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ORIGINAL ARTICLE

Valorization of Post-Supercritical Fluid Extraction Residues of *Olea europaea* L. Leaves: Recovery of Bioactive Compounds

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ABSTRACT

Objectives: Supercritical fluid extraction (SFE) has attracted increasing interest as a sustainable technology for extracting bioactive compounds from plant materials. However, post-SFE plant residues may retain valuable phytochemicals, which remain underutilized. This study aimed to investigate the distribution of phenolic compounds between an industrial SFE extract from *Olea europaea (O. europaea)* leaves and the corresponding post-SFE residue and to evaluate the ability of complementary solvent extraction to recover residual compounds with relevant biological activities.

Materials and Methods: The post-SFE *O. europaea* leaf residue was extracted separately with ethyl acetate and ethanol. The SFE extract and post-SFE residue extracts were characterized by high-performance liquid chromatography with diode-array detection (HPLC-DAD) and comparatively evaluated for antioxidant and selected enzyme-inhibitory activities.

Results: HPLC-DAD analysis revealed distinct phenolic profiles among the extracts. Of the seven quantified compounds, only vanillic acid was detected in OE-SFE (0.24 ± 0.01 mg/g), whereas oleuropein was most abundant in the post-SFE residue ethyl acetate extract (37.77 ± 1.75 mg/g). Luteolin-7-O-glucoside was also detected in both post-SFE residue extracts, with concentrations of 6.74 ± 0.09 mg/g in the ethanol extract and 3.51 ± 0.07 mg/g in the ethyl acetate extract. The post-SFE residue ethyl acetate extract and ethanol extracts showed acetylcholinesterase and butyrylcholinesterase inhibitory activities ranging from 21.56 ± 1.85% to 46.35 ± 3.09%, while OE-SFE exhibited 82.87% lipoxygenase inhibition.

Conclusion: The leaf material of *O. europaea* remaining after SFE represents a potential source of recoverable phenolic compounds rather than merely an extraction residue. Thus, complementary extraction of this material may provide a strategy for improving phytochemical recovery and generating value-added fractions, supporting more efficient use of olive leaf biomass within a circular bioeconomy framework.

Keywords: *Olea europaea*, supercritical fluid extraction, post-extraction residues, HPLC-DAD, waste valorization

INTRODUCTION

Olea europaea (O. europaea) L., a member of the Oleaceae family, is a drought-tolerant plant essentially native to the Mediterranean climate and is considered one of the most important agricultural crops.[1,2] Approximately 98% of the world's more than eight million hectares of cultivated olive trees are located in the Mediterranean region. *O. europaea is extensively* studied for its culinary applications; its fruits and oil are significant components of the daily diets of many people worldwide.[3]

O. europaea leaves are among the most abundant by-products generated during olive cultivation and olive oil production. The

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high phenolic compound content of olive leaves has attracted considerable commercial interest, and olive leaves are increasingly used in the nutraceutical, pharmaceutical, cosmetic, and functional food industries.[1,2,4] Several reports have shown that olive leaves have biological activities, such as antioxidant, anticancer, lipid-lowering, antidiabetic (hypoglycemic), antihypertensive, cardioprotective, antimicrobial, antifungal, anti-inflammatory, and neuroprotective effects.[2,4-6]

Phenolic compounds in olive leaves are numerous and diverse. They are mainly classified as simple phenols and phenolic acids (e.g., hydroxytyrosol, tyrosol, caffeic acid, and vanillic acid), lignans, secoiridoids (primarily oleuropein), and flavonoids (e.g., luteolin, apigenin, rutin, and catechin).[1,4] Among the extraction technologies employed for the recovery of olive leaf bioactives, supercritical fluid extraction (SFE) has attracted increasing attention as a green and sustainable approach due to its reduced solvent consumption and mild extraction conditions.[7-9]

The SFE method, defined as a green extraction technique due to its use of non-toxic and environmentally friendly solvents, its ability to operate at relatively low temperatures, thereby helping to preserve sensitive bioactive compounds from thermal degradation, and its rapid and selective extraction performance, has gained considerable popularity in recent years. Notably, while the primary focus of SFE has been on the target compounds, the residual plant material, often regarded as waste, may still contain substantial amounts of valuable phytochemicals.[10,11] In many industrial and experimental processes, these important chemicals are frequently discarded as waste. Nevertheless, little research has examined the bioactive potential of leftover plant waste after SFE.[12-14] In addition to the extract, the residual biomass obtained after SFE represents a potential resource for further valorization.[15] Its utilization may reduce waste, enable the recovery of value-added products, and contribute to the circular economy and sustainable biorefinery applications.[16-18]

Despite the increasing use of SFE for recovering olive leaf phytochemicals, the phytochemical composition and biological potential of the residual biomass remain poorly explored.[15,16] *O. europaea* and its major constituents, particularly oleuropein and hydroxytyrosol, have been widely investigated for their antioxidant, anti-inflammatory, neuroprotective, and metabolic effects.[15-17] Therefore, this study aimed to determine whether biologically relevant phytochemicals remain in *O. europaea* residues after SFE and whether they can be recovered by subsequent solvent extraction. The well-established neuroprotective and antidiabetic potential of *O. europaea* leaves provided a framework for the biological characterization of the recovered extracts.[2,6,17]

Therefore, this study evaluated olive leaf extracts obtained by industrial-scale CO_2-based SFE and extracts prepared from post-SFE residues by determining their total phenolic content and characterizing their individual phenolic constituents by high-performance liquid chromatography (HPLC) profiling, and by assessing their antioxidant capacity via 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging assays and ferric reducing antioxidant power (FRAP); their *in vitro* antidiabetic potential through α-glucosidase inhibition; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities relevant to Alzheimer's disease; and lipoxygenase (LOX) inhibitory activity, thereby addressing both the chemical valorization and the multi-target bioactivity of olive leaf SFE-derived materials.

MATERIALS AND METHODS

Extraction material and industrial SFE

The primary supercritical fluid extract (OE-SFE) from *O. europaea* L. leaves and the corresponding post-SFE residual plant material were supplied by LUK Botanik (Gebze, Türkiye), a commercial manufacturer that employs industrial-scale supercritical CO_2 extraction technology.

According to the information provided by the manufacturer, the extraction process employed supercritical CO_2 in combination with a water-ethanol co-solvent system. The process was conducted according to the manufacturer's established industrial operating procedures. The detailed operating parameters, including extraction pressure, temperature, CO_2 flow rate, co-solvent composition and proportion, extraction duration, and downstream processing conditions, were not disclosed by the manufacturer because they constitute proprietary process information.

The industrial SFE procedure was not performed or optimized by the authors. Therefore, the present study does not aim to reproduce or evaluate the efficiency of the proprietary industrial extraction process. Instead, the industrially obtained OE-SFE extract and its corresponding post-SFE residual biomass were used in their as-supplied forms as materials for comparative phytochemical characterization and biological activity assessment. The residual biomass was subsequently subjected to laboratory-scale solvent extraction procedures that were performed independently as described below. Ethics committee approval and patient informed consent were not required for this study, as the research was conducted exclusively using plant materials.

Preparation of post-SFE residue extracts

The residual plant material obtained from SFE of *O. europaea* was extracted with ethyl acetate and ethanol by maceration (OE-REA and OE-RE, respectively). Dry post-extraction waste (10 g per extract) was macerated three times at room temperature with ethyl acetate or ethanol (100 mL each). The filtered extracts were evaporated at 40 °C under reduced pressure and lyophilised.[19] The yields of the ethyl acetate and ethanol extracts were 8.5% and 22.4%, respectively. The extracts were stored in a refrigerator at +4 °C until use.

Determination of total phenolic content

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method with slight modifications.[20] Extracts were prepared in 95% ethanol (EtOH) at 1 mg/mL, and gallic acid was used for calibration. 10 µL of samples or gallic acid standards were mixed with 30 µL of Folin–Ciocalteu reagent

(diluted twofold) and 150 µL of sodium carbonate (3.5%) in microplate wells. After incubation at 40 °C for 30 min, absorbance was measured at 765 nm using an ELISA microplate reader (BMG Labtech SPECTROstar, Germany).[21]

Antioxidant assays

DPPH free radical scavenging activity

DPPH radical scavenging activity was determined according to Hatano et al. [22] with minor modifications. Extracts and Trolox were dissolved in EtOH and mixed with DPPH solution (0.138 mg/mL). After incubation at room temperature for 30 min, the absorbance was measured at 515 nm using a BMG Labtech SPECTROstar (Germany).[21] Trolox was used as the reference standard; antioxidant activity was calculated from the Trolox calibration curve and expressed as mg Trolox equivalents per gram of extract (mg TE/g extract).

ABTS•+ free radical scavenging activity

The ABTS radical scavenging assay was performed according to Re et al. [23] with minor modifications. The ABTS•+ solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture overnight. The solution was diluted to an absorbance of approximately 0.70 at 734 nm. Samples were prepared at 1000 µg/mL, and 10 µL was mixed with 240 µL of ABTS•+ solution. After incubation at room temperature for 6 min, absorbance was measured at 734 nm (BMG Labtech SPECTROstar, Germany). Trolox was used as the reference standard, and antioxidant activity was calculated using the Trolox calibration curve and expressed as mg Trolox equivalents per gram of extract (mg TE/g extract).[24]

FRAP assay

The modified Oyaizu[25] assay was used to measure the FRAP of the extracts, with Trolox as the reference standard. Extracts were mixed with 25 µL of phosphate buffer (pH 6.6) and 25 µL of potassium ferricyanide (1%, w/v), and then incubated at 50 °C for 20 min. $FeCl_3$ (17 µL, 0.1%, w/v), distilled water (85 µL), and trichloroacetic acid (25 µL, 10%) were then added. After incubation at room temperature for 30 min, the absorbance was measured at 700 nm using a BMG Labtech SPECTROstar (Germany).[21] The reducing capacity was calculated using the Trolox calibration curve and expressed as mg Trolox equivalents per gram of extract (mg TE/g extract).

Enzyme inhibition assays

Anticholinesterase activity assay

The inhibitory activities of the extracts against AChE and BChE were determined using the Ellman method with minor modifications.[26] Extracts/controls (20 µL) were mixed with 140 µL of sodium phosphate buffer (0.1 M, pH 8.0) and the respective enzyme solution (AChE from Electrophorus electricus and BChE from equine serum; Sigma-Aldrich, USA), then incubated at room temperature for 10 min. The final enzyme activities were 0.013 U/mL for AChE and 0.0176 U/mL for BChE. The reaction was initiated by adding the respective substrate (acetylthiocholine iodide for AChE or S-butyrylthiocholine chloride for BChE) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), all from Sigma-Aldrich, USA. The final concentrations of acetylthiocholine iodide, S-butyrylthiocholine chloride, and DTNB were 0.7, 0.1, and 0.5 mM, respectively, in a total reaction volume of 200 µL per well. Absorbance was measured at 412 nm (Molecular Devices Spectramax ABS Plus). In both experiments, galantamine hydrobromide (Sigma-Aldrich, USA) was used as the reference.[27]

LOX inhibitory activity assay

The LOX inhibitory activity was determined according to Chung et al.[28] with minor modifications. Extracts, positive control, negative control, and LOX enzyme [500 U/mL, from Glycine max (soybean), Sigma-Aldrich, USA] were mixed in 50 mM Tris-HCl buffer (pH 7.4) and incubated at room temperature for 5 min. The reaction was initiated by adding the substrate linoleic acid (12.5 mM) and was incubated for 20 min at room temperature. The reaction was terminated with freshly prepared FOX reagent (Sigma-Aldrich, USA), and absorbance was measured at 560 nm (Molecular Devices SpectraMax ABS Plus) after 30 min. Baicalein (Sigma-Aldrich, USA) was used as the reference.[29]

Alfa-glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity was determined according to Bachhawat et al.[30] with minor modifications. Samples (10 µL) of different concentrations, prepared in a DMSO:buffer mixture (1:1, v/v), were mixed with 10 µL of α-glucosidase (1 U/mL, from Saccharomyces cerevisiae; Sigma-Aldrich, USA) and 50 µL of potassium phosphate buffer (0.1 M, pH 6.9). After pre-incubation at 37 °C for 5 min, 20 µL of p-nitrophenyl-α-D-glucopyranoside (pNPG; 3 mM; Cayman, USA) was added to the mixture, which was then incubated at 37 °C for 30 min. The reaction was terminated with 50 µL of sodium carbonate (0.1 M) and the absorbance was measured at 405 nm using a BMG Labtech SPECTROstar (Germany). Acarbose (Bayer, Germany) was used as the positive control, and the corresponding solvent mixture at the same volume served as the negative control.[24] Extract-specific blanks were prepared by replacing the enzyme and substrate with buffer while maintaining the same final reaction volume. The background absorbance of each extract was subtracted from that of the corresponding sample before calculating percentage inhibition.

$$\alpha\text{-Glucosidase inhibition \%} = [(A_{control} - A_{sample,\ corrected})/A_{control}] \times 100$$

$A_{control}$: The absorbance value obtained when the assay was performed with the negative control.

$A_{sample,\ corrected}$: The absorbance value obtained for the test sample (extract or positive control) after subtraction of the corresponding sample blank absorbance.

HPLC analysis

HPLC-DAD analysis was performed to determine selected target compounds in *O. europaea* leaf extracts. The extracts were dissolved in 50% acetonitrile at a concentration of 5 mg/mL and filtered through 0.45 µm PTFE membrane filters before injection. Since oleuropein was present at relatively high concentrations in the extracts, its determination was performed using a 0.5 mg/mL extract solution. Each sample was analyzed

in at least triplicate using an Agilent 1260 series HPLC system equipped with a G1311B quaternary pump, a G7115A diode-array detector, and a system controller.

Chromatographic separation was achieved using an ACE 5 C18 column (150 × 4.6 mm, 5 μm) maintained at 25 °C. The injection volume was 20 μL. The mobile phase consisted of acetonitrile (A) and ultrapure water (B), each containing 0.1% formic acid. The gradient elution program was based on the method described by Ekin et al.,[31] with minor modifications.

The target compounds comprised gallic acid, chlorogenic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, fumaric acid, rosmarinic acid, quinic acid, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, catechin, epicatechin, epigallocatechin, rutin, luteolin-7-O-glucoside, apigenin-7-O-glucoside, quercetin-O-glucoside, hesperidin, myricetin, quercetin, luteolin, apigenin, kaempferol, naringenin, oleuropein, tyrosol, and 3-hydroxytyrosol. For quantitative determination, calibration curves were established for each reference compound at six concentration levels. The corresponding regression equations and respective calibration curves were used to calculate the amounts of the detected compounds in the extracts.

Compound identification was performed by comparing the retention times and UV absorption spectra of the detected peaks with those of the corresponding reference standards analyzed under identical chromatographic conditions.

Analytical performance of the HPLC-DAD method

The analytical performance of the HPLC-DAD method was evaluated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), and intra-day precision.

Linearity was assessed using six concentration levels for each analyte within its respective calibration range and was evaluated based on the coefficient of determination (R^2) of the calibration equation.

The LOD and LOQ were estimated using the standard deviation of the analytical response and the slope of the calibration curve, according to the equations LOD = 3.3 × (SD/S) and LOQ = 10 × (SD/S), where SD is the standard deviation of the peak areas obtained from six replicate injections of the lowest calibration standard, and S is the slope of the corresponding calibration curve. Intra-day precision was assessed by analyzing the extract in triplicate (n = 3). The results were expressed as the relative standard deviation (RSD, %) of the determined analyte concentrations.

Statistical and data evaluation

All experiments were performed in triplicate as technical replicates (n = 3), and the results are presented as means ± standard deviation. IC_{50} values of the positive controls for cholinesterase and LOX assays, as well as that of OE-SFE for the α-glucosidase inhibition assay, were calculated using GraphPad Prism 8.0.

RESULTS

Total phenolic content

In the current study, total phenolic contents of the extracts were calculated using the equations y = 0.0031x + 0.0504, R^2 = 0.9997 derived from the corresponding calibration curves, and expressed as equivalents of gallic acid. The total phenolic contents of OE-SFE, OE-REA, and OE-RE were 44.23 ± 0.68, 72.56 ± 2.69, and 155.03 ± 5.60 mg GAE/g extract, respectively.

Antioxidant assays

The DPPH and ABTS•+ radical scavenging activities and FRAP of the *O. europaea* extracts are presented as Trolox equivalents (mg TE/g extract) in Table 1. Among the tested extracts, the ethanolic extract showed the highest DPPH radical scavenging activity. In the FRAP assay, the ethanolic extract demonstrated a higher reducing capacity than the corresponding SFE extract and the ethyl acetate extract of the post-SFE residue.

Enzyme inhibition results

According to the enzyme activity results, the ethyl acetate and ethanol extracts of the post-SFE residue of *O. europaea* showed low-to-moderate AChE and BChE inhibitory activity under the tested conditions, with lower inhibition values than those of the positive control, galantamine, whereas the main SFE extract showed a low level of AChE inhibition. OE-SFE exhibited 82.87% LOX inhibition at 200 μg/mL. Table 2 displays the extracts' inhibitory activity, expressed as percent inhibition.

The α-glucosidase inhibitory activities of the extracts are presented in Table 3 as percentage inhibition. OE-SFE showed a concentration-dependent increase in inhibition, reaching 79.72 ± 5.17% at 1600 μg/mL, whereas the post-SFE residue extracts did not show a similar concentration-dependent response. The IC_{50} value of OE-SFE was calculated as 520.01 ± 25.72 μg/mL.

HPLC analysis

HPLC-DAD analysis revealed marked differences in phenolic compound profiles between the SFE extract and the extracts obtained from the post-SFE residue (Table 4, 5 and Figure 1). Of

Table 1. Antiradical (DPPH, ABTS•+) activities and ferric reducing antioxidant power (FRAP) assay of *Olea europaea* leaf extracts.

Extract codes	Radical scavenging activity		FRAP (mg TE/g extract)
	DPPH (mg TE/g extract)	ABTS•+ (mg TE/g extract)	
OE-SFE	39.52 ± 2.85	8.43 ± 0.52	176.42 ± 7.26
OE-REA	42.66 ± 6.36	43.74 ± 1.75	363.08 ± 29.47
OE-RE	**143.66 ± 2.31**	48.31 ± 1.11	**529.47 ± 35.71**

Values are expressed as mean ± SD (n = 3). TE, trolox equivalents; SFE, supercritical fluid extraction.

Table 2. Enzyme (AChE, BChE, LOX) inhibitory activity of *Olea europaea* leaf extracts.

Extract Codes	Enzyme inhibition (inhibition % ± SD[a])		
	AChE 200 μg/mL[b]	BChE 200 μg/mL[b]	LOX 200 μg/mL[b]
OE-SFE	4.40 ± 0.80	-[c]	**82.87 ± 2.02**
OE-REA	**38.78 ± 0.43**	**46.35 ± 3.09**	4.21 ± 2.91
OE-RE	**35.67 ± 0.43**	**21.56 ± 1.85**	10.32 ± 3.27
References	94.03 ± 0.29[d] (IC_{50} = 0.78 ± 0.01 μg/ml)	76.48 ± 1.90[d] (IC_{50} = 37.72 ± 1.24 μg/ml)	83.13 ± 1.49[e] (IC_{50} = 17.92 ± 1.39 μg/mL)

[a]Standard deviation (n=3); [b]Final concentration; [c]No activity; [d]Galantamine (100 μg/mL), [e]Baicalein (100 μg/mL).
AChE, acetylcholinesterase; BChE, butyrylcholinesterase; LOX, lipoxygenase; SFE, supercritical fluid extraction; SD, standard deviation.

Table 3. α-glucosidase inhibitory activity of *Olea europaea* leaf extracts.

Extract codes	Enzyme inhibition (inhibition % ± SD[a])				
	100 μg/mL[b]	200 μg/mL[b]	400 μg/mL[b]	800 μg/mL[b]	1600 μg/mL[b]
OE-SFE	25.80 ± 2.56	29.02 ± 5.04	41.93 ± 1.23	**64.33 ± 4.07**	**79.72 ± 5.17**
OE-REA	26.25 ± 4.18	28.58 ± 5.41	24.77 ± 0.70	10.24 ± 2.39	-[c]
OE-RE	18.76 ± 2.59	20.76 ± 1.43	24.26 ± 0.25	21.20 ± 0.48	-[c]
Acarbose	50.19 ± 1.35	61.34 ± 1.39	69.54 ± 3.33	73.69 ± 1.79	82.10 ± 0.27

[a]Standard deviation (n = 3); [b]Final concentration; [c]Not detected.
SD, standard deviation; SFE, supercritical fluid extraction.

the seven quantified compounds, only vanillic acid was detected in OE-SFE (0.24 ± 0.01 mg/g), while the other compounds were quantified in one or both post-SFE residue extracts. All seven compounds were detected in OE-RE, whereas six were quantified in OE-REA. Oleuropein was the most abundant compound in both residue extracts, with concentrations of 35.64 ± 2.52 and 37.77 ± 1.75 mg/g in OE-RE and OE-REA, respectively.

Intra-day precision was assessed using three injections of the OE-RE extract performed at different time points during the analytical sequence on the same day. Results are expressed as relative standard deviation (RSD, %).

DISCUSSION

Olive production is increasing worldwide, generating large amounts of olive leaf biomass. Olive leaf, an agro-food by-product of olive oil production, is a source of food bioactive compounds.[1] SFE is increasingly used to recover these high-value compounds.[7]

SFE is generally regarded as a selective technique for the recovery of non-polar constituents; therefore, polar phenolics, flavonoids, and bound phenolic compounds are often expected to remain in the residual biomass after extraction. However, the medium's polarity changes substantially when an ethanol-water co-solvent is introduced, enabling recovery of more polar phytochemicals.[7]

The markedly different phenolic profiles observed between OE-SFE and the post-SFE residue extracts provide important insight differences in the phytochemical composition of the investigated extracts. Under the applied analytical conditions, vanillic acid was the only quantified phenolic compound in OE-SFE, whereas oleuropein, 3-hydroxytyrosol, luteolin-7-O-glucoside, apigenin-7-O-glucoside, luteolin, and apigenin were not detected. The same compounds were nevertheless recovered from the spent leaves when the residual matrix was subjected to a subsequent solvent extraction. The detection of these compounds in the residue-derived extracts demonstrates that phenolic constituents can be recovered from the post-SFE biomass. Although this compound-dependent behaviour appears consistent with the low polarity of supercritical CO_2, which is a poor solvent for the polar polyphenols and glycosides present in plant matrices, the SFE extract of *O. europaea* examined in this study was prepared on an industrial scale with a co-solvent, so a higher content of polar compounds could have been expected.[32] This result should not, however, be interpreted as a failure. Rather, it can be described as a purpose-driven extraction, tuned to target selected compound classes.[8,9,33] Since phenolic compounds were recovered from the post-SFE residue, the residual biomass may be suitable for further extraction with more polar solvents.[18] The two-step approach, SFE followed by solvent extraction of the residue, can thus be considered a potential approach for the valorization of residual olive leaf biomass.

Although *O. europaea* leaf extracts have been shown to have antioxidant, cholinesterase-inhibitory, anti-inflammatory, and α-glucosidase-inhibitory qualities, their strength may vary depending on the extract composition.[5,6] In the present study, we

Table 4. HPLC-DAD quantification of phenolic compounds in *Olea europaea* leaf extracts.

Compounds	OE-SFE (mg/g)	OE-RE (mg/g extract)	OE-REA (mg/g extract)	OE-RE (mg/g dry post-SFE residue)	OE-REA (mg/g dry post-SFE residue)
3-hydroxytyrosol	-[a]	0.2212 ± 0.0004	-[a]	0.0495 ± 0.0001	-
Vanillic acid	0.2356 ± 0.0076	0.0481 ± 0.0000	0.0829 ± 0.0080	0.0108 ± 0.0000	0.0070 ± 0.0007
Luteolin-7-O-glucoside	–	**6.7396 ± 0.0893**	**3.5085 ± 0.0678**	**1.5097 ± 0.0200**	**0.2982 ± 0.0058**
Apigenin-7-O-glucoside	–	0.7510 ± 0.0234	0.5480 ± 0.0198	0.1682 ± 0.0052	0.0466 ± 0.0017
Oleuropein	–	**35.6353 ± 2.5197**	**37.7707 ± 1.7458**	**7.9823 ± 0.5644**	**3.2105 ± 0.1484**
Luteolin	–	1.4551 ± 0.0169	0.8939 ± 0.0174	0.3259 ± 0.0038	0.0760 ± 0.0015
Apigenin	–	0.2266 ± 0.0013	0.2330 ± 0.0105	0.0506 ± 0.0003	0.0193 ± 0.0004

[a]Not detected under the applied analytical conditions.
SFE, supercritical fluid extraction.

Table 5. Analytical performance parameters of the HPLC-DAD method.

Compounds	Range (ppm)	Retention time (min)	Detection wavelength (nm)	Calibration equation	Coefficient of determination (R^2)	LOD (ppm)	LOQ (ppm)	Intra-day RSD, %
3-hydroxytyrosol	1-100	8.805	280	y = 68.112x + 33.485	0.9995	0.0787	0.2384	0.19
Vanillic acid	0.2-100	16.350	260	y = 111.14x - 9.3263	0.9999	0.0084	0.0254	0.00
Luteolin-7-O-glucoside	1-100	32.691	260	y = 56.518x - 19.206	0.9997	0.0128	0.0388	2.37
Apigenin-7-O-glucoside	1-100	35.999	260	y = 58.681x - 28.188	0.9998	0.0385	0.1168	3.11
Oleuropein	1-100	37.884	280	y = 14.986x + 3.8344	0.9995	0.0476	0.1442	5.88
Luteolin	1-100	41.030	350	y = 82.017x - 23.715	0.9997	0.0162	0.0490	1.16
Apigenin	1-100	42.881	350	y = 88.801x - 6.1074	0.9998	0.0254	0.0770	0.56

HPLC-DAD, high-performance liquid chromatography with diode-array detection; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

investigated the potential existence and extent of these biological properties in the olive leaf biomass remaining after SFE.

The antioxidant screening results of the residue-derived extracts were consistent with their phenolic profiles, whereas the LOX and α-glucosidase inhibition observed for OE-SFE may be associated with constituents not included in the targeted HPLC-DAD analysis. The minor cholinesterase inhibitory activity observed in the post-SFE residue extracts also suggests some inhibitory potential under the tested conditions.

The α-glucosidase and LOX inhibition observed for OE-SFE could not be fully explained by the targeted phenolic profile determined by HPLC-DAD. Vanillic acid was the only phenolic compound detected in OE-SFE, yet the extract showed inhibition of both α-glucosidase and LOX under the tested conditions. Other constituents not included in the targeted HPLC-DAD analysis, such as terpenoids, fatty acids, triterpenes, or secoiridoid aglycones, may have contributed to the observed activities. However, these compounds were not characterized in the present study, and their possible contribution remains hypothetical.[8,33-35]

However, the above associations should be interpreted with caution, as none of the extracts are single-compound systems. Phytochemical mixtures can act synergistically or antagonistically,[36] and for olive leaves in particular, biological activity may involve the combined contributions from several phenolic constituents rather than oleuropein alone.[37]

Beyond their biological activities, the phytochemical composition of the post-SFE residue extracts provides further evidence for the valorization potential of the residual olive leaf biomass. Notably, oleuropein was found together with 3-hydroxytyrosol and flavonoids luteolin-7-O-glucoside, apigenin-7-O-glucoside and luteolin, demonstrating that these compounds can be recovered from the post-SFE residue.[7] Although OE-REA contained slightly more oleuropein per gram of extract, OE-RE provided a higher

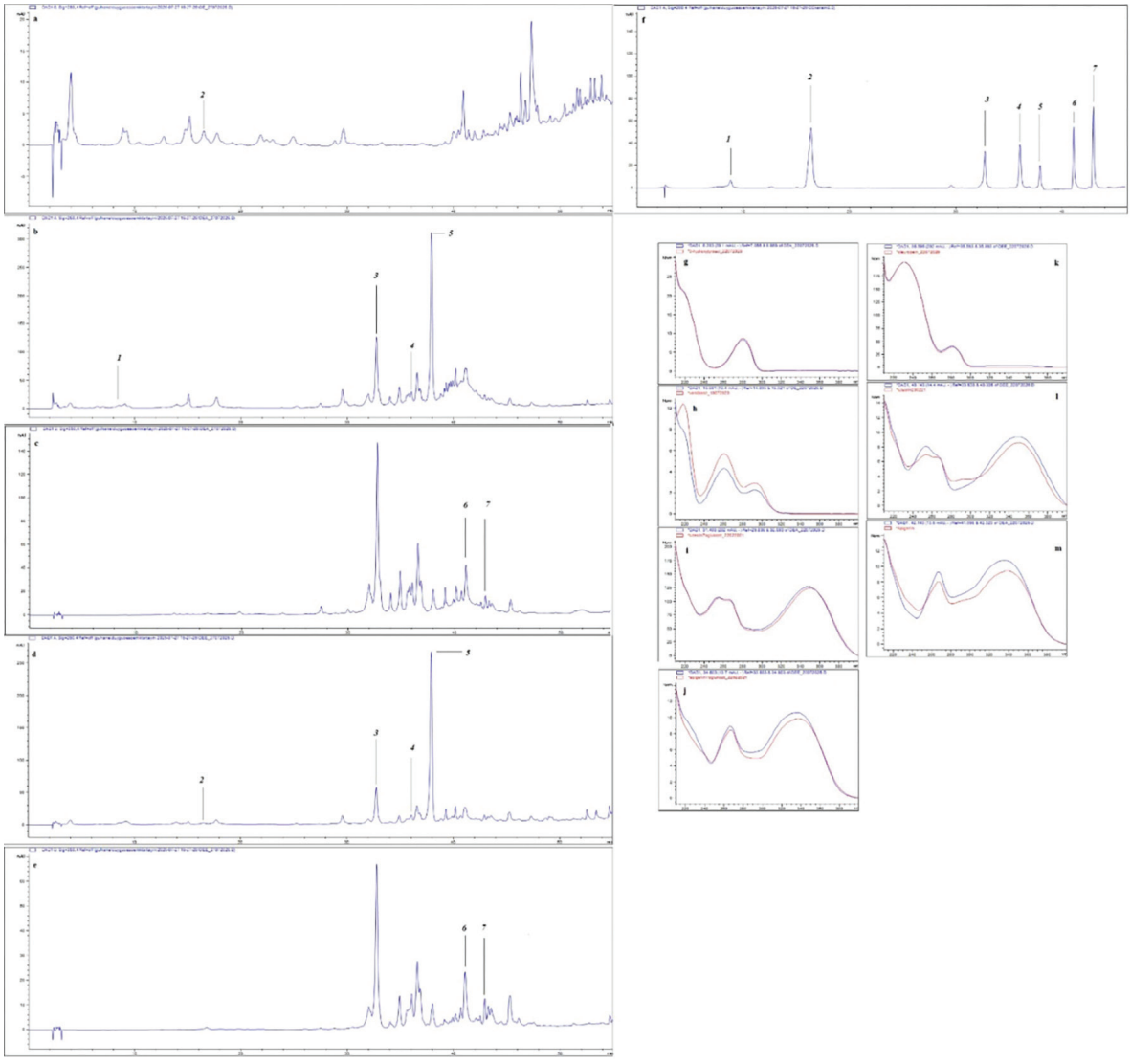

Figure 1. HPLC-DAD chromatographic profiles and UV spectral confirmation of selected compounds in *Olea europaea* leaf extracts. (a) OE-SFE chromatogram at 260 nm; (b) OE-RE chromatogram at 260 nm; (c) OE-RE chromatogram at 350 nm; (d) OE-REA chromatogram at 260 nm; (e) OE-REA chromatogram at 350 nm; and (f) reference standard mixture chromatogram. The identities of the detected peaks were supported by comparison of their UV spectra with those of the corresponding reference standards at the same retention times: (g) 3-hydroxytyrosol, (h) vanillic acid, (i) luteolin-7-O-glucoside, (j) apigenin-7-O-glucoside, (k) oleuropein, (l) luteolin, and (m) apigenin. Numbered peaks indicate the identified compounds: (1) 3-hydroxytyrosol, (2) vanillic acid, (3) luteolin-7-O-glucoside, (4) apigenin-7-O-glucoside, (5) oleuropein, (6) luteolin, and (7) apigenin.

HPLC-DAD, high-performance liquid chromatography with diode-array detection.

amount per gram of dry post-SFE residue due to its higher extraction yield. The remaining compounds are precisely those that make olive leaves an attractive by-product from an industrial point of view. Oleuropein is the most abundant phenolic in olive leaves and the main component responsible for the antioxidant and bioactive properties of hydroalcoholic extracts,[38] with contents reported up to 14% on a dry basis.[39] Among the six Spanish cultivars analyzed, luteolin-7-O-glucoside and apigenin-7-O-glucoside were the most predominant flavonoids.[16] Both oleuropein and luteolin-7-O-glucoside have been targeted in recovery strategies, with up to 69.91 g/kg oleuropein and 1.82 g/kg luteolin-7-O-glucoside obtained with optimized green extractions.[38] Thus, the presence of these compounds in the material after SFE indicates that the residual biomass should not be considered as an exhausted extraction residue but as a secondary source of recoverable phytochemicals. SFE-defatted matrices of other plant materials have been demonstrated to contain recoverable phenolic compounds and to be valuable for subsequent extraction with more polar solvents.[18,32] Sequential extraction of these residual compounds can increase the total use of the initial biomass, reduce the loss of valuable compounds, and help incorporate SFE residues into cascade extraction, biorefinery, and circular economy systems.[38,40]

Study limitations

A limitation of the present study is that the HPLC-DAD analysis was limited to selected phenolic compounds. Further studies with more comprehensive phytochemical profiling and biological models may provide a better understanding of the bioactive potential of post-SFE olive leaf residues.

An additional limitation of the present study concerns the availability of detailed operating parameters for the industrial SFE process. Since the primary SFE extract and corresponding residual biomass were obtained from a commercial manufacturer, the specific extraction conditions were not accessible to the authors due to their proprietary nature.

Consequently, the primary industrial extraction process cannot be independently reproduced solely on the basis of the information presented in this study. Moreover, the absence of detailed operating parameters and a pre-SFE phytochemical baseline prevents quantitative assessment of extraction selectivity, compound depletion, and overall recovery efficiency.

Nevertheless, these limitations do not affect the direct analytical characterization of the supplied extracts or the experimentally demonstrated recovery of selected phenolic compounds from the post-SFE residual biomass.

Accordingly, the findings should be interpreted as evidence of the phytochemical and biological potential of a specific industrially generated residual plant material rather than as a comprehensive evaluation or optimization of the original SFE manufacturing process.

CONCLUSION

This study shows that phenolic compounds can be recovered from *O. europaea* leaf biomass following industrial SFE and that post-SFE biomass has phytochemical and biological potential. Among the targeted phenolics in the primary SFE extract, only vanillic acid was detected, but several valuable phenolic compounds were recoverable from the spent material. In particular, oleuropein, a commercially relevant secoiridoid of olive leaves, was quantified in both residue-derived extracts, while luteolin-7-O-glucoside was recovered at different levels in the residue-derived extracts, thus highlighting the post-SFE biomass as a potential secondary source of phenolic compounds.

Overall, these results indicate that post-SFE olive leaf biomass should not be viewed as an exhausted processing residue but rather as a secondary bioactive matrix that contains recoverable phenolic constituents for high value-added phytochemical recovery. The extraction of this material subsequently, if incorporated in a continuous valorization strategy, could enhance the global utilization of olive leaf biomass and support a more sustainable and resource-efficient processing along the olive agro-industrial chain.

Ethics

Ethics Committee Approval: Ethics committee approval and patient informed consent were not required for this study, as the research was conducted exclusively using plant materials.

Informed Consent: Ethics committee approval and patient informed consent were not required for this study, as the research was conducted exclusively using plant materials.

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Footnotes

A part of this study (excluding the HPLC-DAD results) was presented at the 26th Bitkisel İlaç Hammaddeleri Toplantısı (26th BİHAT), held in İstanbul, Türkiye, on 9–12 September 2026, and its abstract was published in the congress proceedings.

Authorship Contributions

Concept: D.S.T., L.A., Design: D.S.T., Data Collection or Processing: D.S.T., T.B., H.N.G., Analysis or Interpretation: D.S.T., T.B., H.N.G., F.S.Ş.D., Literature Search: D.S.T., Writing: D.S.T., T.B., H.N.G., F.S.Ş.D., L.A.

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