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Green Extraction of Polyphenols from Olive Flesh Pits and Leaves by Supercritical CO₂

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Abstract

Olive flesh, pits, and leaves obtained from the olive tree (*Olea europaea L.*) are rich sources of polyphenolic compounds. Oils and extracts derived from olive flesh, olive pomace, and olive leaves are complex mixtures containing more than a hundred compounds with diverse chemical structures. Freeze drying was employed as an effective preservation technique to maintain the integrity of heat-sensitive compounds while ensuring the production of high-quality dried materials.

This study aimed to identify and quantify the active phenolic constituents in oils and extracts obtained from olive flesh, pits, and leaves using supercritical fluid extraction (SFE). The SFE process was also conducted with the inclusion of an ethanol co-solvent to enhance extraction efficiency. The extraction yields and total polyphenol contents were determined as follows: olive flesh CO_2 (OF-1) 32.11%, 527 mg/kg; olive flesh CO_2 + ethanol (OF-2) 54.21%, 1470 mg/kg; olive pit CO_2 (OP-1) 5.79%, 354 mg/kg; olive pit CO_2 + ethanol (OP-2) 5.85%, 1180 mg/kg; olive leaf CO_2 (OL-1) 4.46%, 485 mg/kg; and olive leaf CO_2 + ethanol (OL-2) 6.26%, 1275 mg/kg. LC-MS analysis of the olive leaf extracts revealed that several phenolic compounds—including 3-hydroxytyrosol, protocatechuic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillin, ferulic acid, pinoresinol, oleuropein, kaempferol, and 2,5-dihydroxybenzoic acid—were present in higher concentrations in the OL-2 sample compared to OL-1. Kaempferol was not detected in OL-1, while the remaining compounds were found in relatively small quantities in both extracts. Both OL-1 and OL-2 demonstrated notable antioxidant activity, further confirming the bioactive potential of the extracted compounds.

In all tested materials, extraction yields, total polyphenol contents, and phenolic compound levels were relatively high, indicating the effectiveness of the supercritical CO_2 (SC- CO_2) process. The addition of ethanol as a co-solvent significantly enhanced phenolic compound recovery, achieving up to a 3.3-fold increase compared with CO_2 -only extraction. These results demonstrate that supercritical fluid extraction systems represent a promising, sustainable, and environmentally friendly technology for the efficient recovery of phenolic compounds from olive by-products, contributing to the valorization of agricultural residues and the development of high-value natural antioxidants for food, cosmetic, and pharmaceutical applications.

Key Words: Olive flesh, olive pit, olive leaf, phenolic compounds, freeze drying, Supercritical extraction

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1. Introduction

Olea europaea L. leaves are well known for their high content of bioactive polyphenolic compounds, which contribute to their diverse biological activities (Cavaca et al., 2018; Clodoveo et al., 2022). Oleuropein is the dominant polyphenolic compound in Olea europaea, and it is also present across various genera within the Oleaceae family, including Syringa, Jasminum, Phillyrea, and Fraxinus (Soler-Rivas et al., 2000; Khalil et al., 2023). Within olive trees, oleuropein is found in leaves, fruit, pulp, and seeds, with the highest concentrations generally observed in unripe fruits and leaves, ranging from 60 to 90 mg per gram of dry leaf weight (Rahmanian et al., 2015). Its content is influenced by climatic conditions, genetic variability, cultivar, agricultural practices, harvest time, plant part, and extraction method (Rahmanian et al., 2015; Otero et al., 2021).

Olive seeds are a major component of solid waste from olive processing, particularly in Europe, which accounts for nearly 67% of global olive oil production (European Commission, n.d.). These seeds can be separated from the fruit pulp during oil extraction and have potential as a by-product for renewable energy or bioactive oil production (Leone et al., 2015). The oil derived from seeds is rich in polyunsaturated acids (PUFAs), especially fatty 1.2dilinoleoyl-3-oleoyl-glycerol, while total aliphatic long-chain and triterpene alcohols are present at lower concentrations (Ranalli et al., 2002; Rodríguez et al., 2008). Oxidative stability is a key factor for seed oil quality, as PUFA oxidation negatively affects food safety and shelf life (Mosibo et al., 2022).

Olive leaf extracts are recognized for their potent antioxidant properties due to a wide range of phenolic compounds, including phenolic acids, hydroxytyrosol, tyrosol, flavonoids (e.g., luteolin 7-0-glucoside, rutin, apigenin 7-0-glucoside), and secoiridoids such as oleuropein. Oleuropein exhibits antioxidant, antimicrobial, antiviral,

cardioprotective, anti-inflammatory, hypocholesterolemic, and hypoglycemic effects (Sahin et al., 2011). Using the whole extract rather than isolated compounds may provide enhanced health benefits due to synergistic interactions among phenolics (Pereira et al., 2007). Olive leaves also contain other bioactive molecules such as verbascoside, apigenin, luteolin, diosmetin, oleanolic and maslinic acids, hydroxytyrosol, tyrosol, caffeic, vanillic, and ferulic acids, making them promising sources functional foods and cosmetic applications (Dauber et al., 2022; Kyriakoudi et al., 2024).

SFE has emerged as an efficient technique for isolating high-value compounds from plant matrices. Compared to conventional Soxhlet extraction, SFE provides higher selectivity, cleaner extracts, shorter extraction time, and lower solvent usage, with process selectivity adjustable through pressure temperature (Wang et al., 2006; Sahin et al., 2011). Co-solvents such as ethanol or water can enhance recovery of polar phenolics, and both SC-CO₂ and these modifiers are generally recognized as safe (GRAS) for food and pharmaceutical applications (da Silva et al., 2016).

Previous studies have explored the effects of different drying treatments and co-solvents on olive leaf extracts, showing that extraction parameters significantly influence phenolic recovery (Canabarro et al., 2019; Dauber et al., 2022; Baldino et al., 2018; Kyriakoudi et al., 2024).

In this study, freeze-dried olive fruits, pits, and leaves from Aydın Province (Türkiye) were subjected to SFE using CO_2 as the primary solvent. The physical and chemical properties of the resulting extracts were evaluated, focusing on maximum extraction yields, total polyphenol content, and potential applications as functional food ingredients.

2. Material and Methods

2.1. Raw material and sample preparation

Olive flesh, pits, and leaves were collected during September and October from orchards in the Aydın region (Türkiye). The pitted and fleshy portions of the olive fruit were manually separated. Olive leaves were shade-dried under ambient conditions.

Approximately 15 kg of olive flesh and pits were lyophilized using a freeze dryer (Liyolife FD5CT, 900 W, Türkiye) and subsequently milled with a grain mill (Emir Industrial Kitchen Products EMR-Ö-01, 1.5 kg capacity, Türkiye). The particle size was standardized to <0.30 mm using a stainless-steel sieve. All prepared samples were stored at +4 °C in airtight containers until analysis.

2.2. Supercritical fluid extraction (SFE)

The SFE system (Polat Makina, Türkiye) consisted a CO_2 storage of recirculating chiller, high-pressure CO2 and co-solvent pumps, extraction vessel, heat exchanger, separation unit, automated back-pressure regulator, and programmable controller logic (PLC) (Figure 1).

For pure CO_2 extractions, sample loading amounts were 1075 g for olive flesh (OF-1), 1180 g for olive pits (OP-1), and 900 g for olive leaves (OL-1). For CO_2 + ethanol cosolvent runs (OF-2, OP-2, OL-2), 1050 g, 1160 g, and 1030 g were used, respectively.

All extractions were performed in triplicate (n = 3). The four main operating parameters—temperature (°C), pressure (bar), ethanol flow rate (qEtOH, kg/h), and CO_2 flow rate (qCO₂, kg/h)—were selected based on (i) preliminary optimization trials and (ii) literature reporting parameter ranges that enhance phenolic solubility and extraction efficiency in SC-CO₂ systems.

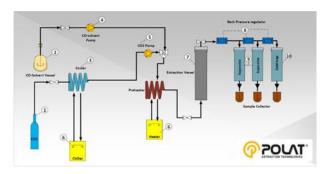


Figure 1. Diagrammatic overview illustrating the components and workflow of the Polat SFE system (Burgaz et al., 2024)

2.3. Supercritical CO₂ (SC-CO₂) extraction of oil and extract of olive flesh, olive pit and olive leaf

SC-CO₂ extraction was applied as environmentally friendly technique obtaining oils and bioactive compounds from olive flesh, pits, and leaves. CO₂ was brought above its critical point (31 °C and 73 atm), enabling it to behave simultaneously as a gaslike and liquid-like solvent. Freeze-dried samples were extracted using either pure CO₂ or CO₂ with ethanol as a co-solvent. **Ethanol-containing** extracts were concentrated using a rotary evaporator bundle system) (BUCHI R300 under controlled temperature and vacuum conditions. All oils and extracts were stored at +4 °C until further analysis. Each extraction condition was conducted in triplicate to ensure reproducibility and statistical validity.

2.4. Design of experiments (DoE) and process optimization

DoE approach was applied to systematically evaluate the effects of extraction parameters on yield and phenolic recovery. Within SC-CO₂ extraction, DoE enables optimization of variables such as temperature, pressure, CO₂ flow rate, extraction time, and co-solvent concentration. Freeze-dried olive flesh, freeze-dried olive pits, and shade-dried olive leaves were loaded into the extractor following the procedure described above. Six experimental runs were conducted under different parameter combinations and

extraction times (Table 1). All extracts were stored at +4 °C before analysis. The parameter sets selected for the DoE were derived from preliminary experiments showing improved extraction efficiency and supported by relevant literature. The

resulting extracts demonstrated high yields and significantly increased polyphenol contents—particularly in ethanol-assisted runs—confirming the suitability of the selected parameters.

Table 1. Optimal SFE conditions for obtaining higher global yields and phenolic compounds from olive by-products.

Sample	Pressure (bar)	Temperature (°C)	Time (min)	Sample (g)	qEtOH (kg/h)	qCO ₂ (kg/h)
OF-1	350	45	180	1075	-	9
OP-1	350	45	180	1180	_	9
OL-1	300	45	180	900	_	9
OF-2	350	45	180	1050	2	9
OP-2	350	45	180	1160	2	9
OL-2	300	45	180	1030	2	9

Values represent means ± SD of three replicates; different letters within columns indicate significant differences, p < 0.05

2.5. Analysis of phenolic compounds

Phenolic compounds were quantified using HPLC-UV and LC-MS/MS. HPLC-UV was employed for olive flesh and pit extracts, while olive leaf extracts (OL-1 and OL-2) were further characterized using LC-MS/MS (Agilent 1260 Infinity LC coupled with an Agilent 6420 Triple Quadrupole MS).

Chromatographic separation was performed using a reversed-phase C18 column with water and methanol/acetonitrile mobile phases under typical analytical conditions (flow 0.2–1.0 mL/min, 25–40 °C). LC-MS/MS acquisition was conducted using ESI or APCI ionization in multiple reaction monitoring (MRM) mode.

Authentic standards—oleuropein, hydroxytyrosol, tyrosol, luteolin, and apigenin—were used to prepare calibration curves. All analyses were conducted in triplicate, and results were expressed as mean ± SD.

2.6. Calculation of extraction yields

Extraction yields for olive flesh, pit, and leaf materials were calculated on a dry-matter basis according to (Felicia et al. 2024).

Yield of olive flesh, olive pith and olive leaf extracts (%) =

$$\left(\frac{\text{Weight of collected extract (g)}}{\text{Total weight of the sample (g)}}\right) \times 100$$

Following each extraction, the mass of recovered oil or extract was measured using an analytical balance. Yield values were normalized to the initial dry sample mass.

All yield measurements were conducted in triplicate to ensure accuracy and reproducibility.

2.7. Antioxidant capacity by DPPH method

The DPPH radical scavenging activity of olive leaf extracts (OL-1 and OL-2) was determined according to Skupień et al. (2004) with minor modifications. A 0.1 mM DPPH solution (2.9 mL) was mixed with 0.1 mL of extract and incubated for 30 min in the dark at ambient temperature. Absorbance was measured at 517 nm. Antioxidant activity was expressed as μ mol trolox equivalents per gram (μ mol TE/g). Each assay was performed in triplicate, and results were reported as mean \pm SD.

2.8. Statistical analysis

All analyses of olive flesh, pit, and leaf extracts were performed in triplicate on independent aliquots. Results were expressed as mean \pm SD. Differences among sample groups were assessed using one-way ANOVA followed by Tukey's post hoc test. Student's t-test was used where applicable. Statistical significance was set at $\alpha = 0.05$. Analyses were performed using IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Oil and extract yield of olive flesh, olive pits and olive leaves

The oil and extract yields obtained from olive flesh, pits, and leaves depend on several factors, including cultivar, growing conditions, maturity stage, and extraction technique. In the present study, all three matrices produced measurable yields and quantifiable levels of phenolic compounds, demonstrating their potential as viable raw materials for SC-CO₂ extraction. Olive pits and leaves—typically considered industrial by-products—showed notable extractability, supporting their potential conversion into value-added products. Literature consistently reports that olive leaves contain substantially higher phenolic concentrations than olive fruits and extravirgin olive oil.

Fresh olive leaves may reach approximately 1450 mg total phenolics per 100 g, compared with 110 mg/100 g in olive fruit and 23 mg/100 mL in extra-virgin olive oil (Bonacci et al., 2020; Kountouri et al., 2007). Reported phenolic levels in dried leaves range even higher, such as 2733.33 \pm 0.15 mg GAE/100 g (Pazir et al., 2019). Olive pits have also been shown to yield polyphenols under SC-CO₂, albeit at lower levels (0.36 g GAE/kg; Mosibo et al., 2022).

Previous studies have demonstrated that extraction parameters strongly influence phenolic recovery. For example, Sahin et al. (2011) reported that adding 20% co-solvent significantly improved oleuropein extraction from olive leaves, with solvent critically affecting efficiency. type Kyriakoudi et al. (2024) observed that increasing temperature from 35 to 90 °C enhanced SC-CO₂ extraction yields $(2.1\% \rightarrow$ promoted 4.3%) and higher polyphenol recovery when methanol or aqueous ethanol were used as co-solvents. Similarly, high phenolic levels (up to 300 mg GAE/g) were achieved with ethanolassisted extraction at elevated temperature (C/E/90).

For olive fruit, previously reported SC-CO₂ extraction yields vary widely depending on pretreatment and operating conditions. Belbaki et al. (2017) obtained 33.35 wt% at 300 bar and 60 °C, while uncrushed olives yielded 12.3 wt% compared with 21.89 wt% for crushed olives under similar pressures (Al-Otoom et al., 2014), highlighting the importance of particle size reduction for improving mass transfer.

In the present study, freeze-dried olive flesh and pits, and shade-dried olive leaves were extracted using an SFE system (PEX1002C). The resulting oil and extract yields, together with their total polyphenol contents, are presented in Table 2. Compared to values reported in the literature, the current results indicate that the selected extraction conditions—particularly those involving ethanol as a co-solvent-enabled higher recovery of polyphenols while maintaining efficient overall extraction (Figure 2). These findings confirm that SFE, when optimized, can effectively recover bioactive compounds from olive-derived materials.

Table 2. Yield of total extracts from supercritical fluid extraction of olive flesh, olive pits and olive leaves and total polyphenol analysis (mg/kg, in tyrosol)

Sample	Yield (%) (mean ± SD)	Total polyphenol (mg/kg as tyrosol) (mean ± SD)	Statistical significance*
OF-1	32.11 ± 1.25	527 ± 18	a
OP-1	5.79 ± 0.20	354 ± 12	b
OL-1	4.46 ± 0.15	485 ± 16	b
OF-2	54.21 ± 2.10	1470 ± 45	С
OP-2	5.85 ± 0.18	1180 ± 38	d
OL-2	6.26 ± 0.22	1275 ± 40	d

^{*}Statistical significance letters (a-d) indicate groups that are significantly different (p < 0.05, Tukey's post hoc test)

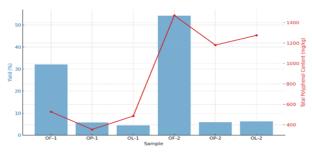


Figure 2. Graph showing yield (%) values and total polyphenol contents (mg/kg tyrosol equivalent) of different olive sample runs (OF-1, OP-1, OL-1, OF-2, OP-2, OL-2)

3.2. Characterization of phenolic compounds in olive leaves

In this study, olive leaf extracts OL-1 and OLobtained at maximum extraction efficiency using the PEX1002C supercritical fluid extraction system, were analyzed by LC-MS to identify and quantify the polyphenolic compounds present (Table 3). results revealed LC-MS differences in the phenolic profiles of extracts obtained under the two extraction conditions. The addition of ethanol as a cosolvent in the OL-2 extraction enhanced the recovery of several bioactive phenolic compounds compared with the OL-1 extract. These compounds included 3hydroxytyrosol, protocatechuic acid, 3hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillin, ferulic acid, pinoresinol, oleuropein, kaempferol, and dihydroxybenzoic acid. Notably, kaempferol was not detected in the OL-1 extract. indicating that co-solvent addition facilitated its extraction from the olive leaf matrix. These results demonstrate that the

use of a polar co-solvent during SC-CO₂ extraction improves the recovery of phenolic compounds from olive leaves, particularly more polar constituents. Quantitative data for each identified compound are summarized in Table 3.

3.3. Antioxidant Activity of Olive Leaf

Antioxidants are molecules that neutralize free radicals, which are highly reactive species with unpaired electrons. If not properly regulated, free radicals can accumulate in the human body, leading to oxidative damage to lipids, proteins, and DNA, and contributing to aging and agerelated diseases (Sahin et al., 2011). In this study, phenolic compounds were extracted from shade-dried olive leaves using SC-CO₂. The primary objective was to evaluate the effect of extraction conditions on total phenolic content and antioxidant activity while maintaining constant temperature and pressure. The antioxidant activity of the extracts was measured using the DPPH assay, and results are summarized in Table 4. The OL-2 extract exhibited significantly higher antioxidant activity than OL-1, which can be attributed to the addition of ethanol as a co-solvent during extraction. Ethanol improved the solubility of polar phenolic compounds, enhancing their recovery and contributing to increased radical scavenging activity. These findings are consistent with previous reports showing that the use of ethanol in SC-CO₂ extraction significantly improves the yield of antioxidant compounds from plant matrices.

Table 3. Phenolic compound content in olive leaf extracts (OL-1 and OL-2) analyzed by LC-MS/MS (ng/mL, mean \pm SD)

Phenolic Compound	RT (min)	OL-1 (ng/mL)	OL-2 (ng/mL)	Statistical significance*
Gallic acid	8.88	98.99 ± 0.49	137.61 ± 0.28	a,b
3-Hydroxytyrosol	10.38	174.31 ± 2.94	8380.94 ± 15.06	С
2,5-Dihydroxybenzoic acid	10.75	38.82 ± 0.47	1704.19 ± 6.88	d
Protocatechuic acid	10.75	36.89 ± 0.71	1737.74 ± 15.32	d
3,4-Dihydroxyphenylacetic acid	11.07	48.00 ± 0.23	68.56 ± 0.68	a
Pyrocatechol	11.25	37.68 ± 7.49	135.33 ± 19.56	b
(+)-Catechin	8.52	4.62 ± 6.53	4.99 ± 4.43	a
Chlorogenic acid	11.81	86.04 ± 3.25	84.40 ± 0.82	a
3-Hydroxybenzoic acid	12.33	25.16 ± 0.07	1865.15 ± 9.88	С
4-Hydroxybenzoic acid	12.33	28.33 ± 0.01	1890.69 ± 20.47	С
(-)-Epicatechin	12.33	30.53 ± 2.20	39.73 ± 14.79	a
Homovanillic acid	12.79	122.92 ± 35.38	260.00 ± 20.93	b
Caffeic acid	12.81	41.85 ± 0.64	121.28 ± 27.04	b
Syringic acid	12.87	58.96 ± 6.58	286.66 ± 9.33	b
Vanillin	13.27	1158.36 ± 0.33	2225.58 ± 94.84	С
Verbascoside	13.59	44.85 ± 2.50	42.69 ± 0.14	a
Taxifolin	13.92	2.06 ± 0.91	252.07 ± 3.66	b
Sinapic acid	13.95	27.42 ± 0.73	244.13 ± 1.56	b
p-Coumaric acid	13.96	15.32 ± 0.19	862.06 ± 1.71	С
Ferulic acid	14.06	145.91 ± 7.87	1798.30 ± 7.07	С
Luteolin 7-glucoside	14.32	70.45 ± 2.11	86.65 ± 0.13	a
Hesperidin	14.42	12.95 ± 1.64	16.90 ± 3.97	a
Hyperoside	14.53	52.52 ± 1.57	53.45 ± 0.11	a
Rosmarinic acid	14.59	85.15 ± 2.25	84.02 ± 0.37	a
Resveratrol	14.68	9.08 ± 0.06	8.97 ± 0.05	a
Oleuropein	14.71	22.75 ± 1.68	68360.10 ± 1584.50	d
Apigenin 7-glucoside	14.82	10.56 ± 0.32	47.81 ± 3.14	b
2-Hydroxycinnamic acid	14.97	13.66 ± 0.88	17.31 ± 0.37	a
Ellagic Acid	14.96	107.61 ± 152.18	105.46 ± 2.03	a
Pinoresinol	15.09	439.22 ± 133.11	1010.18 ± 147.29	b
Eriodictyol	15.27	21.44 ± 1.71	226.55 ± 3.78	b
Quercetin	15.71	79.06 ± 14.89	462.53 ± 15.66	b
Luteolin	15.92	74.69 ± 11.09	667.58 ± 53.79	b
Kaempferol1	15.92	102.12 ± 0.81	491.83 ± 56.99	b
Kaempferol	16.27	nd	611.57 ± 212.74	b
Apigenin	16.36	5.41 ± 1.15	485.70 ± 42.32	b

^{*}Statistical significance letters (a-d) indicate groups that are significantly different (p < 0.05, Tukey's post hoc test). "nd" = not detected.

Table 4. Antioxidant activity of supercritical fluid extracts of olive leaves (mean ± SD, μmol TE/g)

Sample	DPPH (μmol TE/g) (mean ± SD)	Statistical significance*	
OL-1	5.98 ± 0.10	a	
OL-2	19.72 ± 1.04	b	

^{*}Statistical significance letters (a, b) indicate groups that are significantly different (p < 0.05, Tukey's post hoc test)

4. Conclusion

This study demonstrates that olive flesh, pits, and leaves are promising raw materials for the recovery of oils and bioactive compounds through SC-CO₂ extraction. Extraction yields and total polyphenol contents were consistently higher when ethanol was used as a cosolvent. Specifically, olive flesh yield increased from 32.11% with CO₂-only to 54.21% with CO₂+ethanol, with total polyphenol content rising from 527 ± 18 to 1470 ± 45 mg/kg. Olive leaves showed an increase from 4.46% to 6.26% yield and 485 ± 16 to 1275 ± 40 mg/kg polyphenols, while olive pits increased from 5.79% to 5.85% yield and 354 ± 12 to 1180 ± 38 polyphenols. Ethanol-assisted mg/kg extraction significantly enhanced the recovery of key phenolic compounds in olive leaves, including oleuropein (22.75 \rightarrow 68360.10 ng/ml), 3-hydroxytyrosol $(174.31 \rightarrow 8380.94 \text{ ng/ml})$, vanillin $(1158.36 \rightarrow 2226.58 \text{ ng/ml})$, ferulic acid $(145.91 \rightarrow 1798.30 \text{ ng/ml}), \text{ p-coumaric}$ acid (15.32 \rightarrow 862.06 ng/ml), kaempferol1 $(102.12 \rightarrow 491.83 \text{ ng/ml})$, luteolin (74.69 \rightarrow 667.58 ng/ml), and apigenin (5.41 \rightarrow 485.70 ng/ml). These increases directly translated to enhanced antioxidant potential, as demonstrated by DPPH assay $(5.98 \rightarrow 19.72 \mu mol TE/g)$. Although CO_2 only extraction yielded considerable amounts of oil and phenolic compounds, the addition of ethanol consistently improved both yield and phenolic recovery. These findings confirm that SFE, particularly when combined with a polar co-solvent, enables efficient and selective extraction of bioactive compounds from olive-derived materials. The results also highlight the importance of parameter optimization in maximizing extraction performance while preserving compound integrity.

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Author Contribution

Osman **BURGAZ:** Conceptualization. validation, formal analysis, investi-gation, writing manuscript. data curation, supervision, visualization, extraction, project administration. funding acquisition. Ilker YILDIRIM: Validation, investigation, datacuration, review and editing, supervision. Ferhat SANA: Formal analysis, investigation. Alper BAYCAN: Formal analysis, investigation. Hilal SAHIN NADEEM:Data analysis, review. Ibrahim POLAT: Conceptualization, validation. funding, funding acquisition.

Conflicts of Interest

The authors claim that they have got no conflict of interest.

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