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Effects of oleuropein-rich olive leaf extract on the oxidative stability of refined sunflower oil

Y. M'Rabet [a], K. Hosni [a] and K. Khwaldia [a,✉]

[a]Laboratoire des Substances Naturelles, Institut National de Recherche et d'Analyse Physico-chimique (INRAP), BiotechPole Sidi Thabet, 2020, Tunisia.

✉Corresponding author: khaoula.khwaldia@inrap.rnrt.tn; khaoula_khwaldia@yahoo.fr

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SUMMARY: The aim of this study is to investigate the ability of oleuropein-rich olive leaf extract (OLE) to improve the quality and oxidative stability of sunflower oil subjected to accelerated thermal oxidation. Oleuropein was the major phenolic compound determined by HPLC-DAD with a content ranging from 20.81 to 32.56 mg·g^{-1} of dry extract (DE). The evaluation of the *in vitro* antioxidant capacity of OLE showed good scavenging capacity of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydrogen peroxide (H_2O_2) (1.01 and 0.96 mmol Trolox equivalents (ET·g^{-1} DE, respectively). The enrichment of sunflower oil with 0.1, 0.25 and 0.5% OLE (w/v) significantly inhibited thermal-induced peroxidation in a dose-dependent fashion. 0.25% OLE was the most effective concentration and showed a significant reduction in peroxide value and conjugated dienes by 61.4 and 17.4%. These results indicate that OLE can be considered a good natural alternative for extending the shelf-life of polyunsaturated vegetable oils.

KEYWORDS: *Antioxidants; Oils oxidative stability; Oleuropein; Olive leaf extract*

RESUMEN: ***Efectos del extracto de hojas de olivo rico en oleuropeína sobre la estabilidad oxidativa del aceite de girasol refinado.*** El objetivo de este estudio es investigar la capacidad del extracto de hoja de olivo (EHO) rico en oleuropeína para mejorar la calidad y la estabilidad oxidativa del aceite de girasol sometido a oxidación térmica acelerada. La oleuropeína fue el compuesto fenólico mayoritario determinado por HPLC-DAD con un contenido que varió de 20,81 a 32,56 mg·g^{-1} de extracto seco (ES). La evaluación de la capacidad antioxidante in vitro de EHO mostró una buena capacidad de captura del radical 2,2-difenil-1-picrilhidrazilo (DPPH) y peróxido de hidrógeno (H_2O_2) (1,01 y 0,96 mmol Trolox equivalentes (ET·g^{-1} ES, respectivamente). El enriquecimiento de aceite de girasol con 0,1, 0,25 y 0,5 % de EHO (p/v) inhibió significativamente la peroxidación inducida térmicamente de forma dependiente de la dosis. El EHO al 0,25 % fue la concentración más eficaz que mostró una reducción significativa del índice de peróxido y dienos conjugados en un 61,4 % y un 17,4 %. Estos resultados indican que el EHO puede considerarse una buena alternativa natural para prolongar la vida útil de los aceites vegetales poliinsaturados.

PALABRAS CLAVE: *Antioxidantes; Estabilidad oxidativa de los aceites; Extracto de hojas de olivo; Oleuropeína.*

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1. INTRODUCTION

The oxidation of lipids is among the main causes of nutritional quality deterioration and alteration of sensory attributes of commercial oils and fats. Dietary lipid hydroperoxides, the unstable end products of the peroxidation of polyunsaturated fatty acids (PUFA), are considered harmful and are involved in various diseases including atherosclerosis, rheumatoid arthritis, neurodegenerative disorders and aging (Miyazawa, 2021). Consequently, the use of antioxidants to prevent or delay lipid peroxidation has received a great deal of attention. In this context, various additives such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisol (BHA), tert-butylhydroquinone (tBHQ) and propyl gallate (PG) have been widely used as autoxidation inhibitors to improve the stability of oils and fats. Recent studies have raised concerns over the safety and side effects of, supposedly harmless, synthetic preservatives which are possibly involved in serious disorders and diseases (Naidenko *et al.*, 2021). Seeking novel sources of natural antioxidants is a challenging task which has received considerable attention from the scientific community (Blasi and Cossignani, 2020). Previous studies involving namely, polyphenols from pomegranate peel (El-Hadary and Taha, 2020) and essential oils from common aromatic herbs (Benkhoud *et al.*, 2022) showed to effectively prevent the oxidative deterioration of refined soybean and sunflower oils.

Olive (*Olea europaea* L.) leaves (OL) are common by-products from the olive oil industry and tree-pruning in Mediterranean countries. They were traditionally recycled to provide raw material for cattle food, fertilizers, soap making and energy generation. Nevertheless, OL represent an excellent source of valuable bioactive compounds including secoiridoids (oleuropein, oleuropein aglycone, and metoxyoleuropein), flavonoids (rutin, taxifoline, diosmetin, diosmin, quercetin, luteolin and its glycosylated isomers, apigenin and its glycosylated isomers,) phenyl alcohol (tyrosol and hydroxytyrosol), phenolic acids (quinic, gallic, protocatechuic, vanillic, *p*-coumaric, ferulic and syringic acids), triterpenoids (oleanolic acid and its glucosidic isomers, uvaol), tocopherols (α-tocopherol), tannins and pigments (chlorophylls and carotenoids) (Taâmalli *et al.,* 2012; Tarchoune *et al.,* 2019), which are suitable for many applications such as cosmetics, pharmaceuticals, nutraceutical and functional foods. Integrating olive leaf extract (OLE) as an antioxidant and antimicrobial agent is an advantageous alternative to synthetic food preservatives, contributing to a circular model economy. Thereby, OLE (leaf juice) has been proposed as a cheap, natural and efficient solution which improves oxidative stability and extends the shelf-life of various oils (Blasi and Cossignani, 2020).

The "Chétoui" cultivar is the second most common variety of *O. europaea*, in Tunisia. Growing mainly in northern territories, it generally produces bitter and highly pigmented olive oil with relatively important amounts of secoiridoids and good oxidative stability (Hassine *et al.*, 2015). Consonantly, the leaves of this variety were also characterized, among others, as having higher contents of phenolic acids, hydroxytyrosol, oleuropein and oleuroside (Ben Salah *et al.*, 2012). The supplementation of extra-virgin olive oil with "Chétoui" fresh leaves was reported to increase its phenolic content with higher loading on oleuropein and ligstroside aglycones (Ammar *et al.*, 2017).

Therefore, this study was devoted to examining the effect of the addition of a food-compatible aqueous-ethanol extract obtained by ultrasound-assisted extraction (UAE) of the "Chétoui" cultivar leaves on the quality and oxidative stability of sunflower oil subjected to accelerated oxidation. The extraction of bioactive ingredients from olive leaves using UAE has been optimized for some cultivars like Istrska Belica (Cifá *et al.,* 2018), Arbequina, Arbosana, Changlot Real, Frantoio, Picual, Koroneiki, and Sikitita (Martín-García *et al.,* 2022). The UAE of olive leaves has been described in the enrichment process and the incorporation of active extracts into oils (Samli *et al.,* 2020; Sousa *et al.,* 2022). This emerging, low-cost, and environmentally-friendly method shows added advantages in minimizing the time of extraction and volume of solvent and improving the extraction yield. (Cifá *et al.*, 2018).

It is anticipated that the results will contribute to the sustainable use of this agro-industrial waste, provide a safe alternative for preventing the oxidative deterioration of edible oils and thus minimize loss in their nutritional and economic values.

2. MATERIALS AND METHODS

2.1. Standards and reagents

Gallic acid (≥ 97%), syringic acid (≥ 98%), quercetin hydrate (≥ 95%), oleuropein (≥ 97%), ty-

rosol (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Rutin (≥ 90%) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), 3-hydroxytyrosol (≥ 98%) from TCI America (OR, USA). Reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) from Sigma-Aldrich, iron (III) chloride (FeCl3), aluminum chloride (AlCl3), Folin-Ciocalteu's phenol reagent from Loba Chemie (Mumbai, India). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was provided by Acros Organics (NJ, USA). All solvents used were HPLC grade and provided by VWR International (Darmstadt, Germany). Sunflower oil was obtained from a local market in Sidi Thabet (Ariana, Tunisia).

2.2. Olive leaf material

Olive leaves were obtained from "Chétoui" variety olive groves in Raf-Raf during February 2020. Raf-Raf is in the North-East region of Tunis (latitude of 37°11' N; longitude of 10°11' E) at an altitude of 136 m above sea level. A representative sample of old leaves (1 kg) was randomly collected from ten individuals (1 kg/batch). The olive leaves were washed thoroughly with tap water and then dried for 48 h at 40 °C in a Venticell oven (MMM Medcenter Einrichtungen GmbH, Munich, Germany). The dried leaves were ground using a Polymix MFC 90D mill (Kinematica, Couëron, France) to a fine powder and passed through a sieve of 1mm mesh size.

2.3. Preparation of OLE

Powdered olive leaves (1 g) were dispersed in 20 mL of a 50% ethanol-water solution (v/v) at a solid/liquid ratio of 1:20 (w/v). Aqueous ethanol was chosen as the extracting solvent due to its efficient recovery of bioactive compounds from olive leaves (Cifá *et al*., 2018). The suspension was sonicated for 20 min using an ultrasonic probe (Sonics & Materials Inc., Kolkata, India) at 20 kHz with a power of 500 W. During sonication, the dispersion was held in a thermostatic bath at 20 °C. The resulting suspension was centrifuged for 15 min at 2000 rpm. The supernatant was filtered using a 0.45 µm syringe filter and concentrated in a rotary evaporator (Schwabach, Germany). The obtained OLE was freeze-dried (Christ-Alpha, Osterode, Germany) and stored in amber glass bottles at -20 °C until use. The extraction yield of OLE was expressed as % of dry olive leaf weight.

2.4. Characterization of OLE

2.4.1. Determination of the total phenolic and total flavonoid contents

The total phenolic content (TPC) of OLE was determined according to the method described by (Ghasemi *et al.*, 2018). An aliquot of 0.5 mL of the OLE diluted in ethanol (1 $mg \cdot mL^{-1}$) sample was mixed with 2.5 mL of Folin-Ciocalteu reagent (10%) followed by 2 mL of a sodium carbonate solution (7.5 $g \cdot L^{-1}$). Afterward, the samples were held at room temperature for 1 hour for incubation. The absorbance of samples incubated for 1 h at room temperature was measured at 765 nm by a JASCO V-630 UV/visible spectrophotometer (Tokyo, Japan). Gallic acid was used for calibration (0-300 $\mu g \cdot mL^{-1}$; $R^2 = 0{,}9943$) and the TPC is expressed as mg of Gallic acid equivalent per g of freeze-dried extract (mg $GAE \cdot g^{-1}$).

The total flavonoid content (TFC) was measured according to the aluminum trichloride colorimetric method (Miliauskas *et al.*, 2004). 0.1 mL of OLE solution was mixed with 100 µL $AlCl_3$ (10%), 100 µL of sodium acetate solution (5%) and 1.8 mL of distilled water. After 30 min incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used as a calibration standard (0-250 $\mu g \cdot mL^{-1}$; $R^2 = 0{,}9969$) and TFC is expressed as mg of Quercetin equivalent $\cdot g^{-1}$ of freeze-dried extract (mg $QE \cdot g^{-1}$).

2.4.2. Identification and quantification of phenolic compounds by HPLC-DAD

The analysis of OLE phenolic composition was performed according to the International Olive Council method (COI/T.20/ Doc. No. 29) for the determination of biophenols by HPLC. Briefly, OLE (5 mg) and standards were dissolved in 1 mL of methanol:water 80:20 (v/v), sonicated for 10 min, filtered through a 0.45 µm syringe filter and then 20 µL were injected into an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a diode array detector (DAD) (G1315B, Agilent). The separation was achieved on a reversed-phase C18 Spherisorb ODS-2 column (5 µm, 250 x 4.6 mm; Kromasil Co., Sweden) with a flow rate of $1 mL \cdot min^{-1}$ at room temperature. Compounds were eluted with a ternary mobile phase gradient consisting of de-

gassed aqueous 0.2% H_3PO_4 (v/v) (A), methanol (B) and acetonitrile (C). Elution was performed in the following steps: 0-40 min: 96-50% A, 2-25 % B and 2-25% C; 45 min: 40% A, 30% B and 30% C; 60-70 min: 0% A, 50% B and 50% C; followed by column re-equilibration with initial conditions for 4 min. Chromatograms were recorded at 230, 280 and 350 nm. All data were acquired and processed using Agilent Chemstation software.

The quantification of phenolic compounds, including oleuropein, hydroxytyrosol, tyrosol, rutin and quercetin was achieved using 5 point external calibration solutions of 10, 20, 40, 60, 100 $\mu g \cdot mL^{-1}$ analyzed under the same conditions. The determination coefficient (R^2) of calibration curves relating integrated areas at 280 nm and concentrations was between 0.9996 and 0.9998. The quantification of phenolic acids (caffeic acid and *p*-coumaric acid) was carried out according to the COI method using syringic acid as internal standard (IS) added to OLE solution with a final concentration of 15 $\mu g \cdot mL^{-1}$. The response factor of IS was used in the calculation of phenolic acid concentrations, as well as an additional criterion for peak identification using the relative retention time calculated with respect to the IS retention time. The results were expressed as mg of phenolic compound per g of freeze-dried OLE.

2.4.3. Antioxidant activity

The antioxidant activity of OLE was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and hydrogen peroxide (H_2O_2) assays. For the DPPH assay (Edziri *et al.*, 2019), 1 mL of an ethanol solution incorporating different OLE concentrations (0.2 – 1 $mg \cdot mL^{-1}$) was added to 2 mL of DPPH methanolic solution (0.1 mM). The obtained mixture was thoroughly homogenized and kept in the dark for 20 min before measuring its absorption at 517 nm. A FRAP assay was carried out according to the method of (Benzie and Strain, 1996) with some modifications. The FRAP reagent was prepared by mixing 25 mL of acetate buffer 300 mM (pH 3.6), 2.5 mL of TPTZ (10 mM), and 2.5 mL 10 mM of $FeCl_3$ (20 mM) and incubating in a water bath at 37 °C for 30 min. OLE (150 µL) was mixed with 2850 µL of FRAP solution in the dark for 30 min before measuring the absorbance at 593 nm. Hydrogen peroxide (H_2O_2) scavenging capacity was performed according to the method of (Ruch *et al.*, 1989). 100 µL of olive leaf extract (200-1000 $\mu g \cdot mL^{-1}$) were mixed with 300 µL of 50 mM phosphate buffer (pH 7.4) and 600 µL of 2 mM hydrogen peroxide solution. After incubation for 10 min, the absorbance at 230 nm was measured against a blank (phosphate buffer solution).

The potential to scavenge DPPH and H_2O_2 was determined using the following equation:

$$\% \text{ scavenging} = (1 - A_{sample} / A_{blank}) \times 100$$

where A_{blank} is the absorbance of the blank and A_{sample} is the absorbance of the extract.

The positive control Trolox was used to construct calibration curves for all assays, and the results were expressed as mmol $TE \cdot g^{-1}$ of freeze-dried extract.

2.5. Application of OLE for controlling sunflower oil oxidation

Three different concentrations of OLE (0.1, 0.25, and 0.5%, w/v) were mixed with 25 mL sunflower oil. The sunflower oil initially showed (at T0, time corresponding to its production) 0.59 mg $KOH \cdot kg^{-1}$ acid value, 2.1 meq $O_2 \cdot kg^{-1}$ peroxide value, conjugated dienes (K_{232}) and conjugated trienes (K_{270}) at 0.93 and 0.3, respectively. It was a linoleic acid-rich oil with 8.2% C16:0; 3.4% C18:0, 27.6% C18:1 and 59.3% C18:2 as main fatty acids. It also contained α-tocopherol and carotenoids with average values of 39.6 $mg \cdot 100g^{-1}$ oil and 0.34 $mg \cdot 100g^{-1}$ oil, respectively (data not shown). The different mixtures were homogenized for 20 min at 13500 rpm with an Ultra-Turrax T25 (IKA, Labortechnik GmbH, Munich, Germany). Then, the obtained samples of sunflower oil incorporating OLE were incubated in a forced air circulation oven (Venticell) to accelerate their oxidation at 70 °C for 1 week according to the Schaal oven test. The oxidative stability of treated sunflower oil was evaluated in terms of quality indices and fatty acid composition. Sunflower oil without OLE was used as a control.

2.6. Quality indices

Acid value (AV), peroxide value (PV) and ultraviolet spectrophotometric indices at 232 nm (K_{232} value) and 270 nm (K_{270} value) were determined according to the standard protocols of the association of official analytical chemists (AOAC, 2003).

2.7. Fatty acid composition of sunflower oil (GC-FID)

Fatty acid methyl esters (FAME) of sunflower oil fatty acids were obtained by cold transesterification in alkaline conditions. Briefly, 0.1 g of sample dissolved in 2 mL of n-hexane were mixed with 0.5 mL of sodium methoxide in methanol (3 %), followed by methanolic H_2SO_4 (1 N). The hexane layer containing fatty acid methyl esters (FAMES) was washed with 10% NaCl solution and concentrated under a nitrogen steam before analysis. FAME analysis was carried out on a Hewlett-Packard HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and polar TR-FAMEs (Thermo Fisher Scientific Inc., Bordeaux, France) capillary column (60 m × 0.25 mm, 0.25 µm film thickness). The column temperature was initially held at 100 °C for 5 min, raised to 240 °C (4 °C·min^{-1}), and then kept isothermal for 15 min. The temperatures of the injector and detector were held at 240 °C and 260 °C, respectively. Fatty acids were identified by comparison of their retention times with those of standard FAME mixtures injected in the same conditions (Supelco, 37 Component FAME Mix, PA, USA). The area of each fatty acid peak was integrated by Agilent. ChemStation software and the result is expressed as a percentage of total peak areas.

The Iodine value (IV) and oxidative susceptibility (OS) were determined from the percentages of fatty acids using the following equations (Torres and Maestri, 2006):

$$IV = (\%\ \text{palmitoleic acid} \times 1.001) + (\%\ \text{oleic acid} \times 0.899) + (\%\ \text{linoleic acid} \times 1.814) + (\%\ \text{linolenic acid} \times 2.737)$$

$$OS = \%MUFA + (\%\ \text{linoleic acid} \times 45) + (\%\ \text{linolenic acid} \times 100)$$

Where MUFA is the % of monounsaturated fatty acids.

2.8. Statistical analysis

All experiments were performed in triplicate and results are reported as mean ± standard deviation (SD). One-way ANOVA with Tukey's HSD (Honestly Significant Difference) test was used to assess significant differences between means with a significance level of $p < 0.05$. Data were treated using SYSTAT (Systat Software, San Jose, CA).

3. RESULTS AND DISCUSSION

3.1. OLE extraction yield

Olive leaves, which can be obtained during olive tree pruning or harvesting as well as from olive mills, are a low-cost source of active compounds. An OLE extraction yield of 25 ± 1.7% was obtained using UAE for 20 min at room temperature and a solid/liquid ratio of 1/20 (w/v). Under similar conditions (same extraction procedure (UAE), 50% ethanol and 1:20 solid/liquid ratio) an average extract yield ranging from 16.2-20.12% was recorded by Şahin and Şamli (2013) for olive leaves from an unidentified variety. The observed discrepancy might be attributed to varietal differences as has been reported by Martín-García *et al.* (2022). Variable extraction yields depending on olive variety and extraction procedure have also been described for Tunisian olives (Taâmalli *et al.,* 2012). Extraction yields ranging from 5.2 to 22.4% were obtained for the varieties Jarboui and Chemchali, using supercritical fluid extraction and pressurized liquid extraction, respectively. Regarding the variety Chétoui, a maximum extract yield (19.5 %) was achieved with pressurized liquid extraction using ethanol as extracting solvent and a temperature of 150 °C (Taâmalli *et al.,* 2012). At this point, we can postulate that UAE (operating at low temperature) was more suitable for the extraction of bioactive compounds from olive leaves without possible thermal alterations, a fact that could justify the orientation of most of the research teams to the use of UAE as a method of choice for extracting useful nutraceuticals from olive leaves (Cifá *et al.*, 2018). Indeed, the acoustic cavitation generated by ultrasound treatment induces cuticle erosion and fragmentation of leaf surface protrusion (hair), the polyphenol-rich structures of olive leaves lead to an increased release of phenolic compounds.

3.2. TPC, TFC, main phenolic compounds and antioxidant activities of OLE

The TPC, TFC and antioxidant activities of OLE are summarized in Table 1. In this study, The TPC of 50% (v/v) ethanol OLE obtained by UAE was 135 mg

GAE·g^{-1} extract (Table 1). TPC values ranging from 73 to 144 mg GAE·g^{-1} extract have been reported for OLE obtained with 70% (v/v) ethanol from eight Tunisian olive leaf varieties (Ben Salah *et al.*, 2012).

Table 1. Phytochemical and antioxidant properties of ethanolic olive leaf extract.

Parameters	Values (g^{-1} of extract)
Phenolic content (g^{-1} DE)	
TPC (mg GAE)	134.73 ± 1.05
TFC (mg QE)	62.48 ± 0.43
Antioxidant activities (mmol TE·g^{-1} DE)	
DPPH scavenging	1.01 ± 0.01
FRAP	1.36 ± 0.15
H_2O_2 scavenging	0.96 ± 0.04
Phenolic compouds (mg·kg^{-1} DE)	
Hydroxytyrosol	2406.02 ± 245.21
Tyrosol	797.05 ± 135.50
Caffeic Acid	915.58 ± 121.03
p-coumaric acid	537.70 ± 156.12
Rutin	2033.95 ± 247.83
Oleuropein	32568.31 ± 143.17
Quercetin	1272.09 ± 252.05

Results expressed as mean ± SD (n = 3); DE: dry weight extract, TE: Trolox equivalent, GAE: Gallic acid equivalent, QE: Quercetin equivalent, 1-7: compounds identified by HPLC-DAD and quantified using external standard calibration curves.

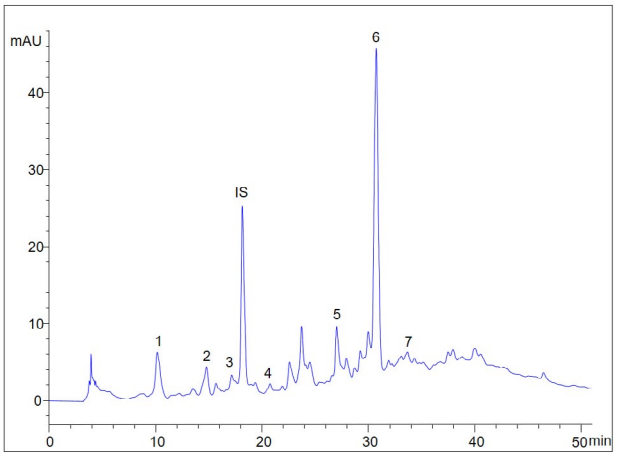

Figure 1. Chromatographic profile of the OLE (280 nm). Identification of compounds: (1) Hydroxytyrosol, (2) Tyrosol, (3) Caffeic acid, (IS) Internal standard, (4) *p*-Coumaric acid, (5) Rutin, (6) Oleuropein, (7) Quercetin.

This value is higher than that reported by Edziri *et al.* (2019) for the methanol OLE of the "Chétoui" cultivar (47.5 mg GAE·g^{-1} extract) obtained by regular maceration. Higher TPC was achieved with MAE by Mohammadi *et al.* (2016) for "Mission" variety (206.8 mg GAE·g^{-1} extract) but using methanol as extraction solvent. The TFC value for the "Chétoui" variety OLE is highly variable and fluctuates between 7.29 – 94 catechin equivalent CE·g^{-1} extract (Edziri *et al.*, 2019; Ben Salah *et al.*, 2012). The difference between the obtained value (Table 1) could be attributed to the type of standard used for calibration (CE vs. QE in this study) and to the known limits of this method (Shraim *et al.*, 2021). In fact, Shraim *et al.* (2021) showed significant variation in the $AlCl_3$-flavonoid complex response according to the flavonoid type used and the composition of the extract, leading to an under or an overestimation of this parameter.

The profiling of phenolic compounds by HPLC-DAD resulted in the identification of 7 compounds by comparison of retention times and UV-visible spectra with those of standards analyzed under the same conditions (Figure 1). These compounds have been previously reported in the literature (Edziri *et al.*, 2019; Quirantes-Piné *et al.*, 2013) and can be grouped, as shown in Table 1, into simple phenols (hydroxytyrosol, tyrosol), secoiridoids (oleuropein), flavonols (quercetin; rutin) and phenolic acids (caffeic acid; *p*-coumaric acid). The OLE obtained by UAE is rich in secoiridoids with oleuropein being the major phenolic compound (87.9% of total identified compounds) (Figure 1). This agrees with studies that demonstrated that oleuropein is the main phenolic compound in ethanolic extracts from olive leaves; while hydroxytyrosol and phenolic acids are the most prevalent phenolic compounds in aqueous extracts (Quirantes-Piné *et al.*, 2013). The oleuropein content (32.56 mg·g^{-1} freeze-dried OLE) is in the range of the oleuropein contents reported for OLE obtained by maceration in ethanol:water (70:30 v/v) (27.3 mg oleuropein·g^{-1} extract) and those obtained by UAE using aqueous ethanol as a solvent (35.6 – 39.2 mg oleuropein·g^{-1} extract) (Cifá *et al.*, 2018). Other phenolic compounds were identified, such as caffeic acid (3.0%), hydroxytyrosol (2.4%), tyrosol (2.2%), rutin (2%), *p*-coumaric acid (1.4%) and quercetin (1.2%). However, due to the lack of analytical standards for confirmation, other known compounds previously identified in olive leaves: lu-

teolin, apigenin, verbascoside and ligstroside derivatives were not assigned (Quirantes-Piné *et al*., 2013). The differences observed in the phenolic composition of OLE may be related to many factors including variety, cultivar, geographic origin, solvent type and polarity, solvent-solid ratio, extraction method and parameters (Ghasemi *et al.*, 2018).

The antioxidant activity of OLE was evaluated by three complementary tests. As shown in Table 1, average values of 1.01, 1.4 and 0.96 mmol TE·mg^{-1} for freeze-dried extract have been observed for DPPH, FRAP and H_2O_2 assays, respectively. The ability of OLE to scavenge DPPH radicals and reduce Fe^{3+} to Fe^{2+} suggests that its antioxidant activity involves a single electron transfer (SET) mechanism (Ivanova *et al.,* 2020). Moreover, OLE also has an effective H_2O_2 scavenging activity. Being a hydrogen atom transfer-based (HAT) mechanism (Ivanova *et al.,* 2020), the result of the H_2O_2 assay indicates that OLE might act as a hydrogen donor. Collectively, the antioxidant activity of OLE could be attributed to the simultaneous presence of electron and hydrogen donors.

The antioxidant activity was found to be correlated with TPC and TFC values (Miliauskas *et al.*, 2004). The phenolic compounds of OLE, mainly oleuropein, may directly contribute to its antioxidant capacity. In a comparative study among four Tunisian olive varieties, the OLE from "Chétoui" was the most effective DPPH radical scavenger (Edziri *et al.,* 2019). Moreover, the ability of OLE to scavenge H_2O_2 and reduce Fe^{3+} has also been evidenced (Martín-García *et al.,* 2022), confirming our findings. The antioxidant activity of OLE appears to be intimately linked to the presence of putative antioxidants which have strong electron and hydrogen donating ability. Among the identified components, some have received particular attention because of their well-known antioxidant properties, including oleuropein, hydroxytyrosol, rutin, gallic and caffeic acids, tyrosol, elenolic acid, luteolin, apigenin and their glycosylated forms (Taâmalli *et al.,* 2012; Tarchoune *et al.,* 2019; Martín-García *et al.,* 2022). In addition to phenolic components, other fat-soluble components such as pigments (carotenoids and chlorophylls) and tocopherols have also been reported as strong radical scavengers and reducing agents, thereby conferring a potent antioxidant activity to OLE (Tarchoune *et al.,* 2019). It should be noted that the overall antioxidant activity resulted from synergistic interactions between the active ingredients present in OLE (Lee *et al.,* 2009).

3.3. Oxidative stability of sunflower oil samples incorporated with OLE

In this study, the ability of OLE to improve the oxidative stability of sunflower oil was assessed through the determination of some analytical indices and fatty acid composition of sunflower oil. At the beginning of the experiments (before thermal treatment), the acid value, peroxide value, K_{232} and K_{270} of sunflower oil were 1.16 ± 0.07 mg KOH·g^{-1}, 11.67 ± 0.58 meq O_2·Kg^{-1}, 2.14 ± 0.18 and 1.28 ± 0.24, respectively (Table 2). The thermal treatment of sunflower oil for 7 days resulted in a 2.9-fold increase in acid value compared to the untreated oil sample at the start of the experiment. This may be attributed to extended lipid peroxidation leading to excessive production of hydroperoxide. The incorporation of OLE significantly decreased ($p < 0.05$) the acid value with 0.25 and 0.5% concentrations being the most effective in reducing the content of free fatty acids. The ability of OLE to delay the thermal-induced production of free radicals and lipid peroxidation could be due to its antioxidant capacity (Şahin *et al*., 2017). So, the presence of putative antioxidants in the OLE (e.g. tyrosol, hydroxytyrosol, oleuropein, quercetin, and rutin) could effectively inhibit the formation of free radicals at the initial stage of lipid peroxidation, break the chain reaction during the propagation stage, and/or scavenge the free radicals in the oil by donating a hydrogen atom (Blasi and Cossignani, 2020). Peroxide value (PV) as a key parameter reflecting the early stage of lipid oxidation was determined in all sunflower oil samples. As shown in Table 2, the PV of thermally treated-control oil was increased significantly owing to an extended autoxidation of the oil and its subsequent increase in hydroperoxides. In contrast, the incorporation of OLE reduced peroxide formation and delayed the thermal oxidation of sunflower oil in the early stage, thereby delaying the onset of oil autoxidation. Sunflower oil incorporated with 0.25 and 0.5% OLE showed the lowest PV value among the enriched oil samples. As the PV is closely related to the polyunsaturated fatty acid (PUFA) content, it was clear that the decrease in the PV in sunflower oil incorporated with 0.25 and 0.5% OLE was mainly

Table 2. Quality indices and fatty acid composition (%) of sunflower oil incorporating different concentrations of OLE.

Parameter	Sunflower oil before heat treatment	Thermally-treated sunflower oil (70 °C, 1 week)			
		Sunflower oil control (without OLE)	+ OLE 0.1%	+ OLE 0.25%	+ OLE 0.5%
Acid value (mg KOH·g^{-1} oil)	1.16 ± 0.07^{c}	3.31 ± 0.08^{a}	2.22 ± 0.04^{b}	1.24 ± 0.16^{c}	1.49 ± 0.12^{c}
Peroxide value (meq O_2·Kg^{-1})	11.6 ± 0.5^{d}	33.3 ± 0.5^{a}	22.7 ± 0.3^{b}	12.8 ± 0.1^{d}	14.1 ± 0.5^{c}
K_{232}	2.1 ± 0.1 ^{a}	2.6 ± 0.2 ^{a}	2.2 ± 0.2 ^{a}	2.2 ± 0.1 ^{a}	2.2 ± 0.1 ^{a}
K_{270}	1.3 ± 0.3 ^{a}	1.4 ± 0.2 ^{a}	1.3 ± 0.1 ^{a}	1.3 ± 0.1 ^{a}	1.2 ± 0.2 ^{a}
Palmitic acid (C16:0)	9.09 ± 0.06^{b}	8.76 ± 0.07^{c}	9.05 ± 0.07^{b}	8.39 ± 0.11^{d}	9.49 ± 0.07^{a}
Palmitoleic acid (C16:1)	0.63 ± 0.02^{a}	0.77 ± 0.06^{b}	0.67 ± 0.06^{b}	1.05 ± 0.05^{a}	0.98 ± 0.05^{a}
Stearic acid (C18:0)	3.43 ± 0.04^{c}	3.68 ± 0.04^{b}	3.29 ± 0.04^{c}	4.05± 0.07^{a}	3.76± 0.04^{b}
Oleic acid (C18:1)	32.1 ± 0.3^{a}	30.1 ± 0.3^{b}	29.1 ± 0.4^{c}	30.9 ± 0.5^{a}	31.3 ± 0.4^{a}
Linoleic acid (C18:2)	53.2 ± 0.7^{b}	55.6 ± 0.3^{a}	56.2 ± 0.6^{a}	53.7 ± 1.4^{b}	53.3 ± 0.4^{b}
Linolenic acid (C18:3)	0.46 ± 0.04^{a}	0.17 ± 0.02^{c}	0.32 ± 0.06^{b}	0.36 ± 0.03^{ab}	0.30 ± 0.08^{b}
Arachidic acid (C20:0)	0.072 ± 0.061^{b}	0.290 ± 0.06^{a}	0.092 ± 0.007^{b}	0.093 ± 0.006^{b}	0.095 ± 0.001^{b}
Gadoleic acid (C20:1)	0.17 ± 0.02^{c}	0.083 ± 0.001^{c}	0.65 ± 0.07^{a}	0.61 ± 0.06^{a}	0.25 ± 0.01^{b}
Behenic acid (C22:0)	0.73 ± 0.45^{a}	0.42 ± 0.01^{a}	0.51 ± 0.04^{a}	0.72 ± 0.08^{a}	0.40 ± 0.03^{a}
SFA	12.0 ± 0.4^{c}	13.1 ± 0.1^{b}	12.9 ± 0.06^{ab}	13.25 ± 0.3^{a}	13.74 ± 0.08^{a}
MUFA	27.4 ± 0.3^{c}	31.0 ± 0.4^{b}	30.5 ± 0.3^{b}	32.6 ± 0.6^{a}	32.6 ± 0.5^{a}
PUFA	60.6 ± 0.7^{a}	55.8 ± 0.3^{ab}	56.5 ± 0.7^{a}	54.2 ± 1.3^{bc}	53.6 ± 0.5^{c}
MUFA/PUFA	0.456 ± 0.002^{c}	0.561 ± 0.011^{b}	0.543 ± 0.002^{b}	0.602 ± 0.028^{a}	0.619 ± 0.003^{a}
Oxidative Susceptibility	2475 ± 14^{c}	2552 ± 17^{ab}	2594 ± 36^{a}	2485 ± 60^{bc}	2465 ± 28^{c}
Iodine value	127 ± 1^{a}	129 ± 0.3^{a}	129 ± 0.5^{a}	127 ± 1.9^{a}	126 ± 1.4^{a}

Results expressed as mean ± SD (n = 3); OLE: Olive leaf extract; OLP: Olive leaf powder; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; Different lowercase letters in the same row indicate significant differences (Tukey's HSD; $p < 0.05$).

due to the extended degradation of hydroperoxides to secondary oxidation products such as volatile aldehydes and ketones (Benkhoud *et al.*, 2022). The specific extinction coefficients are indicative of the presence of conjugated dienes (CD) and trienes (CT) (K_{232} and K_{270}, respectively). CD as a measure of the oxidative state of the oil is considered a reliable indicator of the effectiveness of antioxidants to prevent lipid oxidation. The results from Table 2 show that the highest CD contents were found in the thermally treated control owing to their high content in polyunsaturated fatty acids, namely linoleic. In contrast, samples incorporated with OLE showed CD values close to the non-treated control, suggesting the protective function of OLE against thermal-induced lipid peroxidation. The CT contents in all samples did not differ significantly ($p > 0.05$), indicating a similar rate of oxidation. This may be due to the inhibition of the production of CT through the dehydration of CD hydroperoxides and/or the absence of secondary oxidation products (Zhang *et al.*, 2020). Regardless of thermal treatment, Iodine values (IV) were similar in all sunflower oil samples, suggesting that this parameter could not be considered a reliable parameter for estimating the rate of oxidation of sunflower oil. Moreover, the implication of native antioxidants like tocopherols could, at least in part, explain the steady oxidation state of the untreated control and the oil samples incorporated with OLE.

The fatty acid profiles of control untreated and thermally treated sunflower oil samples are presented in Table 2. PUFA made up the highest contribution (53.6-60.6%) with linoleic (53.2-56.2%) and linolenic (0.17-0.46%) acids as the main components. The monounsaturated fatty acids (MUFA) represent the second-most abundant class with percentage contributions ranging from 27.4 to 32.6%. The MUFA fraction was dominated by oleic (29.1-32.1%), followed by palmitoleic (0.63-1.05%) and gadoleic (0.08-0.65%) acids. The saturated fatty acids (SFA) made up the lowest contribution (12.0-13.74%) and the main fatty acids were palmitic (8.39-9.49%),

followed by stearic (3.29-4.05%) and behenic (0.40-0.73%). Despite their high nutritional quality, the oil samples were particularly prone to oxidation as revealed by their high oxidative susceptibility (OS) (2465-2594) in comparison with other edible oils like olive oil (453-922) (Benkhoud *et al.*, 2022). At this point, the incorporation of 0.25 or 0.5% OLE confers better resistance to thermal oxidation of sunflower oil and improves its oxidative stability.

The protective effect of OLE against the thermal oxidation of sunflower oil may be attributed to the presence of putative antioxidants such as polyphenols (Samli *et al.,* 2020) and fat-soluble antioxidants, including tocopherols (Jaski *et al.,* 2022), carotenoids and chlorophylls (Şahin *et al.*, 2017; Sousa *et al.,* 2022). In a previous study, it has been reported that the enrichment of olive oil with OLE containing 272 ppm oleuropein resulted in a 2.5-fold increase in the free-radical scavenging ability, thus resulting in enhanced oil stability (Şahin *et al.*, 2017). In another study, hydroxytyrosol was found to be the most effective in inhibiting the formation of conjugated hydroperoxides in bulk fish oil and fish oil-in-water emulsions at a concentration of 100 ppm, mainly due to its electron-donating and ferrous-chelating properties (Pazos *et al.*, 2008). Using antioxidant activity guided-fractionation, Lee *et al*. (2009) showed that the protective effect of OLE against lipid oxidation was linked to oleuropein, caffeic acid, vanillin, rutin and catechin. More recently, Sousa *et al*. (2022) showed that the incorporation of chlorophyll-rich extract from *Crithmum maritimum* markedly enhanced the oxidative stability of sunflower oil. Another point to be considered is that the incorporation of OLE into sunflower oil might not only improve its oxidative stability through its direct action on retarding the lipid peroxidation process, and/or scavenging free radicals, but also indirectly through the protection of α-tocopherol and carotenoids, the natural antioxidants of sunflower oil from thermal degradation empowering thus its oxidative stability. Further studies are needed to confirm this assumption.

4. CONCLUSIONS

OLE obtained by a green process from olive leaves which represent an abundant, inexpensive, and underutilized by-product, is revealed as a rich source of antioxidant compounds including the valuable oleuropein. The evaluation of its antioxidant activities showed potent anti-radical, ferric reducing and H_2O_2 scavenging capacities. Therefore, the incorporation of OLE into sunflower oil significantly enhanced its oxidative stability. OLE at 0.25 and 0.5% were effective against the development of rancidity induced by thermal oxidation. This beneficial effect could be attributed to the synergistic effect of antioxidant components (secoiridoids, flavonoids, and phenolic compounds) identified and quantified in OLE. Based on these findings, OLE could substitute conventional synthetic antioxidants associated with many environmental and health risks, adding value to olive leaf by-products obtained after harvesting and offering an economic and environmental solution to the management of this agricultural waste. The search for the adequate harvesting stage which provides the highest OLE yield deserves further investigation.

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DATA AVAILABILITY

Data available on request from the authors.

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