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Extraction, Phytochemical Analysis, and Standardization of Oleuropein-Rich Olive Leaf Extracts: A Study Across Diverse Jordanian Regions

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ABSTRACT

This study aims to investigate and standardize olive leaf extract (OLE) from Jordanian olive trees, examining the impact of geographical differences on the extract's characteristics. Olive leaves were collected from Amman, Karak, Ajloun, and Mafraq and processed using a cost-effective hydro-alcoholic extraction method. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC-UV) were employed to identify and quantify oleuropein, the primary phenolic compound. Additionally, the effects of stress conditions (temperature, UV, humidity) on OLE from Karak were evaluated. The highest oleuropein concentration and total phenolic content were found in extracts from Karak. Physical properties, moisture, ash content, heavy metals, minerals, residual solvents, and microbiological purity were assessed for all extracts. The findings highlight that geographical factors such as altitude and rainfall significantly influence the phenolic content of OLE, with Karak yielding the highest quality extract. The study suggests Jordan's potential as a source of high-quality OLE and recommends further research to improve the stability and formulation of these extracts for therapeutic use.

Keywords: Olive leaves extract; Phytochemical analysis; Jordan; Standardization and quality control.

INTRODUCTION

The olive tree (Olea europaea L.) is believed to be the first domesticated fruit tree in the Mediterranean, and it continues to hold significant economic and cultural importance in this region (1). Olive oil is the primary product, with global production reaching approximately 11 million tons annually (1). The cultivation of olive trees also generates a substantial amount of olive leaves as by-products. These leaves are not merely agricultural waste; they are rich in polyphenolic compounds, particularly biophenols, which possess remarkable biological activities (2–6). The broad spectrum of bioactive compounds found in olive leaf extract (OLE) has garnered interest for their potential applications in cosmetics, pharmaceuticals, and as preservatives to extend the shelf life of food products (4,7,8).

Among the polyphenols in OLE, oleuropein is the most abundant and potent antioxidant, typically comprising between 17% and 23% of the total phenolic content (9). Oleuropein's antioxidant properties are primarily attributed to its ability to donate hydrogen atoms to free radicals, effectively neutralizing them and preventing oxidative chain reactions (10). In addition to its antioxidant capacity, oleuropein exhibits a wide range of pharmacological activities, including anti-inflammatory, anti-cancer, anti-viral, anti-microbial, anti-atherogenic, and anti-aging effects (11). These diverse biological activities have sparked increasing scientific interest in the potential pharmaceutical applications of OLE (12,13). The content of oleuropein in olive leaves varies across geographical regions due to differences in climate, soil composition, altitude, and specific cultivation techniques,

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such as irrigation and fertilization, which influence the plant's metabolic responses and secondary metabolite production. Therefore, understanding this variability is crucial for optimizing the therapeutic use of OLE. Reliable analytical methods are essential to accurately quantify oleuropein and other bioactive compounds.

The analysis of olive leaves requires precise methods for the separation, purification, standardization, and identification (both qualitative and quantitative) of their various constituents (14–16). Although the concept of standardization in phytomedicines is relatively recent, it has quickly become essential to ensure the delivery of high-quality botanical products. Standardization ensures that an extract contains a minimum amount of one or more key compounds, often within a specified range. This process is particularly crucial in phytomedicines, where standardization is applicable solely to extracts (17).

Various analytical tools are employed to ensure the quality and consistency of OLEs. High-performance liquid chromatography (HPLC) is widely used for the precise quantification of oleuropein and other phenolic compounds (17). In addition, Thin-Layer Chromatography (TLC) offers a cost-effective method for the preliminary screening and identification of these compounds, allowing for rapid comparison of samples and confirming the presence of specific bioactive compounds before undertaking more detailed quantitative analyses. Together with other techniques, such as mass spectrometry and spectrophotometry, these methods are integral to the quality control process, ensuring the consistency and efficacy of phytochemical extracts (18,19). Numerous studies have investigated the factors influencing the phenolic profile of olive leaves, including leaf age, ripeness, geographical origin, cultivar, phenological stage during sampling, branch proportion on the tree, moisture content, and industrial extraction processes (2). However, there has been no study specifically evaluating the phenolic profile of Jordanian olive leaves.

In this context, our study aims to investigate the extraction, standardization, and quality control of OLEs from Jordanian olive trees, marking the first such comprehensive analysis. To assess the influence of geographical origin and identify the optimal source of olive leaf extract within Jordan, we selected four distinct regions: Amman, Karak, Ajloun, and Mafraq. This study offers a thorough characterization and standardization of olive leaf extracts, providing a foundational basis for the commercialization of Jordanian olive leaf extract. Moreover, our findings could serve as the groundwork for establishing a preliminary certificate of analysis, ensuring product quality and reliability.

Experimental

Handling of Plant Material

In order to observe the effect of the geographical origin, the following four different regions in Jordan were chosen: Amman, Karak, Ajloun, and Mafraq. These regions have different climates, altitudes and total annual precipitation. Fresh olive leaf samples were collected in the middle of April 2018 from different parts of three trees in the same region. All olive trees were of the same age (about ten years old). A taxonomist confirmed the botanical identity of the plant sample. It was authenticated to be *Olea europaea* L. Sativa (Loudon) Arcang, which belongs to the Oleaceae family. All samples were confirmed to be from the same cultivar and variety. A plant specimen from each geographical area was deposited at the University of Petra Herbarium and given the numbers (OL 1-4/2018). Additionally, a voucher specimen number UOP04/2018/Olea europaea of the leaves was kept for future reference. The collected olive leaf samples were dried using a traditional drying method by shading in a dark place at room temperature for about three weeks, and then the dried olive leaf samples were powdered by using a grinding machine (MOULINEX grinder A843, Mexico). The powdered samples were stored at ambient temperature in a dark place until further extraction.

Extraction

Olive leaf powder (100 g) was added to 500 ml of 80%

ethanol (v/v) in a round-bottom flask and refluxed for approximately 4-5 hours at 70ºC (9,13). After refluxing, the extracts were allowed to cool to room temperature for about 1 hour. The cooled extracts were then filtered, and the liquid filtrates were concentrated using a rotary evaporator (HAHNSHIN S&T CO., LTD., Korea) at 70ºC with a rotation speed of 120 rpm under vacuum. The solvent-free OLE was subsequently dried using a freeze dryer system at -52ºC and 0.2 mbar. The resulting dried OLE powder was stored in an opaque glass container in a refrigerator at 2-8ºC until further analysis. The percentage yield (w/w) of OLE from different regions in Jordan was calculated according to Equation 1.

$$\% \text{ Yield} = \frac{\text{weight of dried extract}}{\text{total weight of olive leaves}} \times 100\% \quad \ldots\ldots\ldots\ldots(1)$$

Phytochemical Analysis and Quality Control

Particle Size

Sieve analysis was used to determine the particle size of OLE powder. A sieve shaker (Gilson, USA) with various mesh sizes was used for 10 minutes. The mesh diameter sizes used were 4, 2, 1, 0.5, 0.25, 0.125 and 0.05 mm. D10, D50, D60, and D90 values were determined. The coefficient of uniformity (C_u) was calculated using Equation 2.

$$C_u = \frac{D60}{D10} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Density Measurement and Flowability Test

The flowability of the OLEs was characterized using the graduated cylinder method to determine bulk and tap density ρ_{bulk} *and* $\rho_{tap,}$ respectively. Then, a powder flowability test was carried out using Carr's Compressibility index (CI) or Hausner ratio (HR) according to Equations 3 and 4.

$$CI = \frac{100 \times (V0 - Vf)}{V0} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

$$HR = \frac{V0}{Vf} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

Loss on Drying (LoD)

Loss on drying (LoD) was measured to determine the moisture content of the samples. About 2 g of each sample was weighed and placed in an infrared moisture determination balance (Kett FD-720, Japan) at 180ºC for about 10 minutes.

Ash Content

The residue remaining after ignition of olive leaf extract is the total ash content or ash value. A Muffle furnace (Lenton FURNACES, UK) and crucible were used for this analysis. Crucibles containing the samples of OLEs were placed in the furnace at 600ºC for 24 hr. After the ash was completed, crucibles were cooled in a desiccator, and the total ash was calculated.

Qualitative Analysis of OLE using TLC

OLE fingerprinting was performed using silica gel plates (Merck; Kieselgel 60 F254, 0.20 mm layers). OLE solutions were prepared by dissolving 0.1 g in 1 ml ethanol. Oleuropein was used as the standard reference. TLC was developed using benzene:ethyl acetate:methanol (60:30:10) as a mobile phase. Then, it was visualized under a UV lamp (366 nm and 254 nm). The retention factor (R_f) for oleuropein was finally calculated.

Quantitative Analysis of OLE using HPLC

The high-performance liquid chromatography (HPLC) method was adapted based on a previously published method by R. Jap'on-Luj'an, M.D. Luque de Castro (20). Partial validation was performed in terms of the limit of detection (LOD), the limit of quantification (LOQ), precision (intraday repeatability) and linearity.

A reversed-phase HPLC analysis was used to standardize the extract using oleuropein as a quality marker. Finnigan surveyor system (Thermo Electron Corporation, San Jose, CA, USA) with an autosampler injector. The injection volume (Autosampler Plus) was 20 µl. A hypersil™ BDS C-18 Column (250 mm x 4.6 mm, 5µm) (Thermo Electron Corporation, San Jose, CA, USA) was used at 40 ºC. Detection was carried out at 254 nm (UV-VIS Plus Detector). The mobile phase was a mixture

of acetonitrile:water (2:8 v/v) with 1% acetic acid and KH_2PO_4 (pH 3.0). The flow rate was 1.0 mL/min (LC pump plus).

A calibration curve was obtained using 10, 50, 100, 200, 400, 800 ppm. Each standard solution was injected six times. Regression equations were calculated in the form of y = ax + b, where y and x were the areas under the peak and standard concentrations, respectively. A sample solution (0.5 % w/v) was prepared in a 100 ml volumetric flask by dissolving 0.5 g of each extract in 80 ml of methanol and placed in an ultrasonic bath at 30°C for 40 min. Subsequently, the volume was completed to 100 ml of methanol. Each sample was prepared in duplicates and injected six times.

Quantitative Analysis of OLE using the Folin-Ciocalteau Method

The folin-Ciocalteu method was used for the phenolic quantification assay following the procedure of Beara et al. (21). It was performed in customized 96-well microplates. Elisa reader (GLOMAX multi-detection system) (Promega Corporation, USA) was used. A calibration curve was calculated using pure gallic acid concentrations ranging from 0.01 to 2 ppm with a regression coefficient of 0.9802. The phenolic content was determined by comparing it with the standard calibration curve of gallic acid. The total phenol value was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (DW) (mg GAE/g of DW).

Stress Conditions Effects

The effect of stress conditions—UV light, elevated temperature (40°C), and high humidity (75%)—on olive leaf extract (OLE) from Karak was tested according to ICH guidelines. Karak samples were selected based on their oleuropein and phenolic content. Two grams of OLE were exposed to these stress conditions for 48 hours. The stressed extracts were analyzed by HPLC-UV at 0, 24, and 48 hours. The influence of UV, elevated temperature, and humidity on the OLE was assessed separately using one-way analysis of variance (ANOVA) to compare the means.

Heavy Metals

Heavy metals analysis was implemented by inductively coupled plasma atomic emission spectroscopy (ICPE-9820, Shimadzu, Japan) to measure the amounts of elemental impurities in OLEs. The software used was Smart Analyzer Vision 5.01.0921. The detector was a charge-coupled device (CCD). ICP-AES is used to detect heavy metals class 1 (Lead, Mercury, Cadmium and Arsenic), which have the highest toxicity risk. Heavy metal contents were determined by the following procedure of Pednekar and Raman (22). The calibration curves were obtained based on the results of the intensity of standard solutions with concentrations ranging from 0.01 to 2 ppm. Heavy metals were detected at Pb 220.353 nm, Hg at 194.227 nm, Cd at 226.502 nm and As was detected at 193.759 nm.

Minerals Content

Inductively coupled plasma atomic emission spectroscopy (ICPE-9820, Shimadzu, Japan) was used to measure OLE's minerals (Calcium, Magnesium, Zinc and Iron) content. Mineral contents were determined by following the procedure of Pednekar and Raman (22). The calibration curves were obtained based on the results of the intensity of standard solutions with concentrations ranging from 0.01 to 2 ppm. Minerals were detected at different wavelengths; Ca was detected at 183.801 nm, Mg at 285.213 nm, Zn at 213.856 nm and Fe at 238.204 nm.

Residual Solvent

The residual solvent was estimated by Gas Chromatography (GC-2010, Shimadzu, Japan) on a CP-Wax 58 CB separation column (30 m x 0.53 mm, Chrompack, Netherlands) to measure the amounts of ethanol residue. An FID detector was used. The flow rates of H_2 and air were set at 30 and 300 mL/min, respectively. The temperature of the FID detector and the injection port was set at 285˚C and 225˚C, respectively. Nitrogen (N2) in the flow rate of 2 mL/min was used as the carrier gas. The software used was (Chrom-Card version 1.06 for Trace GC, TermoQuest, Milan, Italy). The calibration

curve was obtained based on the results of the area under the peak of standard solutions. The resulting solutions contained 15.5, 74.8, 149.9, 299.8, 747.9, 1498.9, and 3077.9 ppm.

Determination of Microbiological Purity

The spread plate technique was used to enumerate the microbial contaminants in OLE samples. About 0.6 mg from each sample was weighed aseptically and dissolved into 1 mL of distilled water. About 0.1 mL of each diluted sample was spread aseptically onto MacConkey agar (Mac), Sabouraud Dextrose Agar (SDA) and Salmonella Shigella (SS) agar media plates for the enumeration of enteric Gram-negative bacteria; e.g., *Escherichia coli* and *Pseudomonas aeruginosa*, fungi and yeasts and *Salmonella*, respectively. Inoculated plates were incubated for 48 h at 25ºC. The microbial count was detected.

Statistical Analysis

The results were expressed as means ± standard deviations. The effect of stress conditions on OLE obtained from Karak was analyzed, and all the determinations were carried out in a triplicate. All statistical analyses were carried out using Microsoft Excel (2017). Comparisons of means with respect to the influence of light, elevated temperature (40ºC), and humidity (75%) were carried out and treated separately using one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Yield of Olive Leaves Extract

The percentage yields of olive leaf extracts (OLE) in our study ranged from 21.00% to 27.95%, with the highest yield obtained from Karak and the lowest from Mafraq (Table 1). For comparison, Jamal et al. (2018) reported yield variations from 0.70% to 16.39% for ursolic acid in *Lantana camara L.* leaves, which were attributed to differences in extraction methods (23). In contrast, our study found that the percentage yields across different geographic regions of Jordan were relatively consistent, ranging from 21% to 28%. This suggests that, despite geographical differences, the extraction efficiency was uniformly effective across the regions studied.

Table 1: Characteristics of Olive Leaves Extract from Different Geographic Regions in Jordan.

Region	Amman	Karak	Ajloun	Mafraq
Yield (%)	26.60	27.95	26.40	21.00
Particle size				
D_{90} mm	1.10	0.98	1.10	1.00
D_{50} mm	0.72	0.70	0.70	0.71
D_{10} mm	0.51	0.52	0.50	0.52
D_{60} mm	0.8	0.80	0.80	0.80
C_u	1.57	1.54	1.60	1.54
Density characteristics				
ρ_{tap} (mean±SD)	0.69±0.02	0.76±0.01	0.76±0.02	0.66±0.01
ρ_{bulk} (mean±SD)	0.55±0.02	0.53±0.01	0.62±0.01	0.50±0.01
Compressibility Index and Hausner's Ratio				
HR (mean±SD)	1.266±0.016	1.429±0.020	1.225±0.023	1.325±0.023
CI % (mean±SD)	21.00±0.90	30.00±1.00	18.33±1.53	24.50±1.32
Flow Property	Passable	Poor	Fair	Passable

Region	Amman	Karak	Ajloun	Mafraq
Other characteristics				
Loss on Drying (mean±SD)	3.67±0.096	3.76 ±0.122	4.48±0.170	4.71±0.043
Ash Content (%)	1.76 %	1.63 %	2.99 %	2.43 %
Total Phenolics (mg of GAE/g of DW) (mean±SD)	14.17±1.17	30.29±0.25	21.32±0.39	7.56±1.02
Ethanol Content (ppm) (mean±SD)	190±1.65	768±11.67	659±13.67	363±3.44
Mineral content				
Ca (mg/g)	0.424	0.464	0.335	0.305
Mg (mg/g)	0.713	0.900	0.530	0.401
Zn (mg/g)	0.010	0.003	0.005	0.006
Fe (mg/g)	0.009	0.007	0.005	0.010

C_u: coefficient of uniformity, ρ_{tap}: tapped density, ρ_{bulk}: bulk density, HR: Hausner ratio, CI: Carr's index, Ca: calcium, Mg: magnesium, Zn: zinc, Fe: iron

Phytochemical Analysis and Quality Control

Particle Size

Particle size results, represented by D10, D50, D90 and D60, and coefficient of uniformity (C_u), are shown in Table 1. Particle size analysis was conducted using sieving, a common method valued for its simplicity, low cost, and minimal expertise requirements (24). The particle size of plant extracts can significantly influence their bioactivity. For example, Hussain et al. found that reducing the particle size of *Vinca rosea L.* extracts from 2.007 ± 0.965 µm to 0.753 ± 0.227 µm improved solubility and enhanced antidiabetic activity (25). In contrast, our study observed that the particle size of OLE was larger, with a D50 value of 0.7 mm. This larger size may be attributed to the use of a commercial-scale grinding machine, suggesting a need for more efficient micronization to meet desired specifications. Despite this, the particle size analysis confirmed that all samples had a uniform grading (26), as indicated by C_u values ranging from 1.54 to 1.6.

Bulk and Tap Density

The bulk density of olive leaf extract (OLE) ranged from 0.50 to 0.62, meeting Ph. Eur. and USP specifications (Table 1). Carr's compressibility index (CI) and Hausner's ratio (HR) were calculated to assess powder flowability. OLE from Amman and Mafraq demonstrated passable flowability, while OLE from Ajloun and Karak showed fair and poor flowability, respectively. Both bulk and tapped densities, used to determine flowability via HR and CI, are influenced by particle size and moisture content. However, the combined effect of these factors on flowability is complex and not fully established, complicating comparisons based solely on physical properties (27). Given the multidimensional nature of flow behavior, no single test can fully capture it. Therefore, adding flowability enhancers is recommended for future formulations, even though the bulk density of OLEs meets USP and Ph. Eur. standards.

Loss on Drying (LoD)

Loss on Drying (LoD) was measured to determine the moisture content of olive leaf extract (OLE) (Table 1). All results were below the 5% acceptance limit specified by the European Pharmacopeia (Ph. Eur., 2007). LoD quantifies moisture content, including both water and volatile matter, which must be minimized in crude drugs (28,29). A study by Kaskoos (2018) reported a higher LoD

of 12.41% for OLE obtained in Iraq using reflux extraction with water (30). This difference may be attributed to variations in extraction solvents. Nonetheless, the LoD results in our study were within the acceptable range, confirming the compliance of our samples with established specifications.

Ash Content

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Qualitative Identification of OLE

TLC fingerprinting was employed for the qualitative identification of oleuropein in OLE, using oleuropein as a standard reference. TLC was investigated at 254 nm (Figure 1A) and 366 nm (Figure 1B). The choice of TLC for this study was driven by its effectiveness in separating and identifying compounds in plant extracts. The Rf value obtained for oleuropein was relatively low (0.16), which is consistent with its known properties under the selected solvent system. While this low Rf value provided a clear identification of oleuropein, it also suggested the need for careful consideration of the mobile phase composition. A stronger mobile phase could potentially enhance the separation of other phytochemicals present in OLE, but it could also result in overlapping spots or reduced specificity for oleuropein. Therefore, the current method was optimized to balance resolution and specificity, particularly for oleuropein. However, for more comprehensive analysis in future studies, slight adjustments to the mobile phase or the use of complementary techniques could be considered to improve separation without compromising specificity.

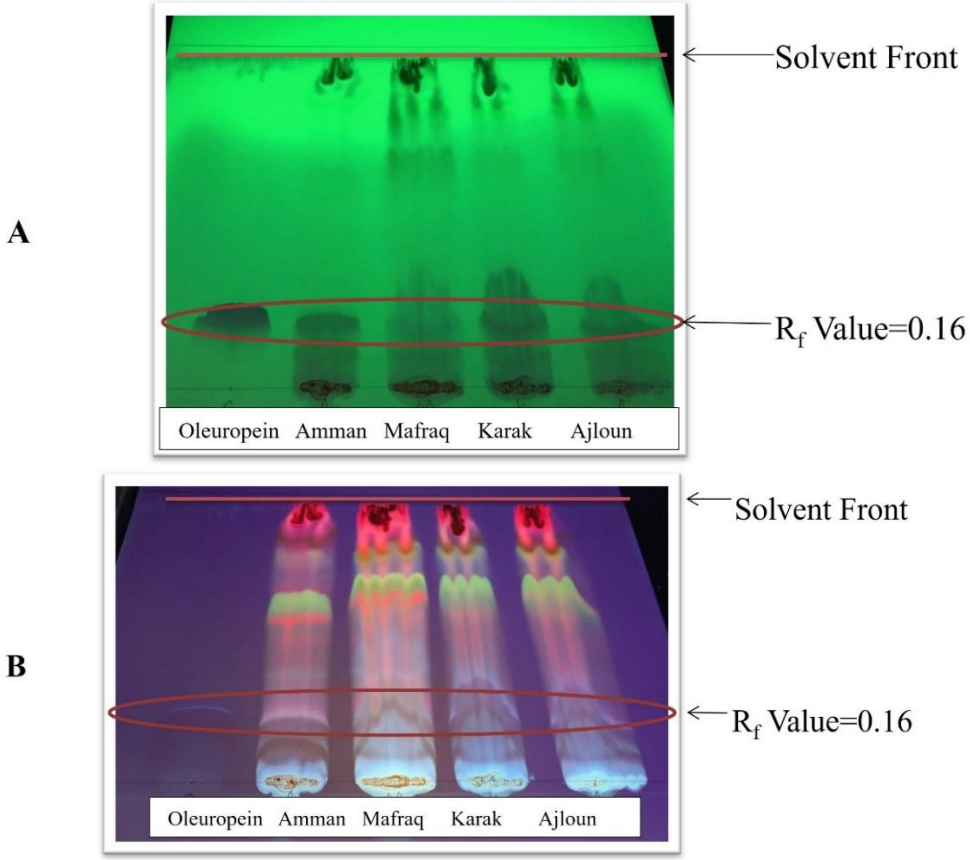

Figure 1: TLC Fingerprinting of Olive leaf extracts at (A) 254 nm and (B) 366 nm.

Quantitative Analysis of OLE using HPLC

High-Performance Liquid Chromatography (HPLC) was employed to quantify oleuropein in OLEs. A calibration curve of oleuropein was plotted (Figure 2) with an R^2 of 0.999. Oleuropein peaks appeared at a retention time of 4.8 min. HPLC assay results revealed notable regional variations (Figure 3). Karak, with its high altitude and substantial annual rainfall, had the highest oleuropein content (19.27%), while Mafraq had the lowest (3.98%). Our results place Karak's oleuropein content above that reported in Turkey (13.4%) (33) but below the levels found in Greece (26.1%) (34). These variations are likely due to differences in climate, soil composition, and altitude.

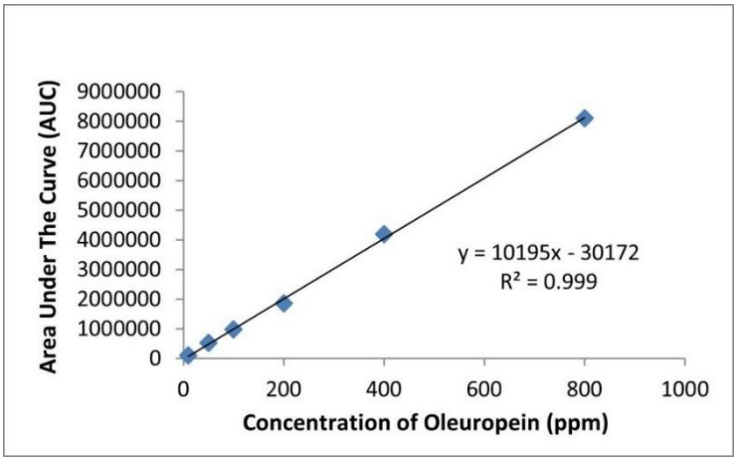

Figure 2: HPLC Calibration Curve of Oleuropein.

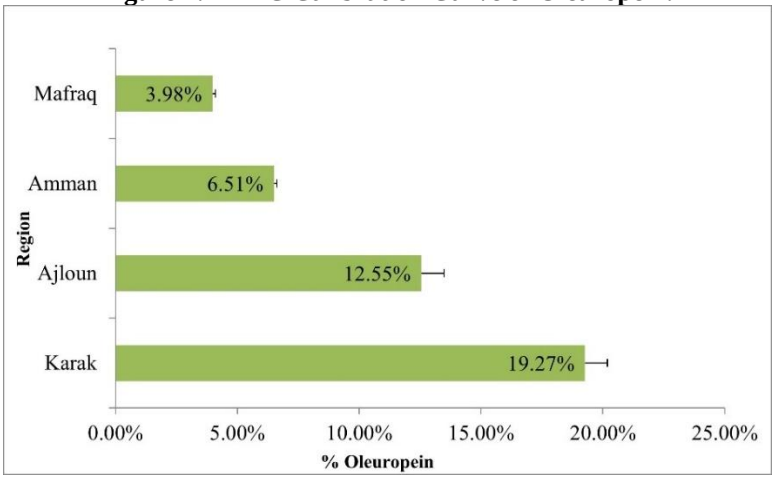

Figure 3: HPLC assay of OLE from Different Geographic Regions in Jordan Represented by Oleuropein.

Quantitative Analysis of OLE using the Folin-Ciocalteau Method

In addition to HPLC, our study employed the Folin-Ciocalteu colorimetric assay to measure the total phenolic content in olive leaves (35). A calibration curve of gallic acid was plotted (Figure 4). This rapid and widely used method revealed significant regional variations in phenolic content across Jordan. The total phenolic content ranged

from 7.56 mg GAE/g DW in Mafraq to 30.29 mg GAE/g DW in Karak (Table 1). This highlights the impact of regional environmental factors, such as soil characteristics, atmospheric conditions, and farming techniques, on the phenolic content.

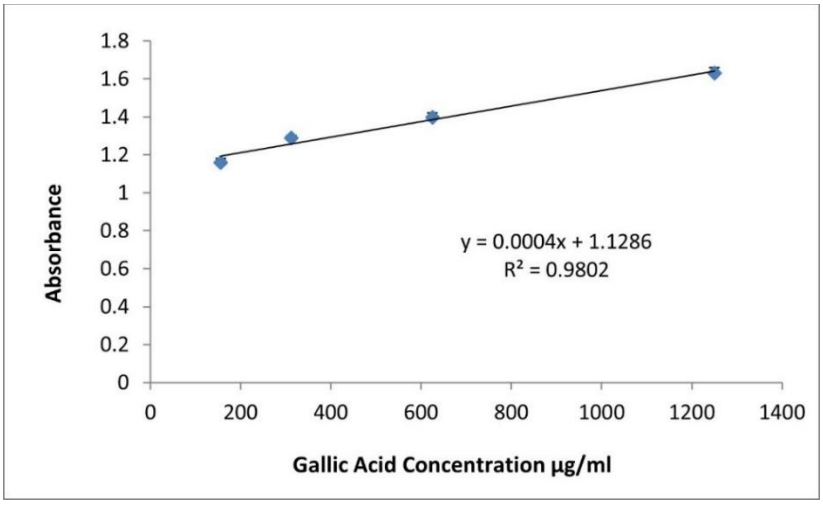

Figure 4: Calibration Curve of Gallic Acid.

Stress Conditions Effects

The effect of stress conditions (UV exposure, elevated temperature of 40ºC, and humidity at 75%) on olive leaf extract (OLE) from Karak (which had the highest oleuropein and phenolic content) was tested (Figure 5). Significant differences were observed under all stress conditions, with a p-value < 0.01, except for the conditions of humidity and elevated temperature after 24 hours, which showed significant differences with a p-value < 0.05. The reduction in oleuropein content exceeded 10% of the initial value.

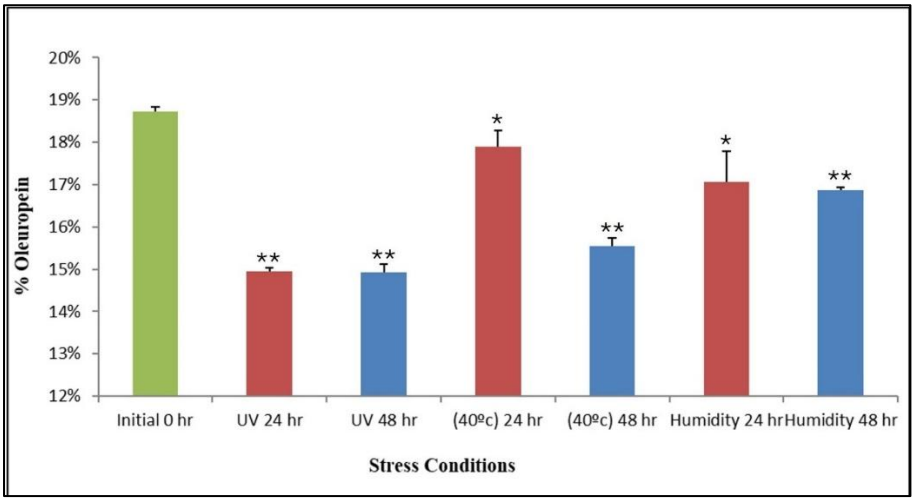

Figure 5: The Effects of Different Stress Conditions on Oleuropein Content in OLE from Karak. *P value <0.05, ** P value <0.01 (level of significance)

Minerals Content

Minerals are essential inorganic substances found in all body tissues and fluids, playing critical roles in maintaining bone health, blood coagulation, acid-base balance, enzyme activity, and osmotic regulation (36). The presence of these minerals in olive leaf extracts (OLE) can support their use for medicinal purposes and as dietary supplements (37). In our study, we measured the levels of calcium, magnesium, zinc, and iron in OLE (reported in Table 1). Our results align with those of Amin et al. who reported similar mineral profiles in their analysis (38). However, research from Brazil evaluating various types of OLE found no significant correlation between phenolic compounds and mineral content (37). This observation is consistent with our findings for OLE from Karak, which exhibited the highest levels of oleuropein and total phenolics, as well as elevated calcium and magnesium concentrations, while zinc and iron levels were relatively low.

Heavy Metals

Heavy metals, even at trace levels, can pose significant risks to human health and the environment (39). Therefore, assessing heavy metal content is crucial in the quality control of medicinal plants to ensure their safety and efficacy (22). In our study, the ICP-AES technique was utilized for its high sensitivity and efficiency in multi-elemental analysis. This method has been recently applied to various plant extracts, as demonstrated by Amin et al. (2016), who used ICP-AES to screen sixty-five elements in Iris persica L. from the Kurdistan region (38). Our analysis of olive leaf extracts (OLEs) from different regions in Jordan revealed that levels of heavy metals, including lead (Pb), cadmium (Cd), and arsenic (As), were within the acceptable limits set by USP and Ph. Eur (Table 2). Mercury was not detected in any OLE samples. Specifically, lead and cadmium were found in OLE from Amman, with concentrations of 0.0109 mg/kg and 0.0006 mg/kg, respectively. Arsenic was present in OLE from Mafraq (0.0114 mg/kg) and Amman (0.0496 mg/kg), but not in Karak or Ajloun. These findings may reflect environmental factors, such as pollution from road traffic and industrial emissions in Amman, which could contribute to higher heavy metal concentrations (40).

Table 2. Heavy Metals Content (ppm) in Olive Leaves Extract from Different Geographic Regions in Jordan.

Region	Pb (ppm)	Pb Specification*	Hg (ppm)	Hg Specification*	Cd (ppm)	Cd Specification*	As (ppm)	As Specification*
Amman	0.0109	≤1.00 ppm	n/d	≤1.00 ppm	0.0006	≤0.50 ppm	0.0496	≤1.00 ppm
Karak	0.0026		n/d		n/d		n/d	
Ajloun	n/d		n/d		n/d		0.0029	
Mafraq	n/d		n/d		n/d		0.0114	

*According to USP and Ph. Eur.

Residual Solvent

Ethanol content in the olive leaf extract (OLE) was evaluated and reported in Table 1. According to the United States Pharmacopeia (USP) 40, 2019, all residual solvent levels were below the acceptance limit. Residual solvents from the extraction process may not be completely removed by standard manufacturing techniques, often resulting in trace amounts remaining in the final product (41). Ethanol, classified as a class 3 residual solvent (42), is commonly used in extraction due to its low toxicity. The USP specifies a maximum allowable residual ethanol level of 5000 ppm in plant extracts. In our study, all samples met this standard, confirming their safety for use.

Determination of Microbiological Purity

Medicinal plant materials often harbor significant amounts of bacteria and molds from the soil, which may

pose pathogenic risks (28). Therefore, assessing microbial contamination is a crucial quality control measure to ensure the safety of olive leaf extract (OLE). The microbial enumeration results, as detailed in Table 3, showed that all OLE samples met the required specifications, confirming they are free from harmful levels of microorganisms.

Table 3. Microbial Count in Olive Leaves Extract from Different Geographic Regions in Jordan.

Microbiological Examination	Specification*	Results
Total Aerobic Microbial Count (cfu/g)	≤5000	Complies
Total Combined Yeast/Molds Count (cfu/g)	≤100	Complies
Pseudomonas Aeruginosa/g	Absent	Complies
Escherichia Coli/g	Absent	Complies
Salmonella/25g	Absent	Complies

*According to Ph. Eur. 5.1.8

DISCUSSION

Olive leaves *(Olea europaea L.)* are a significant by-product of olive cultivation, known for their rich array of polyphenolic phytochemicals with notable biological and pharmacological properties (2,43). Despite Jordan's extensive olive cultivation, with approximately 12 million trees and a strong tradition in both ethnomedicine and rational therapy (44), there has been a lack of studies focusing on the standardization and quality control of olive leaf extract (OLE) in the country. This study represents the first effort to explore the standardization of Jordanian OLE, assess quality control methods through phytochemical analysis, and identify efficient sources within Jordan.

Various extraction techniques, including microwave-assisted extraction, ultrasonication, pressurized liquid extraction, and supercritical fluid extraction, have been used to extract compounds from olive leaves, though these methods can be costly (45). Conventional extraction methods remain preferred due to their cost-effectiveness and efficiency in extracting polyphenolic compounds (46,47). Our study employed a conventional, cost-effective hot extraction method, which allowed for a detailed analysis of the phenolic content and oleuropein yield. The results from this method were then analyzed to assess the quality and potency of the olive leaf extracts. Previous studies have highlighted the impact of extraction parameters such as solvent type, composition, and temperature on phenolic content. For instance, Yateem, Afaneh, and Al-Rimawi (9) identified 80% ethanol as the most effective solvent for extracting oleuropein, while Wissam et al. (48) found that higher temperatures improved extraction efficiency. These findings guided our choice of extraction conditions and helped frame the results of our analyses.

Thin Layer Chromatography (TLC) was utilized for the qualitative identification of oleuropein in OLE, with an Rf value of 0.16. TLC is a widely used technique for phytochemical screening due to its simplicity and effectiveness (30). The low Rf value obtained aligns with the known properties of oleuropein under the selected solvent system. However, the method's resolution and specificity could be improved by adjusting the mobile phase or using complementary techniques in future studies.

HPLC was used in our study to quantify oleuropein in OLE, revealing significant regional variations. The highest oleuropein content, 19.27%, was found in OLE from Karak, a region noted for its high altitude and substantial annual rainfall, which likely enhances oleuropein synthesis. In contrast, OLE from Mafraq had the lowest

oleuropein content at 3.98%. This variation is consistent with Bilgin's (2013) findings that high-altitude regions in Turkey, such as Bursa and Mardin, also show elevated total phenolic content (49). Our results highlight the significant impact of regional environmental factors on oleuropein content in OLEs and underscore the need for regional standardization to ensure consistent quality. Future research should focus on understanding the specific environmental and genetic factors that most significantly affect oleuropein production, to further optimize content across various growing conditions.

The Folin-Ciocalteu colorimetric assay revealed significant regional variations in total phenolic content, with values ranging from 7.56 mg GAE/g DW in Mafraq to 30.29 mg GAE/g DW in Karak. This variation correlates with oleuropein content reported by HPLC, indicating that Karak's olive leaves are particularly rich in both total phenolics and oleuropein. While total phenolic content in Jordanian olive leaves, especially from Karak, is competitive with reported levels from Egypt (34.4 to 39.2 mg GAE/g DW) (50), it is slightly lower.

These findings highlight the significance of geographical origin in determining the quality and potency of OLEs and underscore the need for regional standardization protocols to ensure consistent product quality. Future research should explore how varying environmental conditions and genetic factors, such as genetic variation among olive cultivars, genotype-phenotype relationships, and selective breeding, affect oleuropein production. This investigation could offer valuable insights for optimizing production strategies. Additionally, future studies should assess the impact of different extraction methods and solvents on phenolic profiles to further enhance the understanding of their effects.

5. CONCLUSION

Olive leaves are a valuable but underutilized source of phenolic compounds. This study is the first to standardize olive leaf extract (OLE) from various regions in Jordan using a cost-effective extraction method with 80% aqueous ethanol. Phytochemical and quality control methods were applied to leaves from Amman, Karak, Ajloun, and Mafraq, all of which met USP and Ph. Eur. specifications.

The results revealed that OLE from Karak exhibited the highest levels of oleuropein and total phenolic content. Karak's Mediterranean climate, high altitude, and substantial rainfall are likely contributors to this superior quality. Therefore, controlled cultivation of olive trees in Karak is recommended. This research establishes a foundation for the commercialization of Jordanian OLE and underscores Jordan's potential as a rich source of phenolic and antioxidant compounds from its extensive olive groves. Future research should focus on enhancing extract stability and developing appropriate dosage forms.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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إعداد وتوحيد المستخلصات وتحليل المكونات الكيميائية النباتية لأوراق الزيتون (Olea europaea L) من مناطق جغرافية مختلفة في الأردن

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ملخص

تهدف هذه الدراسة إلى التحقيق في استخلاص وتوحيد مستخلص أوراق الزيتون (OLE)من أشجار الزيتون الأردنية، مع دراسة تأثير الاختلافات الجغرافية على خصائص المستخلص. تم جمع أوراق الزيتون من عمان والكرك وعجلون والمفرق، وتمت معالجتها باستخدام طريقة استخلاص كحولية مائية منخفضة التكلفة. تم استخدام تقنية الكروماتوجرافيا الرقيقة (TLC)والكروماتوجرافيا السائلة عالية الأداء (HPLC-UV)لتحديد وقياس الأوليوروبين، وهو المركب الفينولي الرئيسي. بالإضافة إلى ذلك، تم تقييم تأثير ظروف الإجهاد (الحرارة، الأشعة فوق البنفسجية، الرطوبة) على المستخلص من الكرك. أظهرت النتائج أن أعلى تركيز للأوليوروبين وأعلى محتوى فينولي كلي كان في المستخلصات من الكرك. تم تقييم الخصائص الفيزيائية والمحتوى الرطوبي ومحتوى الرماد والمعادن الثقيلة والمعادن والمذيبات المتبقية والنقاوة الميكروبيولوجية لجميع المستخلصات. أظهرت النتائج أن العوامل الجغرافية مثل الارتفاع وهطول الأمطار تؤثر بشكل كبير على المحتوى الفينولي لمستخلص أوراق الزيتون، مع حصول الكرك على أفضل مستخلص من حيث الجودة. تشير الدراسة إلى إمكانية الأردن كمصدر لمستخلص أوراق الزيتون عالي الجودة، وتوصي بإجراء المزيد من الأبحاث لتحسين استقرار وتشكيل هذه المستخلصات للاستخدام العلاجي.

الكلمات الدالة: مستخلص أوراق الزيتون؛ التحليل النباتي الكيميائي؛ الأردن؛ التوحيد ومراقبة الجودة.

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