075

Bouaziz M, Jemai H, Khabou W, Sayadi S. 2010. Oil content, phenolic profiling and antioxidant potential of Tunisian olive drupes. *J. Sci. Food Agric.* **90**, 1750-1758. https://doi.org/10.1002/jsfa.4013

Ciafardini G, Zullo BA. 2015. Effect of lipolytic activity of *Candida adriatica*, *Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content. *Food Microbiol.* **47**, 12-20. https://doi.org/10.1016/j.fm.2014.10.010

Cillidag S. 2013. Table olive processing technologies. Options Méditerranéennes. Séries A: Mediterranean Seminars. CIHEAM-IAMZ; IOC, International Olive Council.

Ebiad R,Abu-Qaoud H. 2014. Morphological and biochemical characterization of three olive" *Oleaeuropaea* L." cultivars in Palestine. *Jordan J. Agric. Sci.* **10**, 130-143.

Food and Drug Adiminstration. Bacteriological Analytical Manual on line. 2001. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm

Ghanbari R, Anwar F, Alkharfy KM, Gilani AH, Saari N. 2012. Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.)-A review. *Int. J. Mol. Sci.* **13**, 3291-340. https://doi.org/10.3390/ijms13033291

Hatoum R, Labrie S, Fliss I. 2012. Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Front. Microbiol.* **3**, 421. https://doi.org/10.3389/fmicb.2012.00421

Humeid MA, Takruri HR, Daqqaq RF. 1991. Nabali olive oil ripening and oil properties. *Nutr. Health.* **7**, 151-154. PMID:1923072

Hurtado A, Reguant C, Bordons A, Rozes N. 2012. Lactic acid bacteria from fermented table olives. *Food Microbiol.* **31**, 1-8. https://doi.org/10.1016/j.fm.2012.01.006

Issaoui M, Dabbou S, Mechri B, Nakbi A, Chehab H, Hammami M. 2011. Fatty acid profile, sugar composition, and antioxidant compounds of table olives as affected by different treatments. *Eur. Food Res.Tech.* **232**, 867-876. https://doi.org/10.1007/s00217-011-1455-3

Kountouri A, Mylona A, Kaliora A, Andrikopoulos N. 2007. Bioavailability of the phenolic compounds of the fruits (drupes) of *Olea europaea* (olives): impact on plasma antioxidant status in humans. *Phytomedicine* **14**, 659-667. https://doi.org/10.1016/j.phymed.2007.06.001

Kurtzman CP, Fell JW, Boekhout T, Robert V. 2011. Methods for isolation, phenotypic characterization and maintenance of yeasts. *The yeasts, a taxonomic study, 5th edn. Elsevier, Amsterdam* **,** 87-110.

Lanza B, Di Serio MG, Iannucci E, Russi F, Marfisi P. 2010. Nutritional, textural and sensorial characterisation of Italian table olives (*Olea europaea* L. cv. 'Intosso d'Abruzzo'). *Int. J. Food Sci. Tech.* **45**, 67-74. https://doi.org/10.1111/j.1365-2621.2009.02104.x

López A, Garrido A, Montaño A. 2007. Proteins and aminoacids in table olives: relationship to processing and commercial presentation. *It. J. Food Sci.* **19**, 217-228.

López A, García P, Garrido A. 2008. Multivariate characterization of table olives according to their mineral nutrient composition. *Food Chem.* **106**, 369-378.

Lopez-Lopez A, Cortes-Delgado A, Garrido-Fernandez A. 2015. Effect of green Spanish-style processing (Manzanilla and Hojiblanca) on the quality parameters and fatty acid and triacylglycerol compositions of olive fat. *Food Chem.* **188**, 37-45. https://doi.org/10.1016/j.foodchem.2015.04.080

Ministry of Agriculture (MOA). Agricultural Statistical Yearbook. 2016. Amman, Jordan.

Morelló JR, Vuorela S, Romero MP, Motilva MJ, Heinonen M. 2005. Antioxidant activity of olive pulp and olive oil phenolic compounds of the Arbequina cultivar. *J. Agric. Food Chem.* **53**, 2002-2008. https://doi.org/10.1021/jf048386a

National Center of Agricultural Research and Education (NCARE). 2000. Pickled Green Olive Production. Publication of NCARE no **62**. Amman, Jordan.

Othman NB, Roblain D, Chammen N, Thonart P, Hamdi M. 2009. Antioxidant phenolic compounds loss during the fermentation of Chétoui olives. *Food Chem.* **116**, 662-669. https://doi.org/10.1016/j.foodchem.2009.02.084

Pino A, De Angelis M, Todaro A, Van Hoorde K, Randazzo CL, Caggia C. 2018. Fermentation of Nocellara Etnea table olives by functional starter cultures at different low salt concentrations. *Front. Microbiol.* **9**. https://doi.org/10.3389/fmicb.2018.0112

Saibandith B, Spencer JPE, Rowland IR, Commane DM. 2017. Olive polyphenols and the metabolic syndrome. *Molecules* **22**, E1082. https://doi.org/10.3390/molecules22071082

Sakouhi F, Harrabi S, Absalon C, Sbei K, Boukhchina S, Kallel H. 2008. α-Tocopherol and fatty acids contents of some Tunisian table olives (*Olea europea* L.): changes in their composition during ripening and processing. *Food Chem.* **108**, 833-839. https://doi.org/10.1016/j.foodchem.2007.11.043

Savas E, Uylaser V. 2013. Quality improvement of green table olive cv.'domat' (*Olea europaea* L.) grown in Turkey using different de-bittering methods. *Notulae. Botanicae Horti. Agrobotanici. Cluj-Napoca.* **41**, 269

Silva T, Reto M, Sol M, Peito A, Peres CM, Peres C, Malcata FX. 2011. Characterization of yeasts from Portuguese brined olives, with a focus on their potentially probiotic behavior. *LWT-Food Sci. Tech.* **44**, 1349-1354. https://doi.org/10.1016/j.lwt.2011.01.029

Smith IM, Christensen JE, Arneborg N, Jespersen L. 2014. Yeast modulation of human dendritic cell cytokine secretion: an in vitro study. *PloS One* **9**, e96595. https://doi.org/10.1371/journal.pone.0096595

van der Aa Kuhle A, Skovgaard K, Jespersen L. 2005. In vitro screening of probiotic properties of *Saccharomyces cerevisiae* var. *boulardii* and food-borne *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* **101**, 29-39. https://doi.org/10.1016/j.ijfoodmicro.2004.10.039

Wang SY, Chen HC, Liu JR, Lin YC, Chen MJ. 2008. Identification of yeasts and evaluation of their distribution in Taiwanese Kefir and Viili starters. *J. Dairy Sci.* **91**, 3798-805. https://doi.org/10.3168/jds.2007-0468

Zivkovic M, Cadez N, Uroic K, Miljkovic M, Tolinacki M, Dousova P, Kos B, Suskovic J, Raspor P, Topisirovic L, Golic N. 2015. Evaluation of probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia. *J. Intercult. Ethnopharmacol.* **4**, 12-8.

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Shelf-life of Moroccan prickly pear (*Opuntia ficus-indica*) and argan (*Argania spinosa*) oils: a comparative study

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SUMMARY: Cactus seed oil is gaining considerable popularity in the cosmetic industry. To estimate cactus seed oil' industrial as well as domestic ease of use, we investigated the oxidative stability of Moroccan cactus seed oil under accelerated aging conditions. In addition, we compared cactus seed oil stability to that of argan oil, a popular and well-established cosmetic oil, under the same conditions. Cactus seed oil is much more sensitive to oxidation than argan oil. Its shelf-life can be estimated to be no longer than 6 months at room temperature. Such instability means that the preparation process for cactus oil must be carried out with great care and cactus seed oil needs to be protected once extracted.

KEYWORDS: *Cosmetics; Oil preservation; Oxidative stability*

RESUMEN: ***Vida útil de los aceites de cactus marroquí (Opuntia ficus-indica) y de argán (Argania spinosa). Estudio comparativo.*** El aceite de semillas de cactus está ganando considerable popularidad en la industria cosmética. Para estimar la facilidad de uso industrial y doméstico del aceite de semilla de cactus, investigamos la estabilidad oxidativa del aceite de semilla de cactus marroquí en condiciones de envejecimiento acelerado. Además, comparamos, bajo las mismas condiciones, la estabilidad del aceite de semilla de cactus con la del aceite de argán, otro aceite cosmético popular y bien establecido. El aceite de semilla de cactus es mucho más sensible a la oxidación que el aceite de argán. Su vida útil se puede estimar en no más de 6 meses a temperatura ambiente. Tal inestabilidad significa que los procesos preparativos del aceite de cactus deben manejarse con mucho cuidado y el aceite de semilla de cactus debe protegerse una vez extraído.

PALABRAS CLAVE: *Cosmética; Estabilidad oxidativa; Preservación de aceite*

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1. INTRODUCTION

The world of cosmetics is characterized by a constant search for new compounds which are able of satisfy customer demands and high expectations. While the presentation of commercial-only arguments has long been considered satisfactory by the customers, a trend has recently emerged that customers are more inclined to ask for scientific evidences to back up the new cosmetics. A few years ago, argan oil was lauched on the cosmetic market with extensive advertising but also with scientific studies validating its claimed or empirically observed properties (Charrouf and Guillaume, 2008; Guillaume and Charrouf, 2011; Charrouf and Guillaume, 2014). With such an approach, the commercial success of argan oil has been global and its acceptance by the public almost immediate.

The argan tree is only endemically grown in Morocco. Therefore, argan oil is an organic compound originating only from Morocco. It is prepared by simply cold-pressing argan kernels collected following a rigorous process (Charrouf *et al.*, 2002) that has been protected by a geographical indication since 2009 (Charrouf and Guillaume, 2018). The simplicity of the preparation of argan oil ascertains its original chemical purity, a factor particularly appreciated by consumers. Argan oil has high contents in unsaturated fatty acids, as well as tocopherols and phytosterols, which have been repeatedly shown to be responsible for most of its dermocosmetic properties (Guillaume and Charrouf, 2011; Guillaume and Charrouf, 2013; Zaanoun *et al.*, 2014).

The worldwide economic success of cosmetic argan oil has encouraged the search for other oil seeds which present sufficiently similar chemical profile. Tooga (*Balanites aegyptiaca*) and prickly pear (*Opuntia ficus-indica* L.) oil seeds (Guillaume *et al.*, 2015) are currently receiving a lot of attention. Among these two seed oils, the marketing of the latter is much more advanced and, actually, cactus oil has only recently entered the cosmetic market (Ciriminna *et al.*, 2017). It could also be a source of edible oil (Salvo *et al.*, 2002).

Similar to those of argan oil, the triglycerides of cactus oil are mostly unsaturated fatty acids. Cactus oil also contains large amounts of sterols and tocopherols (Ciriminna *et al.*, 2017), two classes of phytocompounds considered to be important and valuable by the cosmetics industry. In both argan and cactus oils, the total content in oleic (monounsaturated) and linoleic (diunsaturated) acids, is around 80% (Labuschagne and Hugo, 2010). However, linoleic acid is the overwhelmingly predominant fatty acid in cactus oil, with a content of around 55% vs only 15-20% for oleic acid (Labuschagne and Hugo, 2010; Ramadan and Mörsel, 2003); whereas in argan oil the oleic acid content slightly surpasses that of linoleic acid (48 vs 32%, respectively) (Charrouf and Guillaume, 1999; Zaanoun *et al.*, 2014). Despite this difference in fatty acid concentrations, cactus seed oil's content in unsaturated fatty acids supports the idea that this seed oil is perfectly suitable for large-scale use as a major ingredient in cosmetology (Sawaya and Khan, 1982) and could follow the path of cosmetic argan oil.

Cactus seed oil is prepared by press-extraction of the seeds. However, cactus seeds are difficult to break and even though a yield in cactus oil of 13.6% has been reported from Moroccan cactus seeds (El Finti *et al.*, 2013), most reported yields for cactus seed oil generally lie between 7.3 and 9.3%, possibly depending on the geographical origin of the seeds (Ciriminna *et al.*, 2017), or on the maturation period (Coskuner and Tekin, 2003). For comparison purposes, argan kernels contain up to 50% of oil (Harhar *et al.*, 2010). Consequently, cactus seeds that have been simply considered as waste for years, now contain the "most expensive oil in the world" with market prices possibly reaching 500 €/L vs 120 €/L for argan oil, which previously held this title.

The prickly pear is a ubiquitous Mediterranean-type cactus which is well-adapted to extreme arid conditions. It is a wild-growing or cultivated plant in Morocco whose cladodes and fruit have received some attention mainly for a use as livestcock feed or human food (Feugang *et al.*, 2006; De Waal *et al.*, 2015). Unlike argan oil, whose traditional uses are well-documented (Charrouf and Guillaume, 1999), cactus seed oil's promising cosmetic and pharmacological properties are almost exclusively a direct consequence of its chemical composition (Sawaya and Khan, 1982) and to our knowledge, there are no reports of a traditional extraction of cactus seed oil. Nevertheless, cactus seed oil could enjoy great commercial success. However, the use of cactus oil by the cosmetic industry could be limited or at least difficult because of its poor preservation properties and chemical

modifications in its composition occurring during aging. Indeed, cactus seed oil induction time calculated by the Rancimat accelerated method, has been found to be of only 7 ± 1 h at 110 °C (Zine *et al.*, 2013). Again, for comparison purposes, in the same experimental conditions, cosmetic argan oil induction time has been calculated to be twice as long (Gharby *et al.*, 2012a). Consequently, with regard to its elevated market price, we decided to investigate further the oxidative stability of the cactus seed oil from Morocco to possibly estimate its shelf-life. We performed our study using storage at 60 °C to amplify and accelerate oxidative processes as we previously did for edible argan oil (Gharby *et al.*, 2012a; Matthäus *et al.*, 2010). Our results on Moroccan cactus seed oil are evaluated in the light of those obtained with a similar set of analyses carried out on cosmetic argan oil samples stored under the same conditions.

2. MATERIALS AND METHODS

2.1. Materials and experimental design

Argan fruit was collected in Tiout (Taroudant County) in August, 2014 and prepared following the methodology used by the local women's cooperative. The fruit was air-dried for 3 weeks then mechanically de-hulled (SMIR Technotour, Agadir, Morocco). Manually opened argan nuts contained the kernels that were ground using an endless press (IBG Monforts Oekotec GmbH, Mönchengladbach, Germany). An aliquot of collected argan oil was immediately analyzed. Remaining oil was stored at 60 °C and analyses were repeated after every week (acidity, peroxide value, and *p*-anisidine index) or after 3, 6, 10, and 12 weeks of storage (tocopherol, sterol, and fatty acid contents).

Cactus fruit was picked in Sidi Ifni in July, 2014. The fruit was manually peeled and cactus seeds were collected, air-dried and finally ground using the same type of endless press as that used for argan oil.

For the study, oil samples were stored in a Memmert UF110 plus oven (Memmert GmbH, Schwabach, Germany) equipped with a Kimo KTT310-RF thermostat at a constant temperature of 60 ± 1 °C.

2.2. Chemical analyses

An aliquot of each oil was analyzed immediately after oil extraction. The remaining oil was stored at 60 °C and an amount necessary for the analysis was subtracted every three weeks. Analyses were performed over a 12 week-period.

The chemical and physical parameters (acidity, peroxide index, *p*-anisidine value, and fatty acid content) were analyzed, in triplicate, following the analytical methods described in Regulations EC 2568/91 (Commission Regulation, 1991).

Fatty acid composition was determined as their corresponding methyl esters by gas chromatography on a CPWax 52CB column (30 m x 0.25 mm i.d., 0.25 µm film thickness) using He (flow rate 1 mL/mn) as carrier gas. Oven, injector, and detector temperatures were set at 170, 200, and 230 °C; respectively. Injected quantity was 1 µL for each analysis (Gharby *et al.,* 2011).

Sterol composition was determined after trimethylsilylation of the crude sterol fraction using a Varian 3800 instrument equipped with a VF-1 ms column (30 m x 0.25 mm i.d., 0.25 µm film thickness) and using Helium (flow rate 1.6 mL/min) as carrier gas. Column temperature was isothermal at 270 °C, the injector and detector temperature was 300 °C. Injected quantity was 1µL for each analysis (Gharby *et al.*, 2011).

On the basis of the AOCS Official method Ce 8-89 (Gharby *et al.*, 2011), tocopherol content was determined by HPLC using Shimadzu instruments equipped with a C18-Varian column (25 cm x 4 mm). Detection was performed using a fluorescence detector (excitation wavelength 290 nm, detection wavelength 330 nm). The eluent used was a 99:1 isooctane/ isopropanol (V/V) mixture, at a flow rate of 1.2 mL/min.

2.3. Statistical analyses

Values reported are the mean values ± SE of 3 replicates. The significance level was set at P = 0.05. Separation of mean values was performed by Tukey's test at the 0.05 significance level.

3. RESULTS AND DISCUSSION

The cosmetic properties of cactus and argan oils are mainly attributed to their high unsaturated fatty acid contents and to the concomittant presence of elevated levels of phytosterols and tocopherols (Ciriminna *et al.*, 2017; Labuschagne and Hugo, 2010; Ramadan and Mörsel, 2003; Charrouf and Guillaume, 1999; Salvo *et al.*, 2002). Therefore, we decided to examine the

variations in the contents of these three types of constituents under accelerated aging conditions. Storage was prolonged for 12 weeks, and analyses of these two markers were performed every 3 weeks.

3.1. Fatty acid content

For both oils, initial fatty acid content was found in the range of published values for cactus seed oil from Morocco (Zine *et al.*, 2013; Taoufik *et al.*, 2015), or of geographically close origin (Monia *et al.*, 2005). Accelerated aging did not induce any significant modification in the fatty acid content or distribution and, even after 12 weeks at 60 °C, the linoleic acid content in cactus seed oil was found to be similar to that of the freshly prepared oil (Table 1).

3.2. Sterol content

Sterols were the second class of compounds investigated. Cactus seed and argan oils are both rich in phytosterols but they only share Δ^7-avenasterol as common sterols. In addition to Δ^7-avenasterol, the sterols in cactus seed oil are campesterol, stigmasterol, β-sitosterol, Δ^5-avenasterol, and Δ^7-stigmasterol (El Mannoubi *et al.*, 2009). In addition, argan oil sterols are schottenol, spinasterol, and stigmasta-8,22-diene (Charrouf and Guillaume, 1999). As observed for fatty acids, no significant variations in sterol content was observed for both oils during 12 weeks of accelerated aging (data not shown). In cactus seed oil, the content of β-sitosterol, as the main sterol, remained constant at over 78% and that of campesterol at around 10%.

3.3. Tocopherol content

Tocopherols, whose anti-oxidant properties are presented as important for the oil's cosmetic properties, were also investigated (Guillaume and Charrouf, 2011). Whereas argan oil contains α-, β-, γ-, and δ-tocopherols (β- being minoritary) (Charrouf and Guillaume, 1999), cactus seed oil does not contain β-tocopherol (El Mannoubi *et al.*, 2009). Therefore, only the changes in those three tocopherols that the two oils have in common were investigated over 12 weeks of accelerated aging conditions.

Tocopherol distribution in freshly prepared cactus seed oil is different from that of argan oil. In argan oil, α- and δ-tocopherol are present in an amount which is 10 times greater than in cactus seed oil (Table 2). The γ-tocopherol content is similar in both oils (Table 2). The concentration in α-tocopherol remained stable in cactus seed oil for 6 weeks, after which it began to significantly decrease, suggesting the occurrence of the oxidative processes. In argan oil, variations in α-tocopherol were not significant over the 12 weeks. The content in γ-tocopherol in cactus seed oil turned out to be more stable over the study period and only began to significantly decrease after the ninth week. At week-12, the γ-tocopherol content in cactus seed oil that had constantly been similar or slightly higher than that of argan oil, became inferior to that of argan oil, likely indicating the intense destruction of γ-tocopherol by oxidizing species. Under the same aging conditions, the γ-tocopherol content in argan oil decreased sooner since a significant variation in γ-tocopherol content was observable after week 3. However, the loss in γ-tocopherol remained moderate throughout the whole study. Initially low δ-tocopherol content remained stable in cactus seed oil over the twelve weeks of study; whereas it had started to significantly decrease in argan oil after week-6. The total amount of tocopherol in both oils became significantly different after 9 weeks of storage.

TABLE 1. Fatty acid (%) composition of cactus and argan seed oils during 12 weeks of storage at 60 °C.

Week	Palmitic acid		Stearic acid		Oleic acid		Linoleic acid		Linolenic acid	
	Cactus	Argan	Cactus	Argan	Cactus	Argan	Cactus	Argan	Cactus	Argan
Initial	12.3±0.1	13.1±0.1	3.4±0.1	5.4±0.1	20.4±0.5	48.2±0.7	61.8±0.9	32.5±0.7	0.3±0.1	0.2±0.1
3	11.9±0.3	13.8±0.4	3.4±0.2	5.7±0.1	21.5±0.5	48.2±0.6	61.8±0.4	32.9±0.9	0.3±0.1	0.2±0.1
6	12.2±0.2	13.9±0.7	3.4±0.2	5.6±0.5	19.2±0.3	47.9±0.6	63.4±0.5	32.9±0.5	0.3±0.1	0.2±0.1
9	12.2±0.2	13.8±0.1	3.4±0.1	5.8±0.2	20.4±0.2	47.9±0.5	61.8±0.2	32.9±0.5	0.3±0.1	0.1±0.1
12	12.3±0.2	13.9±0.2	3.4±0.1	5.6±0.2	20.4±0.2	48.1±0.4	61.8±0.2	32.9±0.3	0.3±0.1	0.1±0.1

Each values is the mean of 3 replicates, Separation of mean values was performed by Tukey's test at the 0.05 significance level

TABLE 2. Tocopherol (mg/kg) composition of cactus and argan seed oils during 12 weeks of storage at 60 °C.

Week	α-tocopherol		γ-tocopherol		δ-tocopherol		Total	
	Cactus	Argan	Cactus	Argan	Cactus	Argan	Cactus	Argan
Initial	8.4± 0.2^{a}	72± 2^{a}	595± 10^{a}	585± 9^{a}	5.7± 0.1^{a}	82± 2^{a}	622± 5^{a}	766± 6^{a}
3	8.2± 0.2^{a}	70± 1^{a}	593± 14^{a}	5812± 11^{a}	5.7± 0.1^{a}	81± 2^{a}	618± 8^{a}	762± 10^{a}
6	8.2± 0.1^{a}	70± 1^{a}	581.2± 8.5^{a}	547± 5^{b}	5.5± 0.1^{a}	77± 3^{a}	604± 7^{a}	703± 10^{a}
9	7.6± 0.2^{b}	68± 1^{a}	573± 5^{a}	549± 11^{b}	5.7± 0.2^{a}	62± 1^{b}	598± 8^{a}	692± 7^{a}
12	6.6± 0.1^{c}	65± 2^{a}	514± 11^{b}	533± 10^{b}	5.2± 0.1^{a}	39± 1^{c}	536± 5^{b}	649± 6^{b}

Each values is the mean of 3 replicates, Separation of mean values was performed by Tukey's test at the 0.05 significance level
$^{a, b, c}$: Values indexed with the different letters within the same column are significantly different.

These differences in tocopherol content variations between the two oils clearly point out that anti-oxidative processes occurring during aging in cactus seed and argan oils are either different or that responses to oxidative processes are different. The differences may reflect the formation of different oxidizing species resulting mainly from oleic (argan oil) or linoleic (cactus oil) acids. They may also reflect a different oxidative behavior of synergestic associations of the fatty acids with other anti-oxidant molecules, possibly the sterols and/or phospholipids (Gharby *et al.*, 2012b; Zaanoun *et al.*, 2014). Despite the observed different reactivity of cactus seed and argan oil tocopherols in accelerated oxidative conditions, it is noteworthy that the initial cactus oil total tocopherol content was 81% of that of argan oil. After 12 weeks of accelerated aging, and different oxidative processes, the total tocopherol content in cactus seed oil was still 81% of that of argan oil, but on a lower total level.

3.4. Acidity, peroxide, and *p*-anisidine values

Once a variation in the content of the anti-oxidant tocopherols was identified, it was decided to further evaluate the shelf-life of argan and cactus seed oils from Morocco, by examining the variations in two other key oxidation markers likely to be modified during prolonged storage at 60 °C. Hence, the peroxide and *p*-anisidine values were determined. The variations in oil acidity, a parameter important for cosmetics, were also examined.

The composition of cactus seed oil depends on its geographical origin (Ciriminna *et al.*, 2017), as does its acidity for which large variations have also been reported. Acidity values as low as 0.56% for oleic acid (Zine *et al.*, 2013) and as high as 5.08% for oleic acid (De Wit *et al.*, 2016) have been reported for cactus oils of various geographical origins. We found an acidity of 1.15% for oleic acid in our cactus seed oil sample (Figure 1). Such acidity, which is twice that found in a previous study on a different batch of Moroccan cactus seed oil (Zine *et al.*, 2013), clearly indicates that the geographical origin is neither the only factor influencing cactus seed oil acidity nor the most influential factor. The maturity of the fruit and/or the seed water-content, two conditions that inevitably superimpose, are two parameters that also likely dramatically influence the acidity of cactus oil. If this later parameter has already been suggested (De Wit *et al.*, 2016) and could be evaluated by a moisture measurement, the huge number of seeds in a cactus fruit and their different degrees of maturity, unfortunately makes a global evaluation of cactus seeds difficult. The presence of enzymes or of unidentified residues formed after oil extraction has also been suggested to explain the high acidity of cactus seed oil (De Wit *et al.*, 2016).

After storage at 60 °C, the calculated acidity of the cactus oil sample increased almost linearly until week 9 to reach 2.87% for oleic acid (Figure 1). The slope of the straight line, which describes hydrolysis, followed a kinetic second order. Such a value corresponds to a hydrolysis rate estimated to be $2x10^{-3}$ mmol of triglyceride/week. The initial acidity value in argan oil was 0.3% for oleic acid, which is very low, far lower than cactus oil. The trend of hydrolysis followed a linear process, such as the cactus oil, during storage at 60 °C for the 12 weeks of our study but its slope was twice as low as that of cactus seed oil (Figure 1). Accordingly, the hydrolysis of argan oil during the storage period occurred at a rate estimated to be $0.75x10^{-3}$ mmol of triglyceride/week, a rate almost thrice lower than that of cactus oil. Hence, cactus seed oil appears

to be much more sensitive to hydrolysis than argan oil.

Accelerated aging is a good measure to evaluate lipid peroxidation (Stewart and Bewley, 1980). Therefore, we evaluated the oxidation degree of cactus seed oil by examining its peroxide value (Figure 2). Cactus seed oil had an initial peroxide value of 4.59 meq O_2/kg. If much lower peroxide values have been previously reported (Matthäus and Özcan, 2011; Özcan and Al Juhaimi, 2011) for cactus seed oil, this value is similar to that already reported for Moroccan cactus oil (Zine *et al.*, 2013) and much lower than that determined for South African cactus oil for which a peroxide value as high as 33.6 meq O_2/kg has been observed (De Wit *et al.*, 2016). The first weeks of storage of cactus seed oil at 60 °C could be identified as the oxidative propagation phase and the cactus oil peroxide value for cactus seed oil reached a maximum value of 9.43 meq O_2/kg at week 3. After that, some peroxides began to be broken into secondary oxidation products as attested by the decrease for 2 weeks (from week 3 to week 5) in the peroxide value. After 6 weeks of storage, the kinetics of the peroxidation became faster than that of the secondary oxidation formation and large amounts of peroxides were again detected. After 12 weeks, the peroxide value reached a maximum value of 39.41 meq O_2/kg. At that moment, the γ-tocopherol content underwent a large decrease, likely attesting to its destruction by the massive formation of peroxides. Interestingly, we observed that the α-tocopherol content in cactus oil decreased after 6 weeks. This also suggests an active and early involvement of α-tocopherol in the prevention of peroxide formation. Tocopherol intervention could be sequential, α-tocopherol would participate in cactus seed oil preservation in a first step and γ-tocopherol in a second phase.

In argan oil, the propagation phase lasted 6 weeks (twice as long as that of cactus seed oil) and additional peroxides began to significantly appear after 10 weeks. After 12 weeks, the peroxide value of argan oil reached 33.6 meq O_2/kg, a value almost 15% lower than that of cactus seed oil. Consequently, peroxide formation is much faster in cactus oil than in argan oil.

To get a better picture of the secondary oxidation product formation, the *p*-anisidine value for our oils as a function of the time of storage were determined. For cactus oil, a strong increase in the *p*-anisidine value was observed after 2 weeks confirming the decrease in peroxide value observed after 3 weeks. The *p*-anisidine value continued to increase regularly, attesting to the constant formation of secondary oxidation products during storage at 60 °C. In argan oil, secondary oxidation products were formed after 7 weeks of storage, again confirming the plateau observed in peroxide formation after 8 weeks.

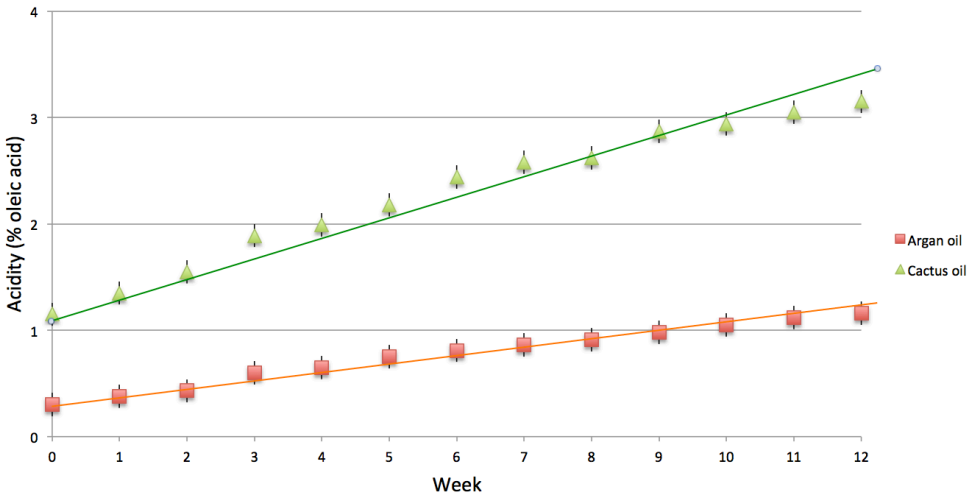

FIGURE 1. Evolution of acidity (in g/100 of oil) in cactus and argan seed oils upon 12 weeks of storage at 60 °C. Values represent the mean of 3 replicates and error bars represent standard variations.

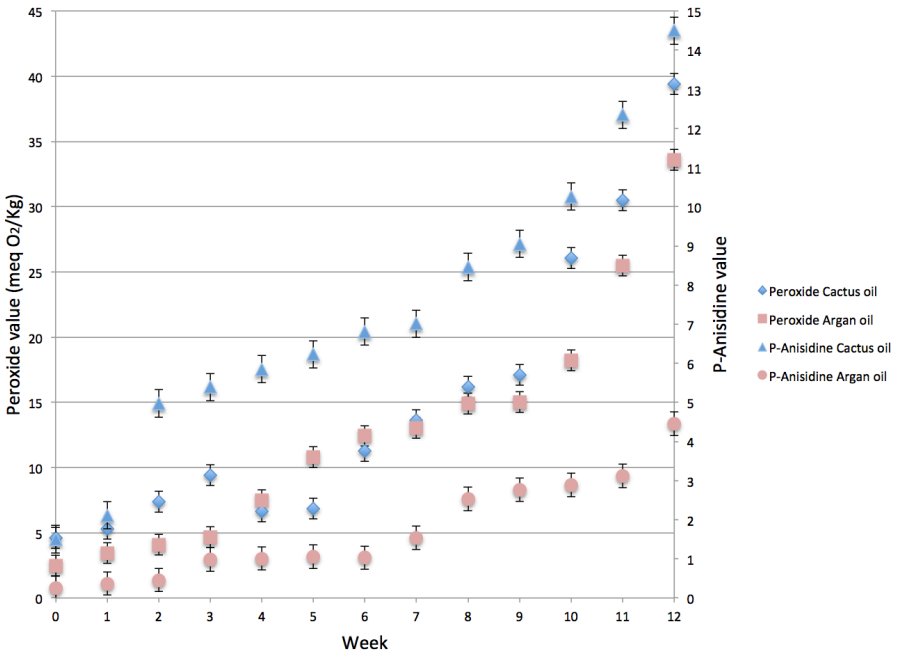

FIGURE 2. Evolution of peroxide value (meq O_2/kg of oil) and *p*-anisidine index of cactus and argan seed oils upon 12 weeks of storage at 60 °C. Values represent the mean of 3 replicates and error bars represent standard variations.

4. CONCLUSION

It should be kept in mind that cactus seed oil is not a product as homogenous as argan oil can be, and its content in fatty acids as well as minor components can undergo large variations, as do its physico-chemical parameters. Consequently, the oil from cactus seeds is susceptible to various oxidative processes and to exhibit high instability, which makes it inadequate for industrial use.

Although we studied cactus seed and argan oils originating from the same geographical area, the oxidative sensitivity of cactus seed oil was fond to be much higher than that argan oil. This difference likely comes from the high content in linoleic acid in cactus seed oil and possibly also from improper technological processes used for cactus seed oil extraction and/or insufficient care in its handling during extraction or storage.

When satisfactorily protected from sunlight and at room temperature, cosmetic argan oil has a shelf-life of one year (Gharby *et al.*, 2014), cactus seed oil's shelf-life can be estimated to be only between 3 and 6 months. Therefore, special care, such as refrigeration or storage under an inert atmosphere, should be seriously considered for cactus seed oil prolonged storage. If adequate precautions are taken, cactus seed oil deserves to find its place in the cosmetics market. Therefore, prickly pear fruit, often not appreciated and even disregarded (Piga, 2004), due to the high number and size of its seeds, may now become highly revalued given its new added value.

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REFERENCES

AOCS Official Method Ce 8e89. 1983. In American Oil Chemist's Society, Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. Uniform Methods Committee. Champaign, Il, AOCS.

Charrouf Z, Guillaume D. 1999. Ethnoeconomical, ethnomedical, and phytochemical study of *Argania spinosa* (L.) Skeels. *J. Ethnopharmacol.* **67**, 7-14. https://doi.org/10.1016/S0378-8741(98)00228-1

Charrouf Z, Guillaume D. 2008. Argan oil: Occurrence, composition and impact on human health. *Eur. J. Lipid Sci. Technol.* **110**, 632-636. https://doi.org/10.1002/ejlt.200700220

Charrouf Z, Guillaume D. 2014. Argan oil, the 35-years-of-research product. *Eur. J. Lipid Sci. Technol.* **116**, 1316-1321. https://doi.org/10.1002/ejlt.201400261

Charrouf Z, Guillaume D, Driouich A. 2002. The argan tree, an asset for Morocco. *Biofutur.* **220**, 54-56.

Charrouf Z, Guillaume D. 2018. The argan oil project: going from utopia to reality in 20 years. *Oilseeds & Fats, Crops and Lipids* **25**, D209. https://doi.org/10.1051/ocl/2018006

Ciriminna R, Bongiorno D, Scurria A, Danzì C, Timpanaro G, Delisi R, Avellone G, Pagliaro M. 2017. Sicilian *Opuntia ficus-indica* seed oil: Fatty acid composition and bio-economical aspects. *Eur. J. Lipid. Sci. Technol.* **119** (11), 1700232. https://doi.org/10.1002/ejlt.201700232

Commission Regulation (EEC) (2568/91) 1991. *Official Journal of the European Community*, L 248, 1.

Coskuner Y, Tekin A. 2003. Monitoring of seed composition of prickly pear (*Opuntia ficus-indica* L.) fruits during maturation period. *J. Sci. Food Agric.* **83**, 846-849. https://doi.org/10.1002/jsfa.1423

El Finti A, El Boullani R, Fallah M, Msanda F, El Mousadik A. 2013. Assessment of some agro-technological parameters of cactus pear fruit (*Opuntia ficus-indica* Mill.) in Morocco cultivars. *J. Med. Plants Res.* **7**, 2574-2583

El Mannoubi I, Barrek S, Skanji T, Casabianca H, Zarrouk H. 2009. Characterization of *Opuntia ficus-indica* seed oil from Tunisia. *Chem. Nat. Comp.* **45**, 616-620. https://doi.org/10.1007/s10600-009-9448-1

Feugang JM, Konarski P, Zou D, Stintzing FC, Zou C. 2006. Nutritional and medicinal use of Cactus pear (*Opuntia* spp.) cladodes and fruits. *Front Biosci.* **11**, 2574-2589.

Gharby S, Harhar H, El Monfalouti H, Kartah B, Maata N, Guillaume D, Charrouf Z. 2012a. Chemical and oxidative properties of olive and argan oils sold on the Moroccan market. A comparative study. *Med. J. Nutr. Metab.* **5**, 31-38. https://doi.org/10.1007/s12349-011-0076-5

Gharby S, Harhar H, Guillaume D, Haddad A, Matthäus B, Charrouf Z. 2011. Oxidative stability of edible argan oil: A two-year study. *LWT-Food Sci. Technol.* **44**, 1-8. https://doi.org/10. 1016/j.lwt.2010.07.003

Gharby S, Harhar H, Guillaume D, Haddad A, Charrouf Z. 2012b. The origin of virgin argan oil's high oxidative stability unraveled. *Nat. Prod. Commun.* **7**, 621-624. https://doi.org/10.1177/1934578X1200700520

Gharby S, Harhar H, Kartah B, Guillaume D, Chafchaouni I, Charrouf Z. 2014. Oxidative stability of cosmetic argan oil: a one-year study. *J. Cosmetic Sci.* **65**, 81-87

Guillaume D, Charrouf Z. 2011. Argan oil and other argan products: Use in dermocosmetology. *Eur. J. Lipid Sci. Technol.* **113**, 403-408. https://doi.org/10.1002/ejlt.201000417

Guillaume D, Charrouf Z. 2013. Argan oil for nutritional and skin care applications. *HPC Today* **8**, 28-30.

Guillaume D, Gharby S, Harhar H, Baba M. 2015. *Opuntia ficus-indica* and *Balanites aegytiaca* oils: two seed oils to watch. *HPC Today* **10**, 45-48.

Harhar H, Gharby S, Guillaume D, Charrouf Z. 2010. Effect of argan kernel storage conditions on argan oil quality. *Eur. J. Lipid Sci. Technol.* **112**, 915-920. https://doi.org/10.1002/ejlt.200900269

Labuschagne MT, Hugo A. 2010. Oil content and fatty acid composition of cactus pear seed compared with cotton and grape seed. *J. Food Biochem.* **34**, 93-100. https://doi.org/10.1111/j.1745-4514.2009.00266.x

Matthäus B, Guillaume D, Gharby S, Haddad A, Harhar H, Charrouf Z. 2010. Effect of processing on the quality of edible argan oil. *Food Chem.* **120**, 426-432. https://doi.org/10.1016/j.foodchem.2009.10.023

Matthäus B, Özcan MM. 2011. Habitat effects on yield, fatty acid composition and tocopherol contents of prickly pear (*Opuntia ficus-indica* L.) seed oils. *Scientia Hort.* **131**, 95-98. https://doi.org/10.1016/j.scienta.2011.09.027

Monia E, Bourret E, Mondolot L, Attia H. 2005. Fatty acid composition and rheological behaviour of prickly pear seed oils. *Food Chem.* **93**, 431-437. https://doi.org/10.1016/j.foodchem.2004.10.020

Özcan MM, Al Juhaimi FY. 2011. Nutritive value and chemical composition of prickly pear seeds (*Opuntia ficus-indica* L.) growing in Turkey. *Internat. J. Food Sci. Nutr.* **62**, 533-536. https://doi.org/10.3109/09637486.2011.552569

Piga A, 2004. Cactus pear: a fruit of nutraceutical and functional importance. *J. Prof. Assoc. Cactus Dev.* **6**, 9-22.

Ramadan MF, Mörsel JT. 2003. Oil cactus pear (*Opuntia ficus-indica* L.). *Food Chem.* **82**, 339-345. https://doi.org/10.1016/S0308-8146(02)00550-2

Salvo F, Galati EM, Lo Curto S, Tripodo MM. 2002. Study on the chemical characterization of lipid composition of *Opuntia ficus-indica* L. seed oil. *Riv. Ital. Sost. Grasse* **79**, 395-398.

Sawaya WN, Khan P. 1982. Chemical characterization of prickly pear seed oil, *Opuntia ficus-indica. J. Food Sci.* **47**, 2060-2061. https://doi.org/10.1111/j.1365-2621.1982.tb12946.x

Stewart RRC, Bewley JD. 1980. Lipid peroxidation associated with accelerated Aging of soybean axes. *Plant Physiol.* **65**, 245-248.

Taoufik F, Zine S, El Hadek M, Idrissi Hassani LM, Gharby S, Harhar H, Matthäus B. 2015. Oil content and main constituents of cactus seed oils *Opuntia Ficus-indica* of different origin in Morocco. *Med. J. Nutr. Metab.* **8**, 85-92. https://doi.org/10.3233/MNM-150036

Waal HO de, Louhaichi M, Taguchi M, Fouché HJ, De Wit M. 2015. Development of a cactus pear agro-industry for the sub-Sahara Africa Region. Proceedings of International Workshop, University of the Free State, Bloemfontein, South Africa, 96 pp.

Wit M de, Hugo A, Shongwe N. 2016. Quality assessment of seed oil from selected cactus pear cultivars (*Opuntia ficus-indica* and *Opuntia robusta*). *J. Food Process. Preserv.* **41**, e12898. https://doi.org/10.1111/jfpp.12898

Zaanoun I, Gharby S, Bakass I, Ait addi E, Ait ichou I. 2014. Kinetic parameter determination of roasted and unroasted argan oil oxidation under Rancimat test conditions. *Grasas Aceites* **65** (3), e033. https://doi.org/10.3989/gya.122713

Zine S, Gharby S, El Hadek M. 2013. Physicochemical characterization of *Opuntia ficus-indica* Seed Oil from Morocco. *Biosci. Biotech. Res. Asia,* **10**, 99-105.

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Catalyst-free production of fatty acid ethyl esters (FAEE) from macauba pulp oil

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SUMMARY: In this study, the production of fatty acid ethyl esters (FAEE) from macauba pulp oil and pressurized ethanol was investigated. The experiments were conducted without the addition of catalyst, at 20 MPa, to determine the effect of temperature (200 to 300 °C) and the oil to ethanol mass ratio (1:1 and 1:2) on the FAEE content and different residence times (10 to 45 min). The effect of the addition of *n*-hexane to the oil (20 wt%) as a co-solvent was also evaluated. The use of high temperatures (275 and 300 °C) resulted in high FAEE content (~90%). Increasing the amount of ethanol in the reaction medium contributed to the formation of esters only at operating temperatures of 200 to 250 °C. It was also observed that with the addition of co-solvent (in the oil) it was possible to obtain high amounts of FAEE in a shorter reaction time. In addition, a low content of unreacted compounds (~8.0%) and the conversion of ~90 and 99% of the free fatty acids and triglycerides were observed, respectively.

KEYWORDS: Acrocomia aculeate*; Catalyst-free; Continuous; Ethanol*

RESUMEN: ***Producción sin catalizador de ésteres etílicos de ácidos grasos (FAEE) a partir de aceite de pulpa de macauba.*** En este estudio, se investigó la producción de ésteres etílicos de ácidos grasos (FAEE) a partir de aceite de pulpa de macauba y etanol presurizado. Los experimentos se realizaron, sin la adición de catalizador, a 20 MPa, para determinar el efecto de la temperatura (200 a 300 °C) y la relación de masa de aceite a etanol (1:1 y 1:2) en el contenido de FAEE, aplicando diferentes tiempos de residencia (10 a 45 min). También se evaluó el efecto de la adición de n-hexano al aceite (20% en peso) como co-disolvente. El uso de altas temperaturas (275 y 300 °C) dio como resultado un alto contenido de FAEE (~90%). El aumento de la cantidad de etanol en el medio de reacción contribuyó a la formación de ésteres solo a temperaturas de funcionamiento de 200 a 250 °C. También se observó que con la adición de co-disolvente (en el aceite) era posible obtener altas cantidades de FAEE en un tiempo de reacción más corto. Además, se observó un bajo contenido de compuestos sin reaccionar (~8,0%) y la conversión de ~90 y 99% de ácidos grasos libres y triglicéridos, respectivamente.

PALABRAS CLAVES: Acrocomia aculeate*; Continuo; Etanol; Libre de catalizador*

Conflict of interest: The authors declare that there is no conflict of interest.

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1. INTRODUCTION

Macauba (*Acrocomia aculeata*) is a promising oleaginous plant for the production of biodiesel, mainly due to its high oil content, with 23-46% in the pulp (Evaristo *et al*., 2016; Lescano *et al*., 2015) and 43-64% in the kernel (Evaristo *et al*., 2016; Nunes *et al*., 2018). Several reports indicate that the oil extracted from the pulp of macauba presents a high content in free fatty acids (FFA), as observed by Dona *et al*., (2013) and Visioli *et al*., (2018), who reported 79.24 and 65.20% of free fatty acids (FFA) in macauba pulp oil, respectively. The oil is often obtained by pressing and filtration without the need for refining steps (Colonelli *et al*., 2017). Thus, the conventional method involving the use of homogeneous alkaline catalysts is not applicable for the transformation of this oil into biodiesel, since the presence of FFA neutralizes the catalyst and also produces soap emulsions, which hinders the separation of glycerol (Patil and Deng, 2009).

Thus, other strategies need to be adopted for the synthesis of biodiesel using low quality raw materials, particularly when high concentrations of free fatty acids are present (Mardhiah *et al*., 2017). Reactions using alcohol under pressurized conditions have been widely investigated for biodiesel production (Visioli *et al*., 2016; Santos *et al*., 2017; Trentini *et al*., 2018). These are associated with the same energy cost as reactions conducted at low pressures using a basic homogeneous catalyst and generate a product with higher purity (Demirbas, 2002), eliminating the need for separation from the catalyst. In this method, high yields of esters are obtained without the use of catalysts in a relatively short time (Nan *et al*., 2015) since, under conditions of high temperature and pressure, the miscibility of the triglycerides with the alcohol increases due to the decreasing polarity of the alcohol (Farobie and Matsumura, 2017; Kusdiana and Saka, 2004; Liu *et al*., 2018; Osmieri *et al*., 2017), which also behaves as an acid catalyst in the reaction besides being a solvent (Kusdiana and Saka, 2004). Another advantage to this method is the efficiency and good results which have been reported for reactions where the oil had a high content in free fatty acids (Postaue *et al*., 2019; Trentini *et al*., 2018; Visioli *et al*., 2018). Thus, this appears to be a viable alternative for studies on macauba pulp oil.

The efficiency of the reaction under pressurized conditions is dependent on the adjustment of the operating variables (pressure, temperature and time), the nature of the raw material (oil and alcohol), the oil-to-alcohol ratio used and the additives added to the reaction medium. The reaction is commonly conducted at pressures in the order of 20 MPa at temperatures between 275 and 350 °C (Silva and Oliveira, 2014).

The amount of alcohol used in the process is higher than that of the stoichiometric oil-to-alcohol molar ratio of 1:3, since an excess of alcohol will favor the formation of the esters, shifting the reaction toward the formation of products (Colonelli *et al*., 2017). This is because there is an increase in the miscibility and the area of contact between the substrates, which benefits the production of esters (Musa, 2016). Some authors have reported that the use of an oil-to-alcohol molar ratio of 1:40 (which equates to a mass ratio of ~1:2) gives the highest ester yield (Silva *et al*., 2007; Trentini *et al*., 2018). However, other authors have found that the formation of esters was favored up to an oil-to-alcohol molar ratio of 1:20 (mass ratio of ~1:1) (Colonelli *et al*., 2017; Silva *et al*., 2010; Trentin *et al*., 2011).

Another aspect to be considered is the addition of a co-solvent to the reaction medium, which can act to improve the operating conditions of the technology using alcohol under sub- and supercritical conditions. This generally involves the reduction of some adjustable parameters of the reaction, such as temperature, pressure, residence time and/or oil-to-alcohol ratio (Akkarawatkhoosith *et al*., 2019c, 2019a; Osmieri *et al*., 2017; Tobar and Núñez, 2018). In addition, the miscibility and solubility of the reaction mixture are also improved (Akkarawatkhoosith *et al*., 2019a, 2019b; Đokić-Stojanović1 *et al*., 2019; Muppaneni *et al*., 2013), ensuring a high production of esters in a short period of time and without the use of a catalyst (Lim and Lee, 2013). Several co-solvents are cited in the literature. Notably, the use of *n*-hexane has proved to be efficient for reactions under supercritical conditions without the use of a catalyst (Abdala *et al*., 2014a; Colonelli *et al*., 2017; Lim and Lee, 2013; Muppaneni *et al*., 2013; Silva and Oliveira, 2014), besides reducing the viscosity of the reaction mixture and allowing the production in a continuous process (Sawangkeaw *et al*., 2011).

Recently, obtaining oil esters from macauba pulp in a catalyst-free medium using pressurized ethanol was reported by Colonelli *et al*., (2017). These authors found that the addition of a co-solvent (*n*-hexane) and the use of higher amounts of alcohol in the reaction medium were the variables that had the greatest influence on the formation of esters. However, the effects of these variables were observed in a fixed residence time.

In this context, the objective of this study was to evaluate the kinetics of ester production from macauba pulp oil (MPO) using ethanol under pressurized conditions and without the use of a catalyst. Experiments were carried out at different temperatures (200 ºC to 300 ºC) and the influence of adding a greater amount of ethanol to the reaction medium was evaluated. The effect of the addition of a co-solvent (*n*-hexane) and the ester yield at different residence times were also investigated.

2. MATERIALS AND METHODS

2.1. Materials

Macauba pulp oil (Cocal - Brasil), ethanol (JT Baker 99.8%) and *n*-hexane (Vetec 98.5%) were used for the reaction. The fatty acid composition of macauba pulp oil used in this study was previously reported (Colonelli *et al*., 2017), with the oil presenting predominance of oleic (70%) and palmitic acids (13%). The oil has FFA content of 70.26 ± 0.05 wt% and water content of 0.76 ± 0.001 wt%. For analysis of the reaction products, standard analytical methyl heptadecanoate (Sigma Aldrich, 99.9%) and heptane (F Maia, 95%) were used.

2.2. Reaction procedure

In order to carry out the reaction between macauba pulp oil and ethanol, the mixture of these substrates was fed by a high-pressure liquid pump in a reactor operated in continuous mode. For reactions with *n*-hexane, the co-solvent was added directly into the reaction mixture before pumping. The reactor was at the test temperature and after filling the system, it was pressurized. The residence time was calculated by the ratio between the reactor volume and the feed rate of the reaction mixture. Before collection, the reaction mixture was cooled to 15 °C by a thermostatic bath. The apparatus and experimental procedure is described in greater detail by Mello *et al*., (2017) and Visioli *et al*., (2016) and the experiment for each experimental condition was performed at least in duplicate.

Samples were collected after the residence time for each reaction had been reached, and the excess ethanol was removed at 80 °C. To remove the glycerol formed the procedure described by Trentini *et al*., (2018) was used.

2.3. Analytical methods

To determine ester content, the samples were diluted in heptane and the internal standard (methyl heptadecanoate) was added and then the samples were injected, in triplicate, in the gas chromatograph (Agilent GC 7890), equipped with flame ionization detector and capillary column (ZB-WAX, 30 m x 0.25 mm x 0.1 µm), using the chromatographic conditions reported by Colonelli *et al*., (2017). The quantification of the ester content in the samples followed the standard UNE-EN 14103 method (2003), using Equation 1 to calculate the ethyl ester content:

$$\text{Esters content (\%)} = \left(\frac{\Sigma A}{AP}\frac{CP}{CA}\right) \times 100 \quad (1)$$

where ΣA is the sum of the peak areas corresponding to the esters, AP is the area for the methyl heptadecanoate, CP and CA are the concentrations of the methyl heptadecanoate and injected sample, respectively.

In order to determine the contents in mono-, di- and triglycerides, 100 mg of sample were derivatized with MSTFA (15 min at room temperature) (Standard UNE-EN 14105, 2003) and then to a concentration of 3 mg·mL^{-1}. The diluted sample (2 µL) was analyzed with a gas chromatograph (Shimadzu, GC-2010 Plus) equipped with a capillary column (Zebron ZB-5HT, 10 m × 0.32 mm × 0.10 µm), flame ionization detector and on-column injector. The information concerning oven temperature gradient, detector temperature and the heating program for the injector were presented by Trentini *et al*., (2019). Calibration curves were constructed using chromatographic standards of triolein, diolein and monolein for the quantification of the compounds. FFA content was determined from the acid-base titration method as recommended by AOCS Ca 5a-40 (American Oil Chemists' Society, 1990).

3. RESULTS AND DISCUSSION

3.1. Ethyl ester content

Figure 1 shows the results from the experiments conducted with a mass ratio of MPO-to-ethanol of 1:1, as well as the effect of increasing the ratio to 1:2 and adding 20 wt% of co-solvent (*n*-hexane) at a ratio of 1:1, at temperatures of 200 to 300 °C. The mass ratios of MPO-to-ethanol of 1:1 and 1:2 are equivalent to molar ratios of triglycerides-to-ethanol of ~40 and 122 and of free fatty acids-to-ethanol of 9 and 18, respectively.

3.1.1. Effect of temperature

In the overall analysis of the data shown in Figure 1 it can be seen that an increase in temperature led to higher ester yields. When the temperature was increased from 200 to 250 °C and from 250 to 300 °C, with a residence time of 10 min, it was possible to obtain an increase in the ester content in the ratios of ~2.35 and ~1.15, respectively, for the three reaction media evaluated. The temperature effect is significant ($p < 0.05$) up to the residence time of 45 min (analysis not shown in Figure 1).

In reactions at high temperatures, changes in the solubility, density, dielectric constant and solvation of the mixture in the reaction medium occur (Farobie and Matsumura, 2017). This favors a reduction in the mass transfer limitations and increases the reaction rate (Abbaszaadeh *et al*., 2012; Pinnarat and Savage, 2010). In addition, near the critical temperature, the polarity of the alcohol decreases, and the alcohol starts to solvate the non-polar triglycerides, forming a practically homogeneous mixture (Srivastava, Paul, and Goud, 2018), which allows higher ester yields to be obtained.

Tobar and Núñez (2018) showed that the reaction kinetics are affected by increasing temperature due to an increase in the kinetic energy of the particles and the relative probability of collisions between them.

In a study conducted by Silva *et al*., (2014), increased ester yields were observed on increasing the temperature from 250 to 300 °C, with a difference of ~42% in only 10 min of reaction. A similar result was reported by Santos *et al*., (2018) with a 30% increase in the ester yield for the same temperature range. In addition, Zhou *et al*., (2017) and Akkarawatkhoosith *et al*., (2019a) obtained an increased ester content of ~60% on increasing the operating temperature from 250 to 300 ºC.

Under the conditions where ethanol was below its critical temperature (243.2 °C), the results demonstrated an FAEE content of 30 to 60%. This is due to the high concentration of FFA in the macauba oil used (70.26%), resulting in the esterification reaction predominating, since FFAs are more reactive than triglycerides (Go *et al*., 2014; Vieitez *et al*., 2012).

Pinnarat and Savage (2010) studied non-catalytic esterification and reported that an ester content of 70% was obtained at 230 °C with a residence time of 80 min and pressure of 5.2 MPa. Go *et al*., (2014) reported an ester yield of ~60% with the reaction conducted at 200 °C for 30 min at a pressure of 2.8 MPa. At the same temperature but with a pressure of 20 MPa, Abdala *et al*., (2014b) achieved ~70% conversion of oleic acids to esters in only 10 min of reaction. Santos *et al*., (2017) obtained an esters content of ~75% at 220 °C and 10 MPa with 80 min of reaction. Jesus *et al*., (2018) observed that the reaction between oleic acid and ethanol yielded ~66% esters at 200 °C with a reaction time of 30 min and pressure of 15 MPa.

3.1.2. Effect of oil to ethanol mass ratio

Increasing the MPO-to-ethanol mass ratio from 1:1 to 1:2 had a more pronounced effect on the formation of esters for the reactions conducted at temperatures of 200 to 250 ºC, particularly at the shortest times evaluated (10 and 15 min). At the highest temperatures considered, that is 275 ºC (Figure 1d) and 300 ºC (Figure 1e), the increase in the amount of alcohol in the reaction medium did not influence the FAEE content.

A greater amount of alcohol in the reaction boosts the formation of the products, since increasing the volume of ethanol available in the medium results in a decrease in the critical temperature of the reaction mixture (Osmieri *et al*., 2017), promoting the occurrence of the reaction occurring in a homogeneous phase region and increasing the reaction kinetics. MPO is mainly composed of FFA and esterification predominates in the reaction medium. As reported by Santos *et al*., (2017), this reaction occurs in a single homogeneous phase at 10 MPa with an FFA-to-ethanol molar ratio of 1:1 to 30:1 from 220 to 280 °C.

The alcohol concentration has little effect at higher operating temperatures, as observed at 275

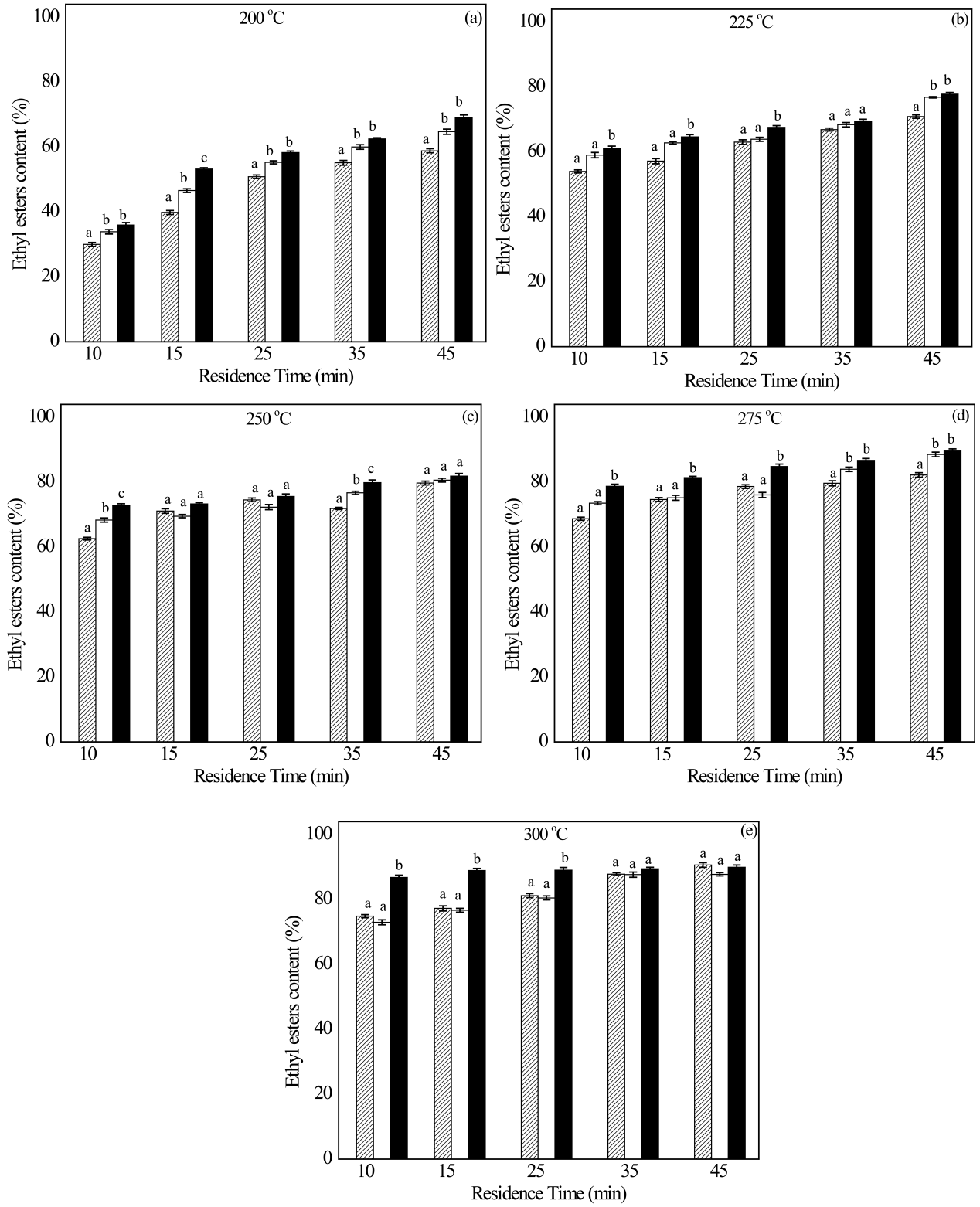

FIGURE 1. Ethyl ester contents of macauba pulp oil obtained at different temperatures: (a) 200 °C, (b) 225 °C, (c) 250 °C, (c) 275 °C and (e) 300 °C at 20 MPa, MPO-to-ethanol mass ratio of 1:1 (▨) and 1:2 (▭), and MPO-to-ethanol mass ratio of 1:1 with 20 wt% of co-solvent (in oil) (■). The values in the graphs represent the means of two determinations, with SD < 2.0%. Means followed by the same lowercase letters (with the same residence time) did not differ statistically ($p > 0.05$) using ANOVA (Tukey's test).

and 300 °C (Figure 1), this effect occurs due to the increase in the critical pressure in the mixture, which makes it necessary to use high pressure to obtain a homogeneous phase, as noted by Nan *et al*., (2015) who obtained similar ester yields at 310 °C, after 30 min at 14 MPa, using microalgae oil-to-ethanol molar ratios of 1:26 and 1:42. The experiments conducted by Bezerra *et al*., (2018) also provided similar conversion to ethyl esters for oil:ethanol molar ratios of 1:20 and 1:40 at 350 °C, after 40 min at 20 MPa. A similar result was obtained by Costa *et al*., (2019) on raising the oil:methanol molar ratio from 1:21 to 1:41, at 300 °C, for 10 min of reaction at 20 MPa.

At lower temperatures (200 to 250 ºC) the effect of the ratio between the lipids (triglycerides or FFA) and the alcohol in the matrix can be noted (Figure 1). Mello *et al*., (2017) reported a 12% increase in the ethyl ester yield on increasing the ratio of alcohol to crambe oil hydrolyzate from 12:1 to 15:1 (molar basis) in a reaction conducted at 275 °C, for 10 min at 15 MPa. Jesus *et al*., (2018) conducted the reaction at 250 °C and 15 MPa and found that on increasing the ethanol:oleic acid molar ratio from 1:1 to 6:1 the conversion of FFA to esters increased by 22%.

3.1.3. Effect of co-solvent

The addition of 20 wt% of co-solvent to the reaction mixture (with an oil-to- ethanol mass ratio of 1:1) promoted an improvement in ester production. Thus, for a residence time of 10 min it was possible to obtain increases of 30 to 36% and 75 to 87% in the ester content at 200 and 300 ºC, respectively. The use of a co-solvent improves the mutual solubility between the alcohol and the oil (Tobar and Núñez, 2018), allowing for the presence of a homogeneous phase (Osmieri *et al*., 2017), increasing the reaction rate and making it possible to obtain high ester yields at moderate temperatures (Maçaira *et al*., 2014).

Zhou *et al*., (2017) performed a coupled extraction and reaction process. When the reaction was conducted at 340 °C, for 120 min, with an *n*-hexane flow of 0.2 $mL \cdot min^{-1}$, there was an increase of 63% in ester yield compared to the reaction without the addition of the co-solvent. In research conducted by Tobar and Núñez (2018), the highest yield of ethyl esters (68%) was obtained with the addition of CO_2 (0.001 g CO_2 per g of ethanol) as a co-solvent at 300 °C and 20 MPa. Akkarawatkhoosith *et al*., (2019c) reported that an iso-propanol:oil weight ratio of 0.1:1 for only 3 min at 350 °C gave a 37% increase in the ester yield. The same research group (Akkarawatkhoosith *et al*., 2019b) also found that in the reaction conducted at 300 °C for 4 min the addition of a co-solvent (55 wt% ethyl acetate) promoted an increase in the ethyl ester production of ~68% compared to the reaction with no co-solvent added.

It should be noted that adding more alcohol to the process would significantly increase the production costs, as the ethanol (99.5%) used for this purpose is ~21% more expensive than *n*-hexane (95%), according to the company Tedia Brazil®. Thus, the costs related to increasing the oil-to-ethanol mass ratio to 1:2 would be ~85% higher than using the mass ratio of 1:1 with 20 wt% of *n*-hexane (basis of calculation: 1 L of reaction mixture). However, it needs to be considered that high ester content (~89%) can be obtained in a relatively short time (15 min) at 300 °C, further enhancing the benefits of using the co-solvent. In addition, as noted by Sawangkeaw *et al*., (2011), *n*-hexane, the solvent most commonly used in the extraction of vegetable oils, can be removed after the oil extraction and employed in the production of biodiesel.

3.2. Triglyceride, diglyceride and monoglyceride contents

In the samples obtained at 300 ºC, the triglyceride (TG), diglyceride (TG), monoglyceride (MG) and free fatty acid (FFA) contents were determined, as shown in Figure 2, considering that the highest levels of FAEE were obtained at this temperature. The contents in these compounds in the MPO were also determined at: 18.42 ± 0.15 wt%, 15.2 ± 0.62 wt%, 3.85 ± 0.12 wt% and 70.26 ± 0.05 wt% of TG, DG, MG and FFA, respectively. In general, the increase in mass ratio and addition of co-solvent did not influence the conversion of TG or the formation of DG. The reaction conducted with a mass ratio of MPO-to-ethanol of 1:1, showed a higher concentration of MG when compared to the others. It can also be seen that there was a higher consumption of FFA for the reaction with a mass ratio MPO-to-ethanol of 1:2.

For all conditions evaluated, high TG conversions were achieved, with contents in these

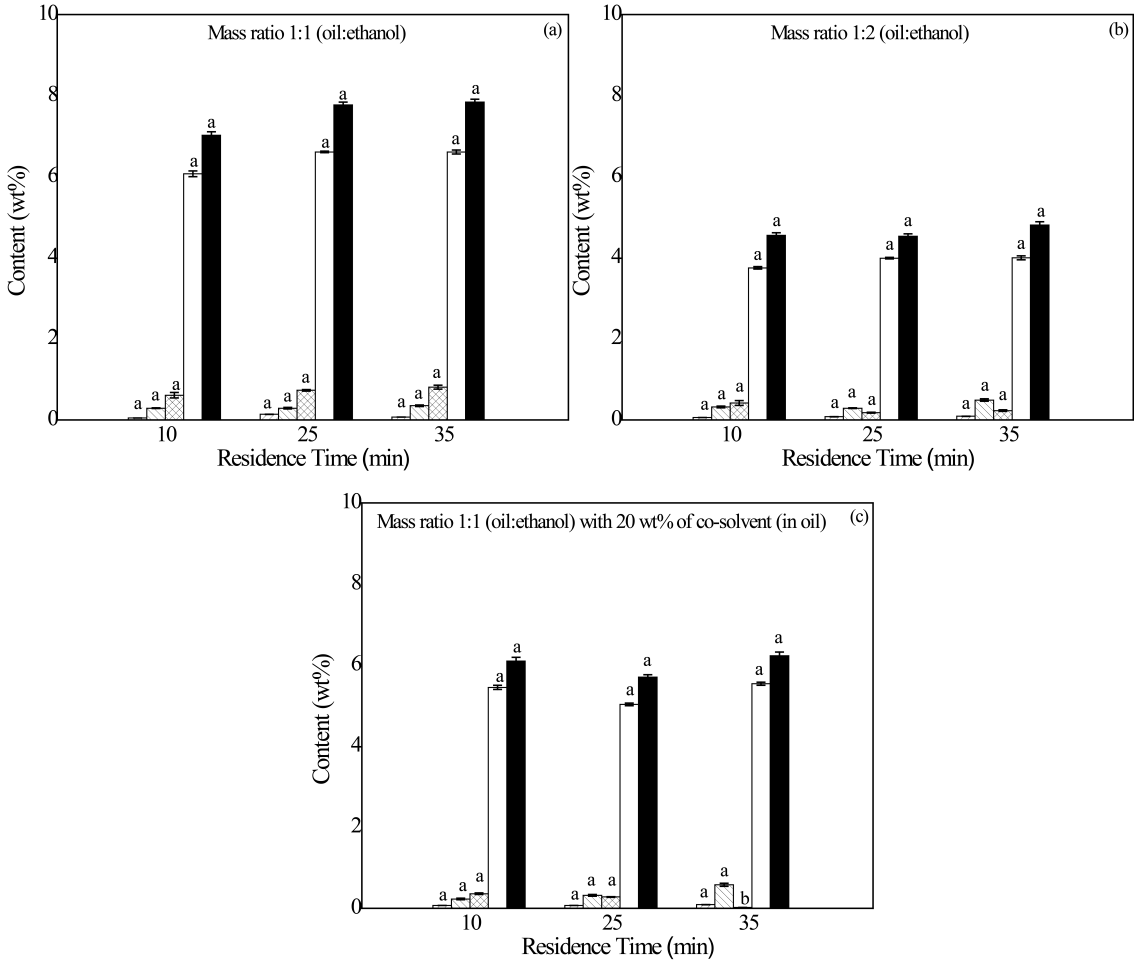

FIGURE 2. Triglyceride (▨), diglyceride (▧), monoglyceride (▩), free fatty acids (□) and total contents obtained (■) at 300 ºC: MPO-to-ethanol mass ratio of (a) 1:1 and (b) 1:2, and (c) MPO-to-ethanol mass ratio of 1:1 with 20 wt% of co-solvent (in oil). The values in the graphs represent the means of two determinations, with SD < 1.0 wt%. Means followed by the same lowercase letters (for the same compound) did not differ statistically ($p > 0.05$) using ANOVA (Tukey's test).

compounds in the samples obtained being below 0.14 wt%. In other studies on the transesterification reaction under pressurized conditions conducted at the same temperature, the TG contents of 0.2 to 22 wt% (Santos *et al*., 2018; Trentin *et al*., 2011; Trentini *et al*., 2019; Vieitez *et al*., 2010) were detected under the conditions that provided maximum ester yields.

The remaining FFA contents in the samples ranged from 4.0 to 6.6 wt%, which correspond to an FFA conversion of above 90%. A similar result was obtained by Visioli *et al*., (2016) for conversion under the thermodynamic equilibrium in the esterification reaction of soybean oil deodorizer distillate with pressurized ethanol. Vieitez *et al*., (2012) performed the supercritical alcoholysis of raw materials with 0 to 100 wt% of FFA and achieved a maximum conversion of FFA to ethyl esters of ~ 90%.

The total amount of unreacted compounds detected was less than 8.0 wt%. Soto *et al*., (2014) found DG, MG and FFA contents of 11, 3 and 2%, respectively, in the reaction between sunflower oil and methanol with 40 min of reaction at 18 MPa. Ortiz-Martínez *et al*., (2016) reported a higher value of unreacted compounds, with ~18 and 27% of MG and DG, respectively, for the reaction between *Pongamia pinnata* oil and methanol, with a reaction time of 20 min at 18 MPa. Low contents in MG (2%), DG (4%) and TG (0.2%) were reported by Trentini *et al*., (2019) for the reaction between grease trap waste lipids and ethanol, with the addition of 2.5 wt% water, with a residence time of 30 min at 20 MPa.

4. CONCLUSIONS

Increasing the operating temperature up to 300 °C for the reaction between macauba pulp oil (MPO) and ethanol led to high ester contents. Moreover, it was found that the reactions conducted at high temperatures (275 and 300 °C) required less alcohol in the reaction medium in shorter residence times, demonstrating that temperature is a key factor to be considered in studies on the production of esters. The addition of *n*-hexane to the reaction, which increased the diglyceride and monoglyceride contents by only 0.6 wt%, allowed for a reduction in the reaction time of ~44% and increased the ester production by up to ~25%. The reaction in a pressurized medium without catalyst was effective, and produced low contents in unreacted compounds (~ 8.0%) and high consumption of triglycerides (~ 99%) and free fatty acids (~90%). The highest FAEE content (~90%) was obtained with an MPO-to-ethanol mass ratio of 1:1 with 20 wt% of co-solvent (in the oil) at 300 °C after 15 min of reaction.

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REFERENCES

Abbaszaadeh A, Ghobadian B, Omidkhah MR, Najafi G. 2012. Current biodiesel production technologies : A comparative review. *Energ. Convers. and Manage*. **63**, 138-148. https://doi.org/10.1016/j.enconman.2012.02.027

Abdala ACDA, Colonelli TADS, Trentini CP, Oliveira JV, Cardozo-Filho L, Silva EA, Silva C. 2014a. Effect of additives in the reaction medium on noncatalytic ester production from used frying oil with supercritical ethanol. *Ener. Fuels* **28**3122-3128. https://doi.org/10.1021/ef402253e

Abdala AC, Garcia VA, Trentini CP, Cardozo-Filho L, Silva EA, Silva C. 2014b. Continuous Catalyst-Free Esterification of Oleic Acid in Compressed Ethanol. *Int. J. Chem. Eng*. **2014**, 1-5. https://doi.org/10.1155/2014/803783

Akkarawatkhoosith N, Kaewchada A, Jaree A. 2019a. Enhancement of continuous supercritical biodiesel production : influence of co-solvent types. *Enrgy Proced*. **156**, 48-52. https://doi.org/10.1016/j.egypro.2018.11.085

Akkarawatkhoosith N, Kaewchada A, Jaree A. 2019b. Production of Biodiesel from Palm Oil under Supercritical Ethanol in the Presence of Ethyl Acetate. *Energ. Fuel* **33**, 5322-5331. research-article. https://doi.org/10.1021/acs.energyfuels.9b00641

Akkarawatkhoosith N, Kaewchada A, Jaree A. 2019c. Simultaneous development of biodiesel synthesis and fuel quality via continuous supercritical process with reactive co-solvent. *Fuel* **237**, 117-125. https://doi.org/10.1016/j.fuel.2018.09.077

American Oil Chemists' Society. Standard Methods for the Analysis of Oils, Fats and Derivatives, International Union of Pure and Applied Chemistry Commission on Oils, Fats and Derivatives § (1990).

Bezerra FWF, Costa WA, Oliveira MS, Andrade HÁ, Carvalho JRN. 2018. Transesterification of palm pressed-fibers (*Elaeis guineensis* Jacq.) oil bysupercriticalfluid carbon dioxide with entrainer ethanol. *J. Supercrit. Fluids* **136**, 136-143. https://doi.org/10.1016/j.supflu.2018.02.020

Colonelli TADS, Trentini CP, Santos KA, Oliveira JV, Cardozo-Filho L, Silva EA, Silva C. 2017. Assessment of process variables on the use of macauba pulp oil as feedstock for the continuous production of ethyl esters under pressurized conditions. *Braz. J. Che. Eng*. **34**, 831-839. https://doi.org/10.1590/0104-6632.20170343s20150768

Costa WA, Bezerra FWF, Oliveira MS, Andrade HA, Santos APM, Cunha VMB, Junior RN. 2019. Supercritical CO_2 extraction and transesterification of the residual oil from industrial palm kernel cake with supercritical methanol. *J. Supercrit. Fluids*, **147**, 179-187. https://doi.org/10.1016/j.supflu.2018.10.012

Demirbas A. 2002. Biodiesel from vegetable oils via transesterification in supercritical methanol. *Energ. Convers. and Manage*. **43**, 2349-2356. https://doi.org/10.1016/S0196-8904(01)00170-4

Đokić-Stojanović1 DR, Todorović ZB, Troter DZ, Stamenković OS, Veselinović LM, Zdujić MV, Manojlović DD, Veljković VB. 2019. Influence of various co-solvents on the calcium oxide-catalyzed ethanolysis of sunflower oil. *J. Serbian Chem. Soc*. **84**, 253-265. https://doi.org/10.2298/JSC180827007D

Doná G, Cardozo-Filho L, Silva C, Castilhos F. 2013. Biodiesel production using supercritical methyl acetate in a tubular packed bed reactor.

Fuel Process. Technol. **106**, 605-610. https://doi.org/10.1016/j.fuproc.2012.09.047

Evaristo AB, Grossi JAS, Pimentel LD, Goulart SDM, Martins AD, Santos VL, Motoike S. 2016. Harvest and post-harvest conditions influencing macauba (*Acrocomia aculeata*) oil quality attributes. *Ind. Crops. Prod*. **85**, 63-73. https://doi.org/10.1016/j.indcrop.2016.02.052

Farobie O, Matsumura Y. 2017. State of the art of biodiesel production under supercritical conditions. *Prog. Energy. Combust. Sci*. **63**, 173-203. https://doi.org/10.1016/j.pecs.2017.08.001

Go AW, Sutanto S, NguyenThi BT, Cabatingan LK, Ismadji S, Ju Y. 2014. Transesterification of soybean oil with methanol and acetic acid at lower reaction severity under subcritical conditions. *Energ. Convers. Manag.* **88**, 1159-1166. https://doi.org/10.1016/j.enconman.2014.03.014

Jesus AA, Souza DF, Oliveira JA, Deus MS, Silva MG, Franceschi E, Dariva C. 2018. Mathematical modeling and experimental esterification at supercritical conditions for biodiesel production in a tubular reactor. *Energ. Convers. Manag*. **171**, 1697-1703. https://doi.org/10.1016/j.enconman.2018.06.108

Kusdiana D, Saka S. 2004. Effects of water on biodiesel fuel production by supercritical methanol treatment. *Bioresour. Technol.* **91**, 289-295. https://doi.org/10.1016/S0960-8524(03)00201-3

Lescano CH, Oliveira IP, Silva LR, Baldivia DS, Sanjinez-Argandonã, EJ, Arruda EJ, Lima FF. 2015. Nutrients content, characterization and oil extraction from *Acrocomia aculeata* (Jacq.) Lodd . fruits. *Afr. J. Food Sci*. **9**, 113-119. https://doi.org/10.5897/AJFS2014.1212

Lim S, Lee K. 2013. Influences of different co-solvents in simultaneous supercritical extraction and transesterification of *Jatropha curcas* L . seeds for the production of biodiesel. *Chem. Eng. J*. **221**, 436-445. https://doi.org/10.1016/j.cej.2013.02.014

Liu J, Nan Y, Huang X, Bond JQ, Tavlarides LL. 2018. Applied Catalysis B: Environmental Continuous esteri fi cation of oleic acid to ethyl oleate under sub / supercritical conditions over γ-Al_2O_3. *Appl. Catal. B* **232**, 155-163. https://doi.org/10.1016/j.apcatb.2018.03.050

Maçaira J, Santana A, Costa A, Ramirez E, Larrayoz MA. 2014. Process Intensification Using CO2 As Cosolvent under Supercritical Conditions Applied to the Design of Biodiesel Production. *Ind. Eng. Chem. Res.* **5** *3*, 3985-3995. https://doi.org/10.1021/ie402657e

Mardhiah HH, Chyuan H, Masjuki HH, Lim S, Pang YL. 2017. Investigation of carbon-based solid acid catalyst from *Jatropha curcas* biomass in biodiesel production. *Energ. Convers. and Manage.* **144**, 10-17. https://doi.org/10.1016/j.enconman.2017.04.038

Mello BTF, Gonçalves JE, Rodrigues GM, Cardozo-Filho L, Silva C. 2017. Hydroesterification of crambe oil (*Crambe abyssinica* H.) under pressurized conditions. *Ind. Crops Prod*. **97**, 110-119. https://doi.org/10.1016/j.indcrop.2016.12.014

Muppaneni T, Reddy HK, Ponnusamy S, Patil PD, Sun Y, Dailey P, Deng S. 2013. Optimization of biodiesel production from palm oil under supercritical ethanol conditions using hexane as co-solvent : A response surface methodology approach. *Fuel* **107**, 633-640. https://doi.org/10.1016/j.fuel.2012.11.046

Musa IA. 2016. The effects of alcohol to oil molar ratios and the type of alcohol on biodiesel production using transesterification process. *Egypt. J. Pet*. **25**, 21-31. https://doi.org/10.1016/j.ejpe.2015.06.007

Nan Y, Liu J, Lin R, Tavlarides LL. 2015. Production of biodiesel from microalgae oil (*Chlorella protothecoides*) by non-catalytic transesterification in supercritical methanol and ethanol: Process optimization. *J. Supercrit. Fluids* **97**, 174-182. https://doi.org/10.1016/j.supflu.2014.08.025

Nunes ÂA, Buccini DF, Jaques JAS, Portugal LC, Guimarães RCA, Favaro SP, Carvalho CME. 2018. Effect of *Acrocomia aculeata* Kernel Oil on Adiposity in Type 2 Diabetic Rats. *Plant. Foods Hum. Nutr*. **73**, 61-67. https://doi.org/10.1007/s11130-017-0648-8

Ortiz-Martínez VM, Salar-García MJ, Palacios-Nereo FJ, Olivares-Carrillo P, Quesada-Medina J, Ríos APDL, Hernández-Fernández FJ. 2016. In-depth study of the transesterification reaction of *Pongamia pinnata* oil for biodiesel production using catalyst-free supercritical methanol process. *J. Supercrit. Fluids* **113**, 23-30. https://doi.org/10.1016/j.supflu.2016.03.009

Osmieri L, Esfahani RAM, Recasens F. 2017. Continuous biodiesel production in supercritical two-step process: phase equilibrium and process design. *J. Supercrit.*

Fluids **124**, 57-71. https://doi.org/10.1016/j.supflu.2017.01.010

Patil PD, Deng S. 2009. Optimization of biodiesel production from edible and non-edible vegetable oils. *Fuel* **88**, 1302-1306. https://doi.org/10.1016/j.fuel.2009.01.016

Pinnarat T, Savage PE. 2010. Noncatalytic esterification of oleic acid in ethanol. *J. Supercrit. Fluids*, **53**, 53-59. https://doi.org/10.1016/j.supflu.2010.02.008

Postaue N, Trentini CP, Mello BTF, Cardozo-Filho L, Silva C. 2019. Continuous catalyst-free interesterification of crambe oil using methyl acetate under pressurized conditions. *Energ. Convers. and Manage*. **187**, 398-406. https://doi.org/10.1016/j.enconman.2019.03.046

Santos KC, Hamerski F, Voll FAP, Corazza ML. 2018. Experimental and kinetic modeling of acid oil (trans)esterification in supercritical ethanol. *Fuel* **224**, 489-498. https://doi.org/10.1016/j.fuel.2018.03.102

Santos PR, Pedersen FA, Ramos LP, Corazza ML. 2017. Esterification of fatty acids with supercritical ethanol in a continuous tubular reactor. *J. Supercrit. Fluids* **126**, 25-36. https://doi.org/10.1016/j.supflu.2017.03.002

Sawangkeaw R, Bunyakiat K, Ngamprasertsith S. 2011. Continuous production of biodiesel with supercritical methanol : Optimization of a scale-up plug fl ow reactor by response surface methodology. *Fuel Process. Technol*. **92**, 2285-2292. https://doi.org/10.1016/j.fuproc.2011.07.014

Silva C, Castilhos F, Oliveira JV, Cardozo-Filho L, Trentin CM, Lima AP, Oliveira JV. 2010. Continuous production of soybean biodiesel with compressed ethanol in a microtube reactor. *Fuel Process. Technol*. **91**, 1274-1281. https://doi.org/10.1016/j.fuproc.2010.12.016

Silva C, Colonelli TAS, Silva EA, Cabral VF, Oliveira JV, Cardozo-Filho L. 2014. Continuous catalyst-free production of esters from *Jatropha curcas* L. oil under supercritical ethanol. *Braz. J. Chem. Eng*. **31**727-735. https://doi.org/10.1590/0104-6632.20140313s00002709

Silva C, Oliveira JV. 2014. Biodiesel production through non-catalytic supercritical transesterification: Current state and perspectives. *Braz. J. Chem. Eng*. **31**, 271-285. https://doi.org/10.1590/0104-6632.20140312s00002616

Silva C, Weschenfelder T, Rovani S, Corazza FC, Corazza ML, Dariva C, Oliveira JV. 2007. Continuous Production of Fatty Acid Ethyl Esters from Soybean Oil in Compressed Ethanol. *Ind. Eng. Chem. Res.* **46**, 5304-5309. https://doi.org/10.1021/ie070310r

Soto G, Hegel P, Pereda S. 2014. Supercritical production and fractionation of fatty acid esters and acylglycerols. *J. Supercrit. Fluids* **93**, 74-81. https://doi.org/10.1016/j.supflu.2014.04.017

Standard UNE-EN 14105. 2003. *Determinación de los contenidos de glicerol libre y total y de mono-, di- y triglicéridos*. Madrid, Spain.

Srivastava G, Paul AK, Goud VV. 2018. Optimization of non-catalytic transesterification of microalgae oil to biodiesel under supercritical methanol condition. *Energy. Conver. Manag*. **156**, 269-278. https://doi.org/10.1016/j.enconman.2017.10.093

Tobar M, Núñez GA. 2018. Supercritical transeterification of microalgae triglycerides for biodiesel production: Effect of alcohol type and co-solvent. *J. Supercrit. Fluids* **137**, 50-56. https://doi.org/10.1016/j.supflu.2018.03.008

Trentin CM, Lima AP, Alkimim IP, Silva C, Castilhos F, Mazutti MA, Oliveira JV. 2011. Continuous production of soybean biodiesel with compressed ethanol in a microtube reactor using carbon dioxide as co-solvent. *Fuel Process. Technol.* **92**, 952-958. https://doi.org/10.1016/j.fuproc.2010.12.016

Trentini CP, Fonseca JM, Cardozo-Filho L, Reis R, Sampaio SC, Silva C. 2018. Assessment of continuous catalyst-free production of ethyl esters from grease trap waste. *J. Supercrit. Fluids* **136**, 157-163. https://doi.org/10.1016/j.supflu.2018.02.018

Trentini CP, Postaue N, Cardozo-Filho L, Reis RR, Sampaio SC, Silva C. 2019. Production of esters from grease trap waste lipids under supercritical conditins: Effect of water addition on ethanol. *J. Supercrit. Fluids* **147**, 16. https://doi.org/10.1016/j.supflu.2019.02.008

Vieitez I, Irigaray B, Casullo P, Pardo MJ, Grompone MA, Jachmanián I. 2012. Effect of free fatty acids on the efficiency of the supercritical ethanolysis of vegetable oils from different origins. *Energy. and Fuels* **26**, 1946-1951. https://doi.org/10.1021/ef201977s

Vieitez I, Silva C, Alckmin I, Borges GR, Corazza FC, Oliveira JV, Jachmanián, I. 2010. Continuous catalyst-free methanolysis and

ethanolysis of soybean oil under supercritical alcohol/water mixtures. *Renew. Energ*. **35**, 1976-1981. https://doi.org/10.1016/j.renene.2010.01.027

Visioli LJ, Castilhos F, Cardozo-Filho L, Mello BTF, Silva C. 2016. Production of esters from soybean oil deodorizer distillate in pressurized ethanol. *Fuel Process. Technol*. **149**, 326-331. https://doi.org/10.1016/j.fuproc.2016.04.038

Visioli LJ, Trentini CP, Castilhos F, Silva C. 2018. Esters production in continuous reactor from macauba pulp oil using methyl acetate in pressurized conditions. *J. Supercrit. Fluids* **140**, 238-247. https://doi.org/10.1016/j.supflu.2018.06.018

Zhou D, Qiao B, Li G, Xue S, Yin J. 2017. Continuous production of biodiesel from microalgae by extraction coupling with transesterification under supercritical conditions. *Bioresour. Technol*. **238,** 609-615. https://doi.org/10.1016/j.biortech.2017.04.097

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Fatty acids, bioactive substances, antioxidant and antimicrobial activity of *Ankyropetalum* spp., a novel source of nervonic acid

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SUMMARY: *Ankyropetalum* extracts were obtained by using two different extractors (Soxhlet and ultrasonic bath). The phenol, flavonoid, DPPH, FRAP, and antimicrobial activity properties of the extracts were investigated. In addition, the fatty acid composition was determined in GC-MS. High values were found in *A. reuteri* and *A. gypsophiloides* for total phenolic and flavonoid contents, respectively. DPPH and FRAP values were high in *A. arsusianum* and *A. gypsophiloides*, respectively. Better results were obtained by using methanol as the solvent and soxhlet as the extractor. The results showed that the extracts seem to be reasonably effective against test organisms including clinical isolates. The most promising results were obtained with all species USB extracts against *Candida parapsilosis*. It is notable that the levels of nervonic acid in *A. arsusianum* and *A. reuteri* reached 40%. Unlike other sources of nervonic acid in the world, the absence of erucic acid in plant oil increases the value of these plants.

KEYWORDS: Ankyropetalum*; Antimicrobial activity; Antioxidant activity; Fatty acid; Nervonic acid*

RESUMEN: ***Ácidos grasos, bioactivos y actividades antioxidante y antimicrobiana de Ankyropetalum spp., una nueva fuente de ácido nervónico.*** Los extractos de *Ankyropetalum* se obtuvieron usando dos medios de extracción diferentes (Soxhlet y baño ultrasónico). A estos extractos se les determinó el contenido fenólico, flavonoides, DPPH, FRAP, y la actividad antimicrobiana. Además, se determinó la composición de ácidos grasos mediante GC-MS. Se encontraron valores altos de contenido fenólico y flavonoide total en *A. reuteri* y *A. gypsophiloides*, respectivamente. Los valores de DPPH y FRAP fueron altos en *A. arsusianum* y *A. gypsophiloides*, respectivamente. Se obtuvieron mejores resultados utilizando metanol como disolvente y Soxhlet como extractor. Los resultados mostraron que los extractos parecen ser razonablemente efectivos contra los organismos ensayados, incluidos los aislados clínicos. Los resultados más prometedores se obtuvieron con todos los extractos USB de especies contra la *Cándida parapsilosis*. Es notable que los niveles de ácido nervónico en *A. arsusianum* y *A. reuteri* alcanzaron el 40%. A diferencia de otras fuentes de ácido nervónico en el mundo, la ausencia de ácido erúcico en el aceite vegetal aumenta el valor de estas plantas.

PALABRAS CLAVE: *Ácido graso; Ácido nervónico; Actividad antimicrobiana; Actividad antioxidante;* Ankyropetalum

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1. INTRODUCTION

Ankyropetalum Fenzl is a small genus which includes only three species in the world. It belongs to the Caryophyllaceae family with 32 genera and 494 species in Turkey (Davis, 1982). The species are *A. arsusianum* Kotschy ex Boiss., *A. reuteri* Boiss. and Hausskn. and *A. gypsophiloides* Fenzl (Barkoudah, 1962). The gene center of the genus is Turkey, and the taxa of this genus are spread mainly throughout South-Eastern Anatolia and partially in the Mediterranean region (Ozcelik and Muca, 2010). While *A. reuteri* is endemic and belongs to the EN category, the other species are rare and found in South-east Anatolia and within the borders of neighboring countries (Ekim *et al*., 2000; Korkmaz and Ozcelik, 2011). The *Ankyropetalum*, *Gypsophila* and *Saponaria* members are known as "Çöven Otu" in Turkey. It is generally difficult to distinguish the *Ankyropetalum* from the perennial *Gypsophila* species. *Ankyropetalum* and *Gypsophyla* are used for similar purposes. *A. gypsophiloides* has been used in the preparation of a local food and *A. reuteri* has been mixed with straw and used as animal feed (Ozcelik and Muca, 2010). They are also used for the production of tahini halvah, foam halvah, Turkish delight, herbed cheese, çöven bread, and the manufacturing of chemical cleaner, fire extinguisher manufacturing, liquor making, and soap making (Korkmaz and Ozcelik, 2011).

Until the development of synthetic medicines from prehistoric times, plants were the basis of almost all medical treatments (Djeridane *et al*., 2006). Because of the less harmful effect than its synthetic counterparts, there is still interest in traditional herbal products today (Mocan *et al*., 2018). Herbal products contain biologically highly active compounds such as phenolic compounds, flavonoids, flavonols, and tocopherols, which play an important role in human nutrition and health (Mocan *et al*., 2018). It has been reported that there is an inverse relationship between the formation of human diseases and the consumption of various antioxidant plants (Dudonne *et al*., 2009). Therefore, research on the identification of antioxidative compounds is an important issue. Due to potential health risks and toxicity, there has also been an increased interest in the use of natural antioxidants in foodstuffs or medical materials in recent times (Djeridane *et al*., 2006). Fatty acids play an essential role in many body functions (Elias, 1983). Nervonic acid, one of the critical fatty acids, has been suggested to help maintain brain health, increase brain function, reduce fatigue, and accumulate less fat in the blood (Mohanty *et al*., 2013). There is a tendency for the use of nervonic acid to be added to foods for the treatment of neurological diseases such as Alzheimer's, multiple sclerosis, adrenoleuko-dystrophy and people with Zellweger's syndrome (Tang *et al*., 2013). It is also used as a medication in the symptomatic treatment of patients with schizophrenia, psychosis and attention deficit (Krishnan, 2009).

Since these species are endemic or rare and furthermore, they are not cultured, they are almost at the point of extinction. There are a limited number of studies on the taxonomy, ecology and economic importance of *Ankyropetalum*. *Ankyropetalum* is an economic plant with its commercial potential due to its addition to food. Despite its medicinal and economic value, there is no published biochemical or bioactivity study on this plant. Therefore, in this study, the total phenolic and flavonoid contents, antioxidant and antimicrobial activity of *A. arsusianum, A. reuteri* and *A. gypsophiloides* were investigated to find new potential sources of natural antioxidants. On the other hand, according to a GC-MS analysis, plant oils were found to be a novel source of nervonic acid.

2. MATERIALS AND METHODS

2.1. Plant materials

Three species of *Ankyropetalum* Fenzl were used in this study. The samples were collected from the natural habitats around Kahramanmaraş, Hatay and Gaziantep in the summer of 2015 (Table 1). The plants were identified according to the Flora of Turkey (Davis, 1982). The plants were kept in the herbarium of Kahramanmaras Sutcu Imam University [YZK-1123 (*A. reuteri*), YZK-1142 (*A. arsusianum*), YZK-1143 (*A. gypsophiloides*)].

2.2. Sample preparation and extraction

The plants were dried in the shade for about a week and then pulverized by grinding in a laboratory blender. Methanol and ethanol were used in two different extraction methods. Total phenolics, total flavonoids, antioxidant activity and antimicrobial activity were determined in these obtained extracts.

TABLE 1. Plant materials used in this study.

Species	Distribution	Altitude (m)	Location
A. arsusianum	narrow spread	10	Arsus/Hatay
A. gypsophiloides	narrow spread	700	Sahinbey/Gaziantep
A. reuteri	Endemic-narrow spread	565	Imali village-Turkoglu/ Kahramanmaras

2.2.1. Extraction method (SOX method)

The extraction was carried out using a Soxhlet apparatus at 60 °C for 6 hours with the addition of solvent (100 ml) to 10 g of plant material. After removal of the solvent in a vacuum rotary evaporator at 40 °C, the extract was kept at -20 °C for further analysis.

2.2.2. Extraction method (USB method)

Solvent (100 ml) was added to 10 g of plant and extraction was carried out in the Ultrasonic Water Bath for 1 hour at room temperature. The samples were centrifuged for 15 min at 3500 rpm. After centrifuging, the eluted liquid fraction was collected in another tube and the plant sample was extracted again as described above. The extracts were combined and the solvent was removed in a vacuum rotary evaporator at 40 °C and the extract was kept at -20 °C for further analysis.

2.3. Antioxidant assay

2.3.1. Determination of total phenolic and flavonoid content

Folin-Ciocalteau colorimetric method was used to determine the total phenolic contents of the fractions (Blainski *et al*., 2013). Flavonoid compounds were extracted in the ultrasonic bath using 50 ml of 80% methanol:water (v/v) with 0.5 g of powdered plant samples for 20 min. Samples were centrifuged at 14000 rpm for 5 min. The total flavonoid content of the extracts was evaluated using spectrophotometry (Chang *et al*., 2002). All extracts were tested in triplicate to confirm the reproducibility of the results.

2.3.2. DPPH and FRAP analysis

Scavenging free radical potentials were analyzed using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Brand-Williams *et al*., 1995). Ascorbic acid was used as positive control. The antioxidant activity was expressed as $IC_{50,}$ which denotes the concentration of sample required to scavenge 50% of the DPPH free radicals. The FRAP (The Ferric reducing antioxidant power) analysis was made according to Benzie and Strain (1996). Plant extracts (50 µl) were transferred to 2 ml eppendorf tubes and 600 µl FRAP agent were added. Absorbance was measured at 593 nm. The results were calculated as µmol ascorbic acid equivalent/g dry plant weight using the ascorbic acid (10-1000 $\mu mol \cdot l^{-1}$) calibration chart. Results were given in µmol/g dry plant weight. All extracts were tested in triplicate to confirm the reproducibility of the results.

2.4. Antimicrobial assay

2.4.1. Microorganisms and culturing

The bacteria and yeast were obtained from the culture collection of the Biotechnology Laboratory in Kahramanmaras Sutcu Imam University. *Bacillus subtilis* ATCC 6633, *Enterobacter cloacae* ATCC 13047D, *Escherichia coli* ATCC 39628, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538P, *Sarcina lutea* ATCC 9341NA, *Klebsiella pneumonia, Candida albicans, Candida parapsilosis, Candida glabrata* and *Saccharomyces cerevisiae* were handled as test organisms. They were maintained and activated on Sabouraud Dextrose and Nutrient Broth/Agar (Oxoid).

2.4.2. Antimicrobial activity assay

The antimicrobial activity of the ethanol and methanol extracts of *A. arsusianum, A. gypsophiloides* and *A. reuteri* were determined by the well diffusion method (Collins *et al.*, 1989). Mueller Hinton Agar (Difco) and Sabouraud Dextrose Agar (SDA) (Oxoid) plates were inoculated with a standardized inoculum, giving $1.5x10^8$ bacteria and 2.1×10^3cfu ml^{-1} yeast (Collins *et al.*, 1989). The wells (6 mm) were prepared with a cork borer and filled with 50 µl of extract (20 mg·ml) dissolved in dimethyl

sulfoxide (DMSO). The plates inoculated with bacteria and yeast were then incubated at 37 and 30 °C for 24 and 48 hours, respectively. The inhibition zones produced were measured and the presence of antimicrobial substances was evaluated after an incubation period.

2.4.3. MIC determination

Minimum inhibition concentrations (MIC) of the extracts were determined according to the micro dilution method (Collins *et al*., 1989) in culture broth media. The extracts presented an inhibition zone in the well-diffusion method dissolved in DMSO and mixed with the Mueller Hinton and Sabouraud dextrose broth in a designed volume. Later, a dilution series was accomplished in micro well plates. As a control, culture medium and DMSO were set as growth control as well as test dilution for sterility control. After inoculation of the test well, 5 µl of organisms were changed, and the plates were incubated for 24/48 hours. The results were stated as $mg \cdot ml^{-1}$.

2.5. Determination of fatty acid content

The fatty acid content was analysed in a Shimadzu 2025 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and a column TR-CN100, 60 m × 0.25 mm × 0.20 mm. He was used as carrier gas at a flow rate of 1.5 $ml \cdot min^{-1}$. Initial column oven temperature was 80 °C for 2 min, then elevated to 140 °C at a rate of 5 $°C \cdot min^{-1}$ (maintained for 2 min at 140 °C), and then elevated to 240 °C at 3 $°C \cdot min^{-1}$ (maintained for 5 min at 240 °C). The detector and injection temperatures were programmed to 250 and 240 °C, respectively. Peak areas were used to calculate the relative percentage of the fatty acids as total fatty acid.

2.6. Statistical analysis

The statistical analysis appropriate for the entirely randomized (3x2x2) x3 factorial design with three replicates was performed. The hypotheses for the means of the primary and interaction effects were tested using the analysis of variance (ANOVA). Since factors of "solvent" and "extractor" have two levels, the direct use of F test is adequate for the related comparisons. If the results of the F test were significant, the means were determined to be statistically different. In addition, for the factors with more than two levels, comparisons of means were made by Tukey's test at the 0.05 significance level (Efe *et al*., 2000).

3. RESULTS

3.1. Antioxidant assay

Oxidative stress is thought to cause the development and progress of diseases as well as ageing. Many phenolic compounds with biologically essential effects are considered to be the most abundant antioxidants in foods (Mocan *et al*., 2018; Abeywickrama *et al*., 2016; Locatelli *et al*., 2017). Since different radicals and oxidants have different mechanisms of antioxidant response, there is no single way to measure antioxidant capacity (Isık *et al*., 2015). Therefore, in this study, different solvents and extractors were applied to compare the total phenolic and flavonoid contents of *Ankyropetalum* species by using Folin-Ciocâlteu and $AlCl_3$ assays and the results are given in Table 2.

When means of the species and two-way interactions of species x extractor and species x solvent were examined, the highest total of phenolic substances was observed for *A. reuteri* extract (52.75, 48.04, 48.51 mg $GAE \cdot g^{-1}$, respectively). Two-way interactions of solvent x indicated the statistical superiority of methanolic extracts over ethanolic extracts in all types. On the other hand, interactions of the x extractor showed that soxhlet was statistically superior to USB in all types (Table 3).

Regarding flavonoid content, *A. gypsophiloides* shows superiority over other species. As in the total phenolic content, flavonoid content was also found to be poorer than *A. arsusianum* in comparison to the other species. Soxhlet and methanol were found to be more effective in eliciting the flavonoid contents in the species. Other researchers working with different species of Caryophyllaceae have also reported that methanolic extracts have higher phenolic and flavonoid contents than other solvents (Nikolova *et al*., 2011; Chima *et al*., 2014).

The lower the IC_{50} in DPPH analysis, the better the free radicals can be scavenged and thus impair the free radical chain reaction (Lim *et al*., 2007). Antioxidant activities were determined by DPPH and FRAP tests in this study and the results are presented in Table 1. The extracts of *A. arsusianum* and *A. gypsophiloides* were more active against DPPH free radical than *A. reuteri*.

TABLE 2. Total phenolic, total flavonoid, FRAP and DPPH results of *Ankyropetalum* species (The results were the mean of three replicates)

		Soxhlet		Ultrasonic Bath (USB)		
Method	**Species**	**Ethanol***	**Methanol***	**Ethanol***	**Methanol***	**Species Mean****
Total Phenolic Content (mg GAE·g^{-1})	***A. arsusianum***	30.94± 1.64^{d}	42.67± 2.02 ^{bc}	11.16± 1.13 ^{e}	41.94± 1.10 ^{bc}	31.68^{c}
	A. gypsophiloides	40.53± 0.78 ^{bc}	46.17 ± 1.92 ^{b}	13.27± 0.50 ^{e}	38.54± 0.21 ^{c}	34.62^{b}
	A. reuteri	43.33± 0.25 ^{bc}	52.75 ± 0.58 ^{a}	15.81± 0.29 ^{e}	44.27± 2.17 ^{bc}	39.04^{a}
		Ethanol**	**Methanol****	**Ethanol****	**Methanol****	**Species Mean****
Total Flavonoid Content (mg QE·g^{-1})	***A. arsusianum***	0.9 ± 0,03 ^{f}	1.10 ± 0.06 ^{ef}	0.66 ± 0.03 ^{g}	1.04 ± 0.04 ^{ef}	0.93^{c}
	A. gypsophiloides	2.97 ± 0.13 ^{b}	3.56 ± 0.01 ^{a}	1.21 ± 0.02 ^{de}	2.24 ± 0.03 ^{c}	2.50^{a}
	A. reuteri	1.21 ± 0.01 ^{e}	1.29 ± 0.05 ^{de}	0.62 ± 0.02 ^{g}	1.47 ± 0.07 ^{d}	1.15^{b}
		Ethanol**	**Methanol****	**Ethanol****	**Methanol****	**Species Mean****
DPPH (mg dw·g^{-1})	***A. arsusianum***	1. 70 ± 0.00 ^{c}	1.47 ± 0.02 ^{ab}	1.91 ± 0.00 ^{e}	1.41 ± 0.03 ^{a}	1.62^{b}
	A. gypsophiloides	1.72 ± 0.01 ^{cd}	1.43 ± 0.01 ^{a}	1.70 ± 0.01 ^{c}	1.42 ± 0.02 ^{a}	1.58^{a}
	A. reuteri	1.90 ± 0.05 ^{e}	1.53 ± 0.01 ^{b}	1.81 ± 0.01 ^{d}	1.51 ± 0.02 ^{b}	1.65^{b}
		Ethanol **	**Methanol ****	**Ethanol ****	**Methanol ****	**Species Mean****
FRAP (µg AAE·g^{-1})	***A. arsusianum***	36.65 ± 0.47 ^{bc}	42.06 ± 0.35 ^{a}	15.61 ± 0.30 ^{e}	33.73 ± 0.55 ^{c}	32.34^{a}
	A.gypsophiloides	39.08 ± 0.52 ^{ab}	41.57 ± 0.34 ^{a}	14.86 ± 0.15 ^{e}	36.35 ± 0.32 ^{c}	32.64^{a}
	A. reuteri	29.86 ± 0.32 ^{d}	35.26 ± 0.71 ^{c}	10.48 ± 0.28 ^{f}	35.34 ± 0.58 ^{c}	27.74^{b}

* P < 0.05, ** P < 0.01

Methanolic extracts were more active than ethanolic extracts; although the extractors did not significantly affect DPPH activity. FRAP results were also found to be similar to DPPH. *A. reuteri* also showed lower activity than the other two species. Methanolic extracts were more active in all three species. However, unlike DPPH, it was statistically significant that the extracts obtained from the soxhlet were more active than USB extracts.

The phenolic compounds and flavonoids obtained from plants were shown to have abundant antioxidant activity in food products (Van Acker *et al*., 1996). In general, extracts with high radical scavenging activity had a high phenolic content. However, there was no significant relationship between total phenolic and flavonoid contents and antioxidant activity in this study. Phenolic content was highest in *A. reuterii*, whereas antioxidant activity was found to be lower than the other two species in both DPPH and FRAP tests. A similar situation was seen in total flavonoid content. According to the results from the DPPH and FRAP tests, *A. arsusianum* and *A. gypsophiloides* species had high activity; while *A. gypsophiloides* alone had superior flavonoid content. This lack of relationship is also present in different studies. For example, Arslan *et al.,* (2013) studied three species of *Gypsophila* (*G. arrostii*, *G. pilulifera*, *G. simonii*) from the same family and obtained the highest total phenolic content in *G. simonii* (15.15 mg·g^{-1}). According to the results from the ABTS and DPPH analyses, *G. pilulifera* had a stronger antioxidant activity compared to the other two species. A similar result was reported by Stankovic *et al.,* (2015). According to their studies, while the phenolic contents in *Hordeum hystrix* and *Puccinella limosa* were found to be low, these plants were shown to have the highest antioxidant activity. On the other hand, the total phenolic content, IC_{50} value and FRAP value of *G. pilulifera* extracts were 6.5 mg·g^{-1}, 4.56 mg·ml^{-1} and 23.5 µg·g^{-1}, respectively (Yazici and Ozmen, 2017). These values were considerably lower than the values obtained in this study.

There may be several reasons why *A. reuterii* had lower antioxidant potency than the other two species despite its high phenol content and relatively high flavonoid content. The reaction of DPPH or FRAP may have been reversed with some phenols (Percentage of disappearance in antioxidant activity), or the reaction between DPPH or FRAP and substrate molecules may have been slow (Lim *et al.*, 2007; Huang *et al*., 2000).

TABLE 3. Two-way interactions of species x extractor and species x solvent

		Species x extractor		Species x solvent	
Method	**Species**	**Species x SOX****	**Species x USB****	**Species x Ethanol Mean****	**Species x Methanol Mean****
Total Phenolic Content (mg GAE·g^{-1})	***A. arsusianum***	36.81^{c}	26.55^{d}	21.05^{d}	42.305^{b}
	A.gypsophiloides	43.35^{b}	25.91^{de}	26.90^{c}	42.353^{b}
	A. reuteri	48.04^{a}	30.0^{ad}	29.57^{c}	48.513^{a}
		Species x SOX**	**Species x USB****	**Species x Ethanol Mean****	**Species x Methanol Mean****
Total Flavonoid Content (mg QE·g^{-1})	***A. arsusianum***	1.01^{d}	0.85^{e}	0.78^{e}	1.07^{d}
	A. gypsophiloides	3.27^{a}	1.723^{b}	2.10^{b}	2.90^{a}
	A. reuteri	1.25^{c}	1.05^{d}	0.91^{de}	1.38^{c}
		Species x SOX**	**Species x USB****	**Species x Ethanol Mean***	**Species x Methanol Mean***
DPPH (mg dw·g^{-1})	***A. arsusianum***	1.59^{ab}	1.66^{c}	1.81^{c}	1.44^{a}
	A.gypsophiloides	1.58^{ab}	1.56^{a}	1.71^{b}	1.43^{a}
	A. reuteri	1.72^{c}	1.66^{c}	1.86^{c}	1.52^{a}
		Species x SOX**	**Species x USB****	**Species x Ethanol Mean****	**Species x Methanol Mean****
FRAP (µg AAE·g^{-1})	***A. arsusianum***	39.35^{a}	25.33^{c}	26.13^{c}	38.55^{a}
	A.gypsophiloides	40.32^{a}	24.95^{c}	26.97^{c}	38.31^{a}
	A. reuteri	32.56^{b}	22.91^{d}	20.17^{d}	35.30^{b}

* P < 0.05, ** P < 0.01, SOX: Soxhlet, USB: Ultrasonic bath

3.2. Antimicrobial assay

The inhibitory activity of plant extracts was assayed against seven bacteria and four yeasts. The results showed that both solvent extracts of *A. arsusianum, A. gypsophiloides* and *A. reuteri* had substantial inhibitory activity against all the Gram-positive bacteria tested (Table 4). As *E. coli,* a member of gram-negative bacteria, was inhibited with all extracts, *Enterobacter cloaca* was the only one inhibited with the *A. reuteri* extract. However, *K. pneumonia* was not affected by any of the extracts. According to the general opinion, Gram-negative bacteria are already more resistant than Gram-positive organisms (Stickler and King, 1992). Among the 4 yeast strains, *Candida parapsilosis* was inhibited by three plant extracts, while *C. albicans* was inhibited only by *A. gypsophiloides* with the extract. On the contrary, *C. glabrata* and *Saccharomyces cerevisiae* were not affected. It could be an essential property having an inhibitory and non-inhibitory activity against pathogenic and non-pathogenic strains, respectively, for food and pharmaceuticals. Concerning extraction method, although neither soxhlet and USB nor methanol and ethanol were found superior to each other, the most promising results were obtained from the activity of *A.arsusianum, A.gypsophiloides* and *A.reuteri* USB extracts against *C. parapsilosis* (MIC: 0.781mg·ml^{-1}).

Around the world, numerous plants have been screened by many researchers with different methods against different microorganisms. Here in this study, the extract from *A. arsusianum*, *A. gypsophiloides* and *A. reuteri* obtained with different methods and solvents were tested against common microorganisms. As a result, these extracts seem to be reasonably effective against test organisms, including clinical isolates.

3.3. Fatty acid content

The oil content of *Ankyropetalum* plant extracts was 5.79% for *A. arsusianum*, 6.86% for *A. gypsophiloides* and 5.77% for *A. reuteri*. As a result of the fatty acid analysis of plant extracts, 18, 21 and 26 fatty acids were found in *A. arsusianum*, *A. reuteri* and *A. gypsophiloides*, respectively (Table 5). The major components were nervonic acid (23.66%, 39.76% and 42.88%), butyric acid (10.64%, 19.42% and 21.59%), palmitic, oleic and linoleic acids. Butyric acid was the major SFA in *A. arsusianum* and *A. reuteri* (19.42% and 21.59%, respectively), while it was palmitic acid (13.10%) in

TABLE 4. The antimicrobial activity of *Ankyropetalum* spp. against test microorganisms. (The results were the mean of three replicates)

			A. arsusianum		***A. gypsophiloides***		***A. reuteri***		
			Inhibition Zone (mm)	**MIC (mg·ml^{-1})**	**Inhibition Zone (mm)**	**MIC (mg·ml^{-1})**	**Inhibition Zone (mm)**	**MIC (mg·ml^{-1})**	**Gnc**
B. subtilis	SOX	Ethanol	9±1.52	6.25	11±0.57	6.25	10±1.54	25	21
		Methanol	12±0.57	6.25	8±0.54	12.5	8±1.52	12.5	
	USB	Ethanol	12±0.57	12.5	12±1.54	12.5	12±0.54	12.5	
		Methanol	14±1.54	12.5	14±0.52	12.5	12±0.57	12.5	
*E. cloaca**	SOX	Ethanol	-	NT	-	NT	7±1.00	50	16
		Methanol	-	NT	-	NT	9±1.52	50	
	USB	Ethanol	-	NT	-	NT	8±0.57	25	
		Methanol	-	NT	-	NT	9±0.52	25	
E. coli	SOX	Ethanol	10±2.00	25	10±1.15	12.5	11±2.00	25	24
		Methanol	10±1.15	6.25	8±1.15	12.5	10±1.00	50	
	USB	Ethanol	8±1.00	12.5	11±1.52	25	10±1.15	12.5	
		Methanol	12±1.15	12.5	10±1.57	25	12±1.15	25	
*E. faecalis**	SOX	Ethanol	11±0.00	12.5	11±1.52	12.5	12±1.52	25	26
		Methanol	12±1.52	12.5	8±1.00	25	10±1.52	12.5	
	USB	Ethanol	12±0.54	12.5	10±1.15	6.25	12±1.52	12.5	
		Methanol	12±0.54	12.5	11±1.15	6.25	11±1.52	12.5	
*S. aureus**	SOX	Ethanol	12±1.52	12.5	9±1.15	6.25	12±1.54	12.5	25
		Methanol	9±1.72	6.25	8±1.52	25	8±1.52	12.5	
	USB	Ethanol	10±1.15	12.5	9±1.73	12.5	10±1.00	25	
		Methanol	11±2.00	25	8±0.00	25	8±1.00	25	
S. lutea	SOX	Ethanol	14±1.52	25	13±0.00	25	13±1.52	12.5	28
		Methanol	11±1.52	6.25	12±..54	25	9±1.57	12.5	
	USB	Ethanol	10±1.00	12.5	13±1.73	12.5	10±1.00	12.5	
		Methanol	10±1.00	12.5	14±1.52	3.125	9±1.52	>50	
									Nys
*C. albicans**	SOX	Ethanol	-	NT	8±1.15	25	-	NT	18
		Methanol	-	NT	8±1.54	25	-	NT	
	USB	Ethanol	-	NT	8±1.52	25	-	NT	
		Methanol	-	NT	8±1.52	50	-	NT	
*C. parapsilosis**	SOX	Ethanol	11±1.00	6.25	9±0.00	3.125	11±1.52	12.5	12
		Methanol	10±1.00	3.125	12±0.52	0.781	8±1.54	25	
	USB	Ethanol	11±1.15	1.562	12±0.52	0.781	12±1.54	0.781	
		Methanol	12±1.15	0.781	11±0.57	0.781	9±0.00	3.125	

*Clinical isolate, NT: Not tested, -: No inhibition zone, MIC: Minimum inhibition concentration, Gnc: Gentamicin, Nys: Nystatine, *SOX: Soxhlet, USB: Ultrasonic bath

TABLE 5. Fatty acid compositions (%) of the plant extract of *Ankyropetalum* species (The results were the mean of three replicates)

Number of Carbon Atoms		Fatty acids	*A. arsusianum* %	*A. gypsophiloides* %	*A. reuteri* %
1	C4:0	Butyric acid	**19.42 ± 0.03**	**10.64 ± 0.03**	**21.59 ± 0.04**
2	C6:0	Caproic Acid	0.14 ± 0.02	0.05 ± 0.01	0.13 ± 0.00
3	C8:0	Caprylic Acid	0.21 ± 0.00	0.09 ± 0.00	0.14 ± 0.00
4	C10:0	Capric Acid	-	0.04 ± 0.00	0.13 ± 0.00
5	C12:0	Lauric Acid	0.46 ± 0.00	0.45 ± 0.02	0.76 ± 0.01
6	C13:0	Tridecanoic Acid	-	0.159 ± 0.01	-
7	C14:0	Myristic Acid	0.91 ± 0.00	1.25 ± 0.01	0.92 ± 0.01
8	C15:0	Pentadecanoic Acid	-	-	0.21 ± 0.01
9	C16:0	Palmitic Acid	**9.11 ± 0.01**	**13.10 ± 0.03**	**6.95 ± 0.03**
10	C17:0	Heptadecanoic Acid	-	0.15 ± 0.00	-
11	C18:0	Stearic Acid	1.87 ± 0.02	3.13 ± 0.02	1.50 ± 0.01
12	C20:0	Arachidic Acid	2.29 ± 0.02	6.71 ± 0.02	2.58 ± 0.01
13	C22:0	Behenic Acid	-	0.49 ± 0.01	0.36 ± 0.00
14	C23:0	Tricosanoic Acid	-	0.22 ± 0.00	-
15	C24:0	Lignoceric Acid	-	0.37 ± 0.00	0.28 ± 0.00
16	C15:1	*Cis*-10-Pentadecanoic Acid	-	0.18 ± 0.00	-
17	C16:1	Palmitoleic Acid	-	0.37 ± 0.00	-
18	C17:1	*Cis*-10-Heptadecanoic Acid	2.23 ± 0.01	1.86 ± 0.02	2.48 ± 0.01
19	C18:1	Oleic Acid **Ω9**	**5.92 ± 0.02**	**13.88 ± 0.03**	**9.97 ± 0.03**
20	C20:1	*Cis*-11-Eicosenoic Acid **Ω9**	0.48 ± 0.00	0.38 ± 0.01	1.06 ± 0.01
21	C24:1	Nervonic Acid **Ω9**	**42.88 ± 0.03**	**23.66 ± 0.04**	**39.76 ± 0.04**
22	C18:2	Linoleic Acid **Ω6**	**6.09 ± 0.01**	**17.50 ± 0.03**	**3.31 ± 0.02**
23	C18:3	Gamma-Linolenic Acid **Ω6**	1.66 ± 0.01	1.34 ± 0.01	1.84 ± 0.01
24	C18:3	Alfa-Linolenic Acid **Ω3**	0.39 ± 0.00	0.60 ± 0.0	-
25	C20:4	Arachidonic Acid **Ω6**	1.43 ± 0.01	1.15 ± 0.02	2.66 ± 0.01
26	C20:5	*Cis*-5.8.11.14.17-Eicosapentaenoic **Ω3**	1.01 ± 0.01	0.60 ± 0.00	1.22 ± 0.01
27	C22:6	*Cis*-4,7,10,13,16,19-Docosahexaenoic **Ω3**	3.50 ± 0.02	1.62 ± 0.01	2.15 ± 0.01
		SFA (Saturated Fatty Acid)	34.41	36.85	35.52
		MUFA (Monounsaturated Fatty Acid)	51.51	40.33	53.27
		PUFA (Polyunsaturated Fatty Acid)	14.08	22.81	11.18
		Total	**100.00**	**99.99**	**99.24**

A. gypsophiloides. There is a need for saturated fats for energy, hormone production, cellular membranes and organs. Butyric acid reduces virulence (a disease-causing effect) and it is used both in hygiene measures and in protection measures (Van Immerseel *et al*., 2005). In addition, since butyric acid esters have pleasant odors or flavors, they are often used as food and perfume additives. Some saturated fatty acids are also necessary for important signalling and stabilization processes in the body. Saturated fatty acids that play an important role in these processes are known as palmitic acid, myristic acid and lauric acid (Mohanty *et al*., 2013). *Ankyropetalum*, which contains all three fatty acids, contains palmitic acid predominantly.

Parameters associated with significant risk factors for cardiovascular disease have been associated with dietary habits. Olive oil rich in MUFA is one of the main components of the Mediterranean diet (Teres *et al*., 2008). On the other hand, the literature is increasingly showing the benefits of PUFAs for alleviating cardiovascular, inflammatory, heart diseases, atherosclerosis, autoimmune disorders, diabetes and other diseases (Finley and Shadidi, 2001). It has been observed that MUFAs contribute to the majority of the unsaturated fatty acid content in *Ankyropetalum* species. In all three species, the major MUFA is nervonic acid and the major PUFA is linoleic acid. It is known that some unsaturated fatty acids, such as nervonic acid, linoleic acid and *cis*-11 eicosenoic acid are suitable for human nutrition (Carvalho *et al*., 2006). The studied species contain all three fatty acids, two of which are dominant.

Interestingly, the amount of nervonic acid, especially in *A. arsusianum* plant oil, was higher than *Tropaeolum speciosum* (NA: 42.5%, EA: 17.3%) (Carlson *et al*., 1993), *Lunaria annua* (NA: 30%, EA: 45%) (Guo *et al*., 2009); *Lunaria biennis* L. (NA: 36-48%, EA: 14-25%) (Katavic *et al*., 2012); transgenic *Brassica napus* (NA: 30%, EA: 20%) (Napier and Graham, 2010); *Cardamine graeca* L. (NA: 9-10%, EA: 43-54%) (Katavic *et al*., 2012); *Acer truncatum* (NA: 5.8%, EA: 17.2%) (Wang *et al*., 2006) seed oils, which are known as nervonic acid sources. However, as you can see, these plants contain erucic acid. Nervonic acid is abundant in the white matter of the brain and the peripheral nervous tissue. Nervonic acid, which plays a role in nerve cell myelin biosynthesis, is one of the major fatty acids that make up about 40% of the total fatty acids in the brain sphingolipids (Sandhir *et al*., 1998; Taylor, 2010).

Interest in dietary therapy with nervonic acid-containing oils and fats has increased with the suggestion of Sargent *et al*., (1994) that dietary nervonic acid may support the normal synthesis and function of myelin in brain and nerve tissues. This recommendation encouraged the development of refined, nervonic acid-enriched vegetable oil to make experiments on humans and animals. Nervonic acid can be evaluated as a bioactive lipid supplement for the promotion of human and animal health, but it has been reported that nervonic acid-rich vegetable oil with the minimal amount of erucic acid has to be developed to be able to carry out these applications (Guo *et al*., 2009). In this context, *Ankyropetalum* oil which does not contain erucic acid at all and which contains nervonic acid at a satisfactory level will be preferred among other sources of nervonic acid.

4. CONCLUSIONS

Ankyropetalum is consumed locally as food and food additives. However, this plant has not been studied thoroughly enough. For this reason, the bioactive properties of *Ankyropetalum* have been examined in this study. The results show that *Ankyropetalum* is rich in phenols and flavonoids, as well as antioxidants and antimicrobials. The effects of extraction methods were evaluated and soxhlet extraction was found to be more effective in total phenolic content and FRAP. Methanol was superior to ethanol as solvent in extraction methods. With high MUFA and PUFA contents, *Ankyropetalum* oils can be evaluated in various fields as a valuable raw material that can be used in the pharmaceutical, cosmetic, perfume, and food and medicine industry. *Ankyropetalum* oil is rich in nervonic acid, so it has great potential for the symptomatic treatment of many neurodegenerative diseases such as multiple sclerosis, schizophrenia and Parkinson's disease. Further work on the isolation and identification of bioactive compounds will be beneficial for a better and specifically directed application.

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REFERENCES

Abeywickrama G, Debnath SC, Ambigaipalan P, Shahidi F. 2016. Phenolics of selected cranberry genotypes (*Vaccinium macrocarpon* Ait.) and their antioxidant efficacy. *J. Agric. Food Chem.* **64**, 9342-9351. https://doi.org/10.1021/acs.jafc.6b04291

Arslan I, Celik A, Melzig MF. 2013. Nebulosides A-B, novel triterpene saponins from underground parts of *Gypsophila arrostii* Guss. var. *Nebulosa. Bioorgan. Med. Chem.* **21**, 1279-1283. https://doi.org/10.1016/j.bmc.2012.12.036

Barkoudah YI. 1962. A Revision of *Gypsophila, Bolanthus, Ankyropetalum* and *Phryna. Wenti.,* **9**, 1-203.

Benzie IF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **239,** 70-76.

Blainski A, Lopes GC, De Mello JCP. 2013. Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense* L. *Molecules* **18**, 6852-6865. https://doi.org/10.3390/molecules18066852

Brand-Williams W, Cuvelier ME, Berset CLWT. 1995. Use of a free radical method to evaluate antioxidant 45 activity. *LWT-Food Sci. Technol.* **28**, 25-30.

Carlson KD, Kleiman R. 1993. Chemical survey and erucic acid content of commercial varieties of nasturtium, *Tropaeolum majus* L. *J. Am. Oil Chem. Soc.* **70**, 1145-1148.

Carvalho ISD, Miranda I, Pereira H. 2006. Evaluation of oil composition of some crops suitable for human nutrition. *Ind. Crop Prod.* **24**, 75-78. https://doi.org/10.1016/j.indcrop.2006.03.005

Chang CC, Yang MH, Wen HM, Chern JC. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **10**, 178-182.

Chima NK, Nahar L, Majinda RR, Celik S, Sarker SD. 2014. Assessment of free-radical scavenging activity of *Gypsophila pilulifera*: assay-guided isolation of verbascoside as the main active component. *Rev. Bras. Farmacogn.* **24**, 38-43. https://doi.org/10.1590/0102-695X20142413391

Collins CH, Lyne PM, Grange JM. 1989. Collins and Lyne's Microbiological Methods, Sixth Edition, Butterworths Co. Ltd. London.

Davis PH. 1982. Flora of Turkey and East Eagean Island, Vol 7, Edinburg at the University Press.

Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P, Vidal N. 2006. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food Chem*. **97**, 654-660. https://doi.org/10.1016/j.foodchem.2005.04.028

Dudonne S, Vitrac X, Coutiere P, Woillez M, Mérillon JM. 2009. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *J. Agric. Food Chem.* **57**, 1768-1774. https://doi.org/10.1021/jf803011r

Efe E, Bek Y, Sahin M. 2000. SPSS'te çözümleri ile istatistik yöntemler II, Kahramanmaraş Sütçü İmam Üniversitesi Rektörlüğü Yayınları, Kahramanmaraş.

Ekim T, Koyuncu M, Vural M, Duman H, Aytac Z, Adiguzel N. 2000. Türkiye Bitkileri Kırmızı Kitabı (Red Data Book of Turkish Plants), Türkiye Tabiatını Koruma Derneği, Ankara.

Elias PM. 1983. Epidermal lipids, barrier function, and desquamation. *J. Invest. Dermatol.* **80**, 44-49.

Finley JW, Shahidi F. 2001. Introduction-1 The Chemistry, Processing, and Health Benefits of Highly Unsaturated Fatty Acids: An Overview, In *ACS Symposium Series* Washington, DC: American Chemical Society **788**, 2-13.

Guo Y, Mietkiewska E, Francis T, Katavic V, Brost JM, Giblin M. Taylor DC. 2009. Increase in nervonic acid content in transformed yeast and transgenic plants by introduction of a *Lunaria annua* L. 3-ketoacyl-CoA synthase (KCS) gene. *Plant Mol. Biol.* **69**, 565-575. https://doi.org/10.1007/s11103-008-9439-9

Huang D, Ou B, Prior RL. 2000. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **53**, 1841-1856. https://doi.org/10.1021/jf030723c

Isık M, Korkmaz M, Bursal E, Gulcin I, Koksal E, Tohma H. 2015. Determination of antioxidant properties of *Gypsophila bitlisensis* bark. *Int. J. Pharmacol.* **11**, 366-371.

Katavic V, Mietkiewska E, Taylor DC, Guo Y, Brost JM. 2012. *Lunaria annua*, *Cardamine graeca* and *Teesdalia nudicaulis* fae Genes and their Use in Producing Nervonic and Eicosenoic Acids in Seed Oils. *U.S.* Patent No. 8,269,062, Washington, DC: U.S. Patent and Trademark Office.

Korkmaz M, Ozcelik H. 2011. Economic importance of *Gypsophila* L., *Ankyropetalum* Fenzl and *Saponaria* L. (Caryophyllaceae) taxa of Turkey. *Afr. J. Biotechnol.* **10,** 9533-9541. http://dx.doi.org/10.5897/AJB10.2500

Krishnan, H. 2009. (Ed.), Modification of seed composition to promote health and nutrition (No. 51). American Society of Agronomy, Crop Science Society of America, Soil Science Society of America.

Lim PO, Kim HJ, Gil Nam H. 2007. Leaf senescence. Annu. *Rev. Plant Biol.* **58**, 115-136. https://doi.org/10.1146/annurev.arplant.57.032905.105316

Locatelli M, Zengin G, Uysal A, Carradori S, De Luca E, Bellagamba G, Lazarova I. 2017. Multicomponent pattern and biological activities of seven Asphodeline taxa: potential sources of natural-functional ingredients for bioactive formulations. *J. Enzym. Inhib. Med. Ch.* **32**, 60-67. https://doi.org/10.1080/14756366.2016.1235041

Mocan A, Zengin G, Mollica A, Uysal A, Gunes E, Crisan G, Aktumsek A. 2018. Biological effects and chemical characterization of *Iris schachtii* Markgr. extracts: A new source of bioactive constituents. *Food Chem. Toxicol.* **112**, 448-457. https://doi.org/10.1016/j.fct.2017.08.004

Mohanty BP, Bhattacharjee S, Paria P, Mahanty A, Sharma AP. 2013. Lipid biomarkers of lens aging. *Appl. Biochem. Biotech.* **169**, 192-200. https://doi.org/10.1007/s12010-012-9963-6

Napier JA, Graham IA. 2010. Tailoring plant lipid composition: designer oilseeds come of age. *Curr. Opin. Plant Biol.* **13**, 329-336. https://doi.org/10.1016/j.pbi.2010.01.008

Nikolova M, Evstatieva L, Nguyen TD. 2011. Screening of plant extracts for antioxidant properties. *Bot. Serb.* **35**, 43-48.

Ozcelik H, Muca B. 2010. *Ankyropetalum* Fenzl (Caryophyllaceae) cinsine ait türlerin Türkiye'deki yayılışı ve habitat özellikleri, *BIBAD*. **3**, 47-56.

Sandhir R, Khan M, Chahal A, Singh I. 1998. Localization of nervonic acid β-oxidation in human and rodent peroxisomes: impaired oxidation in Zellweger syndrome and X-linked adrenoleukodystrophy. *J. Lipid Res.* **39**, 2161-2171.

Sargent JR, Coupland K, Wilson R. 1994. Nervonic acid and demyelinating disease. *Med. Hypotheses.* **42**, 237-242.

Stankovic MS, Petrović M, Godjevac D, Stevanović ZD. 2015. Screening inland halophytes from the central Balkan for their antioxidant activity in relation to total phenolic compounds and flavonoids: Are there any prospective medicinal plants? *J. Arid. Environ.* **120**, 26-32. https://doi.org/10.1016/j.jaridenv.2015.04.008

Stickler DJ, King JB. 1992. Bacterial sensitivity and resistance. Intrinsic resistance. In: Russell AD, Hugo WB, Ayliffe GAJ, (eds). Principles and practice of disinfection, preservation and sterilisation, Oxford: Blackwell Scientific Publications.

Tang TF, Liu XM, Ling M, Lai F, Zhang L, Zhou YH, Sun RR. 2013. Constituents of the essential oil and fatty acid from *Malania oleifera. Ind. Crop. Prod.* **43**, 1-5. https://doi.org/10.1016/j.indcrop.2012.07.003

Taylor, DC. 2010. New very long-chain fatty acid seed oils produced through introduction of strategic genes into *Brassica carinata. Inform* **21**, 602-605.

Teres S, Barceló-Coblijn G, Benet M, Alvarez R, Bressani R, Halver JE, Escribá PV. 2008. Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. *P. Natl. Acad. Sci. Usa.* **105**, 13811-13816.

Van Acker SA, Tromp MN, Griffioen DH, Van Bennekom WP, Van Der Vijgh WJ, Bast A. 1996. Structural aspects of antioxidant activity of flavonoids. *Free Radical Bio. Med.* **20**, 331-342.

Van Immerseel F, Boyen F, Gantois I, Timbermont L, Bohez L, Pasmans F, Ducatelle R. 2005. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. *Poultry Sci.* **84**, 1851-1856. https://doi.org/10.1093/ps/84.12.1851

Wang XY, Fan JS, Wang SY, Sun RC. 2006. A new resource of nervonic acid from purpleblow maple (*Acer truncatum*) seed oil. *Forest Prod. J.* **56**, 147-150.

Yazici SO, Ozmen I. 2017. Effect of the Crude Saponin Extract from *Gypsophila pilulifera* Boiss. Heldr. on Protease from Bacillus subtilis ATCC 6633 and Antioxidant Properties of the Extract. *Iran J. Sci. Technol.* **45**, 1-7. https://doi.org/10.1007/s40995-017-0366-y

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New insights into the antifungal activity of lactic acid bacteria isolated from different food matrices

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SUMMARY: The anti-mold activity of 397 strains of lactic acid bacteria was evaluated using both the spot method in Petri plates and co-culture in liquid medium. The study led to the selection of 34 strains isolated from table olives or olive brines, 15 strains from dairy products, and 10 strains from sourdoughs, all able to inhibit a strain of *Penicillium crustosum* and/or a strain of *Aspergillus* section *Nidulantes*, prevailing in two Calabrian olive brines. Seven representative strains were identified as *Lactobacillus pentosus* (four strains) and *Lactobacillus sanfranciscensis* (three strains) and are currently under testing for their antifungal activity during table olive fermentation. This research constitutes an initial contribution to the control of fungal growth and mycotoxin accumulation during table olive fermentation. The selected strains could be used as adjunct cultures in table olive fermentation, allowing for the biological control of table olive safety.

KEYWORDS: *Antifungal activity;* Aspergillus *section* Nidulantes*; Lactic acid bacteria;* Penicillium*; Table olive fermentation*

RESUMEN: ***Nuevos conocimientos sobre la actividad antifúngica de las bacterias del ácido láctico aisladas de diferentes matrices alimentarias.*** La actividad antimoho de 397 bacterias del ácido láctico se evaluó utilizando tanto el método puntual en placas de Petri como el co-cultivo en medio líquido. El estudio condujo a la selección de 34 cepas aisladas de aceitunas de mesa o salmueras de oliva, 15 cepas de productos lácteos y 10 cepas de masa madre, todas capaces de inhibir una cepa de *Penicillium crustosum* y/o una cepa de *Aspergillus* sección *Nidulantes*, que prevalecen en dos salmueras de aceituna de Calabria. Se identificaron siete cepas representativas como *Lactobacillus pentosus* (cuatro cepas) y *Lactobacillus sanfranciscensis* (tres cepas) y actualmente se están probando su actividad antifúngica durante la fermentación de aceituna de mesa. Esta investigación constituye una primera contribución para controlar el crecimiento de hongos y la acumulación de micotoxinas durante la fermentación de aceitunas de mesa. Las cepas seleccionadas podrían usarse como cultivos adjuntos en la fermentación de aceitunas de mesa.

PALABRAS CLAVE: *Actividad antifúngica;* Aspergillus *sección* Nidulantes*; Bacterias de ácido láctico; Fermentación de aceituna de mesa;* Penicillium

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1. INTRODUCTION

In Mediterranean countries, table olives are among the most commonly consumed fermented food. They are considered a functional food due to their nutritional value, content in bioactive compounds and dietary fiber, fatty acid composition and presence of several antioxidants (Campus *et al.,* 2018).

In table olive fermentation, mold growth can cause huge economic losses and reduce the product safety (El Adlouni *et al.,* 2006; Ghitakou *et al.,* 2006). Molds of the genera *Aspergillus* and *Penicillium* have been described in different olive fermentation processes (Heperkan *et al.,* 2006) and both are considered undesirable microorganisms. They can produce mycotoxins and cause the softening of fruits because of their cellulase and xylanase activities (Arroyo-López *et al.,* 2016). *Penicillium crustosum* is one of the most prevalent molds in fermented black table olives and is able to produce toxic metabolites such as dehydrocyclopeptin, andrastin A, cyclopenol, penitrem A, roquefortine C, viridicatol (Bavaro *et al.,* 2017) and thomitrem A and E (Rundberget and Wilkins, 2002). *Aspergillus* section *Nidulantes* includes several species able to produce mycotoxins, like aflatoxins, sterigmatocystin, emestrin, fumitremorgins, asteltoxins, and paxillin; four species (*A. astellatus*, *A. miraensis*, *A. olivicola*, *A. venezuelensis*) produce aflatoxin B1 (Chen *et al.,* 2016).

Different chemical, physical and biological methods have been proposed to prevent mold growth or to eliminate or reduce aflatoxins during table olive fermentation. For example, the spoilage of table olive by molds can be reduced using chemical preservatives, such as potassium sorbate and sodium benzoate (Turantaş *et al.,* 1999) or natamycin (Hondrodimou *et al.,* 2011). Değirmencioğlu *et al.* (2014) studied the effect of washing solutions -acetic acid, lactic acid, chlorine dioxide- on dry-salted olives during 7 months of storage at 4 °C. The chlorine dioxide solution (10 ppm) combined with vacuum packaging was the best approach for controlling mold and yeast growth. In addition, the effectiveness of high hydrostatic pressures and citrinin against mold growth in table olives has been demonstrated (Tokuşoğlu *et al.,* 2010; Argyri *et al.,* 2014).

In view of growing consumer concern for food safety issues, including additive content (Bearth *et al.,* 2014), the food industry is seeking biological alternatives in order to inhibit undesirable molds. Among the novel approaches, biopreservation and, more specifically, the selection of protective cultures has been identified as one of the more promising alternatives.

It is well known that lactic acid bacteria (LAB) can produce several antifungal metabolites (Schnürer and Magnusson, 2005). LAB are able to inhibit molds related to bread spoilage (Corsetti *et al.,* 1998); consequently, the use of antifungal sourdough in the bakery industry is now a common practice to ensure the microbiological safety of bread (Gerez *et al.,* 2009). Antifungal lactobacilli can also be found in raw milk (Delavenne *et al.,* 2012); therefore, specifically selected LAB strains can also be used as biopreservatives in fresh cheeses (Fernandez *et al.,* 2017). The antifungal activity of LAB, specifically *Lactobacillus plantarum*, and their ability to reduce aflatoxin B1 during olive storage has also been demonstrated (Kachouri *et al.,* 2014).

Considering the state of the art, the aim of the present study was to find LAB able to inhibit molds commonly associated with table olive fermentation and spoilage.

2. MATERIALS AND METHODS

2.1. Microorganisms

Three hundred ninety-seven strains of LAB from the Collection of the Laboratory of Microbiology (Department of Agraria, *Mediterranea* University of Reggio Calabria, Reggio Calabria, Italy) were used. All the strains were previously classified to genus level, according to Cogan *et al.,* (1997). Each strain was tested for its Gram reaction, catalase using 3 g H_2O_2/L, shape by observation of overnight cultures using a phase contrast microscope Standard 20 (Carl Zeiss, Göttingen, Germany), and for the heterofermentation or homofermentation of sugars (Abd-el-Malek and Gibson, 1948). The growth of coccal-shaped bacteria was examined in M17 broth after incubation at 10 °C for 7 days, at 45 °C for 2 days, and in M17 broth containing 20, 40, and 65 g NaCl/L after incubation at 30 °C for 4 days. The LAB was tested against two mold strains -one *Penicillium* and one *Aspergillus*- prevailing in two different Calabrian olive brines. *Penicillium* was identified as *Penicillium crustosum* according to

Visagie *et al.,* (2014); *Aspergillus* was included in the section *Nidulantes* according to Chen *et al.,* (2016). Out the 397 strains of LAB, 198 strains were isolated from table olives or olive brines, 115 strains from dairy products, and 84 strains from sourdough.

Finally, the seven more representative LAB were identified as *Lactobacillus pentosus* (four strains) and *Lactobacillus sanfranciscensis* (three strains) by molecular methods. DNA was extracted by the InstaGene matrix (Bio-Rad) from LAB and subjected to amplification using specific primers (Young *et al.,* 1991). LAB identification was performed with PCR-ARDRA, comparing the profile isolates to those previously described in the literature and to International Collection reference strains (Aquilanti *et al.,* 2007; Torriani *et al.,* 2001).

2.2. Preliminary screening

The LAB were screened for their antagonistic activity using the agar spot method (Spelhaug and Harlander, 1989) with some modifications. All the strains were stored at −80 °C using a cryopreservative bead storage system Microbank TM (Pro-Lab Diagnostics, Canada). The LAB were cultured in a deMan Rogosa Sharpe (MRS) broth at 30 °C for 48 hours. Subsequently, 0.1 ml of each culture were inoculated in MRS agar plates (60 mm), incubated at 30 °C for 48 hours. For each strain, the biomass was collected using a sterile loop and spotted in triplicate in MRS agar plates (90 mm). Then, the plates were overlaid with 10 ml of Yeast Extract Peptone Dextrose agar (agar 0.7%) containing 0.1 ml of an abundant spore suspension of each mold. After 72 h at 30 °C, the plates were checked for the presence of inhibition zones around the spots of each LAB.

2.3. Test for antifungal activity of LAB by co-culture in a liquid medium

The LAB that exhibited antagonistic activity with the agar spot method were tested by co-culture in a liquid medium against three serial dilutions of spore suspensions of the two molds. *Penicillium crustosum* and *Aspergillus* section *Nidulantes* were inoculated in MRS agar plates and incubated at 30 °C for 48 h in order to test their ability to grow in this medium and adapt them to the subsequent conditions. Spore suspensions were prepared from the same plates as described above and diluted at 1:10, 1:100 and 1:1000 in sterile physiological solution. Co-cultures were prepared by inoculating 0.1 ml of pre-culture of each LAB in tubes containing 10 ml of MRS broth; 0.1 ml of each dilution of the spore suspensions was added. The tubes were incubated at room temperature for sixteen days and the antagonistic activity was evaluated based on the absence of mold growth at the top of the MRS broth.

3. RESULTS

3.1. Preliminary screening

One hundred and fifteen strains (28.97% of the total strains tested) exhibited antagonistic activity in the agar spot method: 60 strains isolated from table olives or olive brines, 20 dairy strains and 35 sourdough strains. The strains which were isolated from the same sample, exhibited the same cellular morphology, and showed an identical antagonistic activity were considered duplicates. Consequently, the 115 strains were reduced to 59:34 strains isolated from table olives or olive brines (listed in Table 1), 15 dairy strains (listed in Table 2), and 10 sourdough strains (listed in Table 3).

3.2. Test for antifungal activity of LAB by co-culture in a liquid medium

The antagonistic activity of the 59 pre-selected strains of LAB against the two molds was studied by co-culture in a liquid medium at three different concentrations of the two molds; all strains exhibited good antagonistic activity against one or both tested molds (Figure 1). When the LAB were inoculated together with the less diluted spore suspensions (1:10), 58 strains (98.3% of all 59 LAB) exhibited antagonistic activity against *Penicillium crustosum* and 57 (96.6% of all 59 LAB) against *Aspergillus* section *Nidulantes* after sixteen days. All the LAB strains were able to inhibit the growth of *Penicillium crustosum* at the spore suspension 1:100; 58 strains (98.3% of all 59 LAB) inhibited the growth of *Aspergillus* section *Nidulantes* at the same concentration. All the LAB tested inhibited the growth of both molds at the most diluted (1:1000) spore suspensions after sixteen days.

TABLE 1. List of the 34 lactic acid bacteria isolated from olives and exhibiting antagonistic activity

Lactic acid bacteria			Activity against olive moulds	
Strain	**Sample**	**First identification**	***Penicillium spp.***	***Aspergillus spp.***
B 200	Olive_1	*Lactobacillus spp.*	+	-
B 212	Olive brine_18	*Lactobacillus spp.*	+	-
B 221	Olive_2	*Lactobacillus spp.*	+	-
B 229	Olive_16	*Lactobacillus spp.*	+	-
B 248	Olive brine_5	*Lactobacillus spp.*	+	-
B 283	Olive brine_13	*Lactobacillus spp.*	-	+
B 284	Olive_14	*Lactobacillus spp.*	-	+
B 318	Olive brine_12	*Lactobacillus spp.*	+	-
B 337	Olive brine_11	*Lactobacillus spp.*	+	-
B 348	Olive_2015_C_15gg	*Lactobacillus spp.*	+	-
B 350	Olive_2015_D_15gg	*Lactobacillus spp.*	+	-
B 354	Olive_2015_E_15gg	*Lactobacillus spp.*	+	-
B 358	Olive_2015_G_15gg	*Lactobacillus spp.*	+	-
B 364	Olive_2015_L_15gg	*Lactobacillus spp.*	+	-
B 366	Olive_2015_N_15gg	*Lactobacillus spp.*	+	+
B 384	Olive_2015_B_30gg	*Lactobacillus spp.*	+	-
B 386	Olive_2015_C_30gg	*Lactobacillus spp.*	-	+
B 389	Olive_2015_D_30gg	*Lactobacillus spp.*	+	+
B 391	Olive brine_MF3_67gg	*Lactobacillus spp.*	+	+
B 524	Olive_2015_G1_240gg	*Lactobacillus spp.*	+	-
B 525	Olive_2015_G2_240gg	*Lactobacillus spp.*	+	-
B 526	Olive_2015_H1_240gg	*Lactobacillus spp.*	+	-
B 527	Olive_2015_H2_240gg	*Lactobacillus spp.*	+	-
B 529	Olive_2015_I1_240gg	*Lactobacillus spp.*	+	-
B 531	Olive_2015_L1_240gg	*Lactobacillus spp.*	+	-
B 534	Olive_2015_M1_240gg	*Lactobacillus spp.*	+	-
B 536	Olive_2015_N1_240gg	*Lactobacillus spp.*	+	-
B 539	Olive_2015_O1_240gg	*Lactobacillus spp.*	+	-
B 540	Olive_2015_O2_240gg	*Lactobacillus spp.*	+	-
B 542	Olive_2015_E1_240gg	*Lactobacillus spp.*	+	-
B 543	Olive_2015_M1_240gg	*Lactobacillus spp.*	+	-
B 545	Olive_2015_D1_240gg	*Lactobacillus spp.*	+	-
B 546	Olive_2015_B1_240gg	*Lactobacillus spp.*	+	-
B 560	Olive brine_2016	*Lactobacillus spp.*	+	-

3.3. LAB identification

Among the 34 LAB listed in Table 1, 91.2% of them exhibited antagonistic activity against *Penicillium crustosum* but only 17.6% exhibited antagonistic activity against *Aspergillus* section *Nidulantes*. The following three strains are representative of the three typologies of behavior against the two molds:

- strain B221, identified as *Lactobacillus pentosus*, which exhibited antagonistic activity against *Penicillium crustosum*.
- strain B283, identified as *Lactobacillus pentosus*, which exhibited antagonistic activity against *Aspergillus* section *Nidulantes*.
- strain B391, identified as *Lactobacillus pentosus*, which exhibited antagonistic activity against both strains of mold.

All 15 LAB listed in Table 2 exhibited antagonistic activity against *Penicillium crustosum* but none of them exhibited antagonistic activity against *Aspergillus* section *Nidulantes*. The strain B167, identified as *Lactobacillus pentosus*, is representative of this typology of behavior against the two molds.

Among the 10 LAB listed in Table 3, 90% exhibited antagonistic activity against *Penicillium crustosum* but only 10% exhibited antagonistic activity against *Aspergillus* section *Nidulantes*. The following three strains are representative of the three typologies of behavior against the two molds:

1. Strain B426, identified as *Lactobacillus sanfranciscensis*, which exhibited antagonistic activity against *Penicillium crustosum*.
2. Strain B551, identified as *Lactobacillus sanfranciscensis*, which exhibited antagonistic activity against the strain of *Aspergillus* section *Nidulantes*.
3. Strain B511, identified as *Lactobacillus sanfranciscensis*, which exhibited antagonistic activity against both strains of mold.

The seven representative strains of LAB are currently under testing for their antifungal activity during table olive fermentation (unpublished data).

4. DISCUSSION

In the present study, 59 LAB exhibited antifungal activity. Both in agar and in co-culture, the LAB exhibited greater inhibitory activity against *Penicillium crustosum* compared to *Aspergillus* section *Nidulantes*. The antifungal

TABLE 2. List of the 15 dairy lactic acid bacteria exhibiting antagonistic activity

Lactic acid bacteria			Activity against olive moulds	
Strain	**Sample**	**First identification**	***Penicillium spp.***	***Aspergillus spp.***
B 3	PP_A03_1	*Lactobacillus spp.*	+	-
B 15	PP_M3	*Lactobacillus spp.*	+	-
B 17	PP_P4	*Lactobacillus spp.*	+	-
B 27	PP_A03_2	*Lactobacillus spp.*	+	-
B 28	PP_A03_3	*Lactobacillus spp.*	+	-
B 54	UK_TH1	*Lactobacillus spp.*	+	-
B 59	UK_SR1	*Lactobacillus spp.*	+	-
B 62	UK_WB1	*Lactobacillus spp.*	+	-
B 76	RAF_M1	*Lactobacillus spp.*	+	-
B 79	PM_M2	*Lactobacillus spp.*	+	-
B 147	M_DN1	*Lactobacillus spp.*	+	-
B 164	M_FP1	*Lactobacillus spp.*	+	-
B 167	P_M3	*Lactobacillus spp.*	+	-
B 172	CC_4	*Lactobacillus spp.*	+	-
B 179	MC_5	*Lactobacillus spp.*	+	-

TABLE 3. List of the 10 lactic acid bacteria isolated from sourdough and exhibiting antagonistic activity

Lactic acid bacteria			Activity against olive moulds	
Strain	**Sample**	**First identification**	***Penicillium spp.***	***Aspergillus spp.***
B 426	Sourdough_SC_SA1	*Lactobacillus spp.*	+	-
B 435	Sourdough_CL_IF1	*Leuconostoc spp.*	+	-
B 455	Sourdough_CL_IF2	*Lactobacillus spp.*	+	-
B 470	Sourdough_CZ_IFAL1	*Pediococcus spp.*	+	-
B 481	Sourdough_CZ_IFAL2	*Lactobacillus spp.*	+	-
B 489	Sourdough_RC_LSDMA1	*Lactobacillus spp.*	+	-
B 503	Sourdough_VV_CF1	*Lactobacillus spp.*	+	-
B 511	Sourdough_GI_C1	*Lactobacillus spp.*	+	+
B 551	Sourdough_VV_SF1	*Lactobacillus spp.*	-	+
B 553	Sourdough_VV_LS1	*Lactobacillus spp.*	+	-

LAB strains so identified belong to two species (*Lactobacillus pentosus* and *Lactobacillus sanfranciscensis*), whose ability to inhibit or reduce mold growth is well known (Corsetti *et al.,* 1998; Schnürer and Magnusson, 2005).

The antifungal activity of LAB is related to their ability to produce antifungal metabolites, e.g. organic acids, proteinaceous compounds, reuterin, and 3-hydroxylated fatty acids (Schnürer and Magnusson, 2005). For example, the inhibitory properties of phenyllactic acid (PLA) produced by LAB against several fungal species isolated from food have been demonstrated (Valerio *et al.,* 2004). Regarding proteinaceous compounds, bacteriocin-producing LAB were already isolated from fermented olives and it was observed that their ability to produce bacteriocins was affected by NaCl, pH and temperature (Hurtado *et al.,* 2011). Moreover, the bacteriocin production by LAB seemed to be affected by the presence of other bacteria during olive fermentation (Ruiz-Barba *et al.,* 2010).

The antifungal activity of LAB could also be due to a synergic effect between the sodium acetate present in the MRS medium and lactic acid and other compounds produced by LAB (Cabo *et al*., 2002). Schillinger and Villareal (2010) demonstrated how sodium acetate in a culture medium can influence the inhibitory activity of LAB; in their experiment, LAB which exhibited antifungal activity in MRS agar with sodium acetate did not have the capacity to inhibit molds in MRS agar without sodium acetate. Also, Cheong *et al*., (2014) reported that LAB which exhibited antifungal activity in MRS agar with sodium acetate did not exhibit the same behavior in MRS agar without sodium acetate; however, the same strains were able to inhibit *Penicillium commune* in cottage cheese.

Lind *et al*., (2005) demonstrated how the absence of sodium acetate in MRS agar did not influence the antifungal activity of *Propionibacterium* against *Penicillium roqueforti* and *Aspergillus fumigatus*. Magnusson *et al*., (2003) performed an HPLC analysis of the supernatants of the LAB exhibiting antifungal activity, previously tested in MRS broth, and the concentration of lactic acid was equal to or even higher than concentrations in strains devoid of inhibitory activity; in addition, the concentration of acetic acid was similar to that found in MRS broth. This demonstrated that the activity was probably due to the production of other antifungal compounds.

In the present study we decided to carry out the tests using MRS with sodium acetate. *Penicillium crustosum* and *Aspergillus* section *Nidulantes* grew well in all the control samples, so the inhibition was probably due to the production of antifungal substances by LAB.

5. CONCLUSIONS

In our opinion, this study makes a useful contribution to solving the problem of fungal growth and potential mycotoxin accumulation during table olive fermentation, thereby improving its safety.

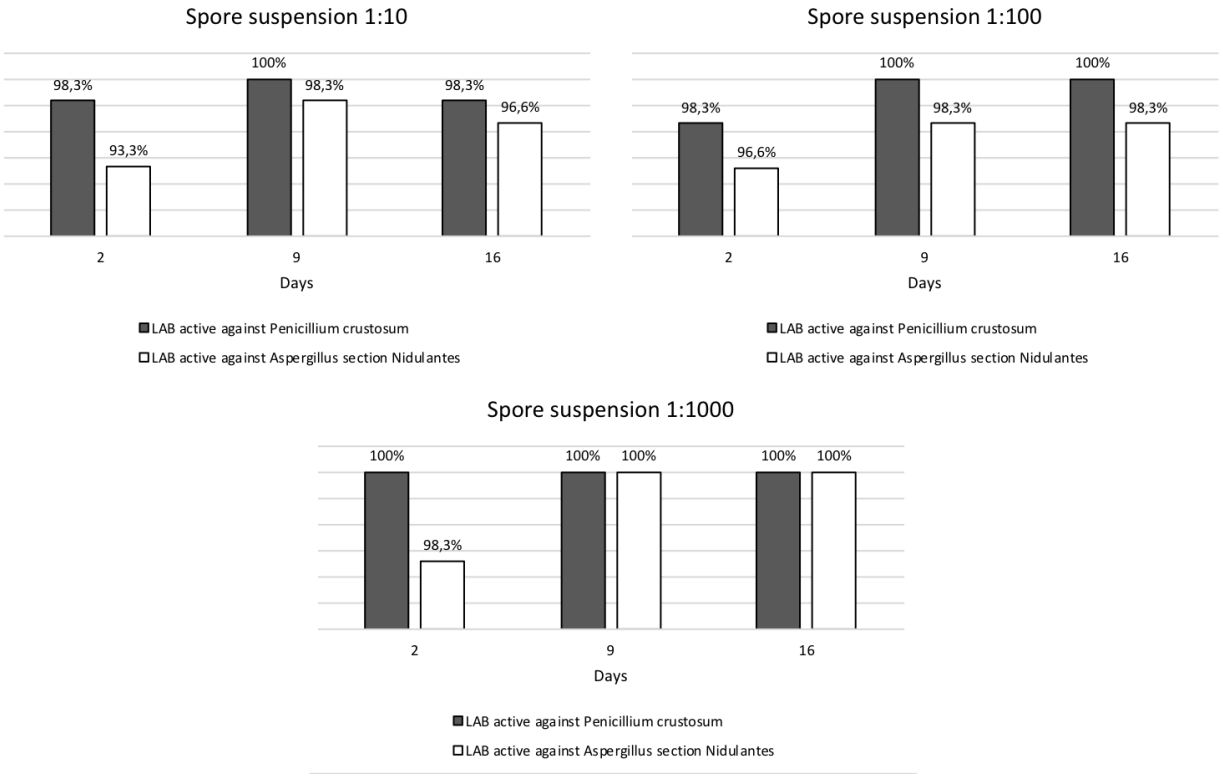

FIGURE 1. Percentage of the selected 59 strains of lactic acid bacteria that confirmed their activity against *Penicillium crustosum* in liquid medium co-culture (■) and *Aspergillus* section *Nidulantes* (□) at the three concentrations of spore suspension.

Summarizing:

Almost 400 strains of LAB were screened for anti-mold activity.

One strain of *Penicillium crustosum* and one strain of *Aspergillus* section *Nidulantes* were used;

Almost 60 strains of LAB resulted in the ability to inhibit one or both molds.

The antagonistic activity was evaluated both using the spot method and by co-culture in liquid medium.

Starting from the present results, a consortium of the best anti-mold LAB could be tested in table olive fermentation.

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REFERENCES

Abd-el-Malek Y, Gibson T. 1948. Studies in the bacteriology of milk: I. The streptococci of milk. *J. Dairy Res.* **15**, 233−248. https://doi.org/10.1017/S0022029900005082

Aquilanti L, Silvestri G, Zannini E, Osimani A, Santarelli S, Clementi F. 2007. Phenotypic, genotypic and technological characterization of predominant lactic acid bacteria in Pecorino cheese from central Italy. *J. Appl. Microbiol.* **103**, 948−960. https://doi.org/10.1111/j.1365-2672.2007.03513.x

Argyri AA, Panagou EZ, Nychas G-JE, Tassou CC. 2014. Nonthermal pasteurization of fermented green table olives by means of high hydrostatic pressure processing. *Biomed. Res. Int.* ID 515623. https://doi.org/10.1155/2014/515623

Arroyo-López FN, Medina E, Ruiz-Bellido MÁ, Romero-Gil V, Montes-Borrego M, Landa BB. 2016. Enhancement of the knowledge on fungal communities in directly brined Aloreña de Málaga green olive fermentations by metabarcoding analysis. *PLoS One* **11** (9), e0163135. https://doi.org/10.1371/journal.pone.0163135

Bavaro SL, Susca A, Frisvad JC, Tufariello M, Chytiri A, Perrone G, Mita G, Logrieco AF, Bleve G. 2017. Isolation, characterization, and selection of molds associated to fermented black table olives. *Front. Microbiol.* https://doi.org/10.3389/fmicb.2017.01356

Bearth A, Cousin ME, Siegrist M. 2014. The consumer's perception of artificial food additives: Influences on acceptance, risk and benefit perceptions. *Food Qual. Prefer.* **38,** 14−23. https://doi.org/10.1016/j.foodqual.2014.05.008

Cabo ML, Braber AF, Koenraad PM. 2002. Apparent antifungal activity of several lactic acid bacteria against *Penicillium discolor* is due to acetic acid in the medium. *J. Food Prot.* **65,** 1309−1316. https://doi.org/10.4315/0362-028x-65.8.1309

Campus M, Değirmencioğlu N, Comunian R. 2018. Technologies and trends to improve table olive quality and safety. *Front. Microbiol.* https://doi.org/10.3389/fmicb.2018.00617

Chen AJ, Frisvad JC, Sun BD, Varga J, Kocsubé S, Dijksterhuis J, Kim DH, Hong SB, Houbraken J, Samson RA. 2016. *Aspergillus* section *Nidulantes* (formerly *Emericella*): polyphasic taxonomy, chemistry and biology. *Stud. Mycol.* **84**, 1−118. https://doi.org/10.1016/j.simyco.2016.10.001

Cheong EYL, Sandhu A, Jayabalan J, Le TTK, Nhiep NT, Ho HTM, Zwielehner J, Bansal N, Turner MS. 2014. Isolation of lactic acid bacteria with antifungal activity against the common cheese spoilage mould *Penicillium commune* and their potential as biopreservatives in cheese. *Food Control* **46**, 91−97.

Cogan TM, Barbosa M, Beuvier E, Bianchi-Salvadori B, Cocconcelli PS, Fernandes I, Gomez J, Gomez R, Kalantzopoulos G, Ledda A, Medina M, Rea MC, Rodriguez E. 1997. Characterization of the lactic acid bacteria in artisanal products. *J. Dairy Res.* **64**, 409−421. https://doi.org/10.1017/S0022029997002185

Corsetti A, Gobbetti M, Rossi J, Damiani P. 1998. Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. *Appl. Microbiol. Biotechnol.* **50**, 253−256. https://doi.org/10.1007/s002530051285

Değirmencioğlu N, Gürbüz O, Değirmencioğlu A, Yildiz S. 2014. Effect of pretreatments on microbial growth and sensory properties of dry-salted olives. *J. Food Prot.* **77**, 1527−1537. https://doi.org/10.4315/0362-028X.JFP-14-036

Delavenne E, Mounier J, Déniel F, Barbier G, Le Blay G. 2012. Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period. *Int. J. Food Microbiol.* **155**, 185−190. https://doi.org/10.1016/j.ijfoodmicro.2012.02.003

El Adlouni C, Tozlovanu M, Naman F, Faid M, Pfohl-Leszkowicz A. 2006. Preliminary data on the presence of mycotoxins (ochratoxin A, citrinin and aflatoxin B1) in black table olives "Greek style" of Moroccan origin. *Mol. Nutr. Food Res.* **50**, 507−512. https://doi.org/10.1002/mnfr.200600055

Fernandez B, Vimont A, Desfossés-Foucault E, Daga M, Arora G, Fliss I. 2017. Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese. *Food Control* **78**, 350−356. https://doi.org/10.1016/j.foodcont.2017.03.007

Gerez CL, Torino MI, Rollán G, Font de Valdez G. 2009. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control* **20**, 144−148. https://doi.org/10.1016/j.foodcont.2008.03.005

Ghitakou S, Koutras K, Kanellou E, Markaki P. 2006. Study of aflatoxin B_1 and ochratoxin A production by natural microflora and *Aspergillus parasiticus* in black and green olives of Greek origin. *Food Microbiol.* **23**, 612−621. https://doi.org/10.1016/j.fm.2005.12.008

Heperkan D, Meric BE, Sismanoglu G, Dalkiliç G, Güler FK. 2006. Mycobiota, mycotoxigenic fungi, and citrinin production in black olives. *Adv. Exp. Med. Biol.* **571**, 203−210. https://doi.org/10.1007/0-387-28391-9_13

Hondrodimou O, Kourkoutas Y, Panagou EZ. 2011. Efficacy of natamycin to control fungal growth in natural black olive fermentation. *Food Microbiol.* **28**, 621−627. https://doi.org/10.1016/j.fm.2010.11.015

Hurtado A, Ben Othman N, Hamdi M, Ferrer S, Reguant C, Bordons A, Rozès N. 2011. Characterization of *Lactobacillus* isolates from fermented olives and their bacteriocin gene profiles. *Food Microbiol.* **28**, 1514−1518. https://doi.org/10.1016/j.fm.2011.07.010

Kachouri F, Ksontini H, Hamdi M. 2014. Removal of aflatoxin B1 and inhibition of *Aspergillus flavus* growth by the use of *Lactobacillus plantarum* on olives. *J. Food Prot.* **77**, 1760−1767. https://doi.org/10.4315/0362-028X.JFP-13-360

Lind H, Jonsson H, Schnürer J. 2005. Antifungal effect of dairy propionibacteria - contribute on of organic acids. *Int. J. Food Microbiol.* **98**, 157−165. https://doi.org/10.1016/j.ijfoodmicro.2004.05.020

Magnusson J, Ström K, Roos S, Sjögren J, Schnürer J. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiol. Lett.* **219**, 129−135. https://doi.org/10.1016/S0378-1097(02)01207-7

Ruiz-Barba JL, Caballero-Guerrero B, Maldonado-Barragán A, Jiménez-Díaz R. 2010. Coculture with specific bacteria enhances survival of *Lactobacillus plantarum* NC8, an autoinducer-regulated bacteriocins producer, in olive fermentations. *Food Microbiol.* **27**, 413−417. https://doi.org/10.1016/j.fm.2009.10.002

Rundberget T, Wilkins AL. 2002. Thomitrems A and E, two indole-alkaloid isoprenoids from *Penicillium crustosum* Thom. *Phytochemistry* **61**, 979−985. https://doi.org/10.1016/s0031-9422(02)00369-2

Schillinger U, Villarreal JV. 2010. Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods. *Food Control* **21**, 107−111. https://doi.org/10.1016/j.foodcont.2008.11.010

Schnürer J, Magnusson J. 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci. Technol.* **16,** 70−78. https://doi.org/10.1016/j.tifs.2004.02.014

Spelhaug SR, Harlander SK. 1989. Inhibition of foodborne bacterial pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceus*. *J. Food Prot.* **52**, 856−862. https://doi.org/10.4315/0362-028X-52.12.856

Tokuşoğlu Ö, Alpas H, Bozoğlu F. 2010. High hydrostatic pressure effects on mold flora, citrinin mycotoxin, hydroxytyrosol, oleuropein phenolics and antioxidant activity of black table olives. *Innov. Food Sci. Emerg. Technol.* **11**, 250−258. https://doi.org/10.1016/j.ifset.2009.11.005

Torriani S, Felis GE, Dellaglio F. 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. *Appl. Environ. Microbiol.* **67**, 3450−3454. https://doi.org/10.1128%2FAEM.67.8.3450-3454.2001

Turantaş F, Göksungur Y, Dinçer AH, Ünlütürk A, Güvenç U, Zorlu N. 1999. Effect of potassium sorbate and sodium benzoate on microbial population and fermentation of black olives. *J. Sci. Food Agric.* **79**, 1197−1202. https://doi.org/10.1002/(SICI)1097-0010(19990701)79:9%3C1197::AID-JSFA349%3E3.0.CO;2-A

Valerio F, Lavermicocca P, Pascale M, Visconti A. 2004. Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. *FEMS Microbiol. Lett.* **233**, 289−295. https://doi.org/10.1016/j.femsle.2004 .02.020

Visagie CM, Houbraken J, Frisvad JC, Hong S-B, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA. 2014. Identification and nomenclature of the genus *Penicillium*. *Stud. Mycol.* **78**, 343−371. https://doi.org/10.1016/j.simyco.2014.09.001

Young JPW, Downer HL, Eardly BD. 1991. Phylogeny of the phototrophic rhizobium strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J. Bacteriol.* **173**, 2271−2277. https://doi.org/10.1128%2Fjb.173.7.2271-2277.1991

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