Assessment of seasonal variation of the bioactive oleuropein in *Olea europaea* L. leaves cultivated in Saudi Arabia

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Received: March 13, 2021  •  Accepted: May 20, 2021

**ABSTRACT**

Plants secondary metabolites undergoes qualitative and quantitative variation due to environmental and growth factors. It is a crucial factor to select the proper time for collection of medicinal plants to assure maximum content of active components reflected as maximum efficacy. Olive leaves (*Olea europaea* L.) are known traditionally for their antidiabetic effect. The secoiridoid glycoside oleuropein is the main active component of Olive leaves responsible for the biological activity. The current study was conducted to monitor the seasonal variation of oleuropein in Olives leaves collected from the same location. To achieve this goal a validated HPLC method following the ICH guidelines was established. Separation was conducted using RP18 column and a mobile phase consisted of ultrapure water containing 20% acetonitrile and 1% acetic acid. Detection was performed at 254 nm with 1 mL/min flow rate. The method was simple, linear, accurate, precise, specific and robust. The analyses revealed considerable variations in the level of oleuropein throughout the year. This variation cannot be explained by temperature variation during the year. Two points of high levels of oleuropein were detected prior to flowering stage and ripening of the fruits. The levels of growth regulators most likely is responsible for the increased production of oleuropein. It is recommended that leaves intended for medicinal use to be collected during the fruiting stage prior to fruit ripening.

**KEYWORDS**

oleuropein, *Olea europaea* L., HPLC, ICH guidelines, seasonal variation

**INTRODUCTION**

Traditional medicine is trusted by considerable percentage of patients suffering from type II diabetes to control their glucose blood level [1]. Herbal products are reputed and gain increasing popularity to manage diabetes and its complications. For centuries olive leaves “*Olea europaea* L.” are highly trusted in folk medicine to control diabetes [2]. Pharmacological studies revealed the potential of olive leaves extract to produces hypoglycemic, antioxidant, hypotensive and antimicrobial effects [3, 4]. Surveys among diabetic patients indicated that the most commonly used herbal product is olive leaves [5]. Recent studies demonstrated the ability of olive leaves extract to protect against diabetic disorders.
targeting the male reproductive system. This effect induced via enhancing tissues antioxidant potentials as well the up-regulation of P450scc and 17β-HSD expression [6].

Phytochemical and pharmacological investigation of olive leaves constituents led to the identification of the major active secoiridoid glycoside oleuropein [7–9]. Oleuropein and its aglycone were detected in olive mill wastewaters by LC-MS [8]. Treatment of type I diabetic mice with oleuropein-rich extract resulted in suppression of the T lymphocytes proliferation leading to decrease in the autoimmune inflammatory process associated with this type of diabetes [10]. Oleuropein was proved to act as free radical scavenger with strong antioxidant potential [11] exceeding that of BHT (butylated hydroxytoluene) [12]. Oleuropein was reported to have genoprotective effect attributed to its potential to scavenge free radical, chelate metal ions and/or activate the indigenous antioxidant defense and DNA repair systems [13, 14]. In vivo study revealed that oleuropein has the ability to enhance the levels and activities of endogenous antioxidant enzymes as glutathione reductase (GRx), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). In addition, it also can enhance the level of non enzymatic antioxidants such as glutathione (GHS), β-carotene, α-tocopherol, and ascorbic acid in alloxan-diabetic rabbits, rats exposed to cholesterol rich diet and rats exposed to acute arsenic intoxication [15–17]. Oleuropein anti-inflammatory potential is well documented via the inhibition of the NF-kB, cyclooxygenase-2 (COX-2), caspase-3 and iNOS [18]. In vivo cardioprotective activity of oleuropein was demonstrated via biochemical and pathological analyses [19, 20]. In several experimental models, oleuropein and olive polyphenols exhibited significant neuroprotective effects pointing out to the possible therapeutic potential against neurodegenerative diseases, including Alzheimer’s disease (AD) [21]. Oleuropein also expressed anti-gout [22], anti-anxiety [23] and anti-ageing activities [24]. Furthermore, oleuropein demonstrated protective effects against UVB radiations [25], age-related bone loss and osteoporosis [26] as well as acceleration of wound healing process via increasing collagen fibre deposition and re-epithelialization in wounded skins [27].

All plants pass through variable stages of biosynthetic activity. The secondary metabolites of plants undergo qualitative and quantitative variations during the different stages of growth [28, 29]. As olive leaves are the most important source of the promising bio-active molecule “oleuropein” and in absence of any synthetic source, it is important to identify the growth stages that provide the highest amount of oleuropein.

In this work we assessed the amount of oleuropein in olive leaves on monthly bases via validated HPLC method [30] to enable the selection of the best timing to obtain oleuropein in correlation with the plant’s growth stage.

### EXPERIMENTAL

#### Standard and solutions

Standard oleuropein (Fig. 1) was obtained from Aktin Chemicals Inc. (Chengdu, China). All solvents utilized in the study were of HPLC grade. Chemicals utilized in the study were of analytical reagent (AR) grade. Water used in the investigation was distilled and then purified using a Milli-Q water purification system (Direct 8, Millipore, USA). A stock solution containing 2000 μg/mL of oleuropein was prepared in HPLC grade methanol. Different concentrations of standard oleuropein containing 5; 10; 100; 250 and 500 μg/ml were obtained by dilutions of the stock solution using HPLC grade methanol (HPLC grade).

#### Plant material

The leaves of *O. europaea* L. were collected monthly during 2019–2020 from Alshmly region 180 km west of Hail, Saudi Arabia. The plants identity was confirmed by Prof. Sanya Kamal, Department of Botany, College of Science, Alexandria University, Alexandria, Egypt. A voucher specimen (#15621) was deposited in the Herbarium of the Department of Pharmacognosy, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, KSA.

#### Extractions procedure

The dried powdered leaves (0.5 g) from each collection were extracted separately, by percolation with 95% methanol (4×10 ml) at room temperature till exhaustion. The solvent was evaporated under reduced pressure using Büchi rotary vacuum evaporator and the concentrated extracts were dissolved in 10 mL HPLC methanol in volumetric flask. The prepared solutions from the different samples were quantified by the developed HPLC method.

#### Chromatographic conditions

The HPLC analysis of standard oleuropein and different extracts of olive leaves samples were carried out in a UHPLC instrument (Dionex UltiMate 3000, Thermo Fisher Scientific®), equipped with a Quaternary pump (Dionex UltiMate 3000, Thermo Fisher Scientific®), a degasser and autosampler (Dionex UltiMate 3000, Thermo Fisher Scientific®). The system is coupled with diode array detector (DAD – 3000; Thermo Fisher Scientific®). Separation was performed...
on a RP18 HPLC column (150 mm × 4.6 mm i.d., particle size 5 µm, Dionex, Thermo Fisher Scientific®), and the column oven was maintained at room temperature. The used mobile phase composed of ultrapure water (A) and acetonitrile (B), each acidified with 1% acetic acid, under 1.0 ml/min flow rate. An isocratic system was selected composed of 80% A and 20% B run for 20 min. Three wavelengths of 240 nm, 254 nm and 280 nm were selected for detection. From each standard solution and sample 25 µL were injected using autosampler. The peak representing oleuropein was detected at RT = 10.27 ± 0.01 min.

**Method validation**

The HPLC quantification complied with the guidelines implemented by international conference on harmonization [30].

**Calibration curves.** Calibration curves were obtained from the chromatographic analysis of oleuropein standard solutions containing 5; 10; 100; 250 and 500 µg/ml in HPLC grade methanol. Each solution was analyzed in triplicated. Regression equation was \( y = 0.148x + 0.5505 \), where Y and X represents area under the peaks and sample concentrations, respectively.

**Linearity.** The calibration plot was linear in the concentrations range between 5 and 500 µg/ml with 0.9992 correlation coefficient (\( R^2 \)). Linearity data was statistically treated using least square linear regression analysis (Table 1, Fig. 2).

**Accuracy.** Recovery of oleuropein was studied following standard addition method. Excess amounts of oleuropein standard (0, 50, 100, and 150%) were added to pre-analyzed samples (100 µg/mL) and the mixtures were reanalyzed using the established HPLC method. Solutions were quantified in six replicates. Percent recovery and percent relative standard deviation (% RSD) were determined at each concentration level (Table 2).

**Precision.** To evaluate the method’s precision both repeatability and intermediate precision were carried out. Repeatability of the developed method was evaluated by carrying out six independent assays of a test sample against oleuropein standard and calculating the percent relative standard deviation (RSD).

Intra- and inter-day precisions were done by repeating the analysis on the same day (intra-day precision) and during the three consecutive days (inter-day precision) for three concentration levels (100, 250, 500 µg/mL). Assay for each analysis was calculated and %RSD was determined (Table 3).

**Robustness.** Robustness of this method was carried out by purposefully altering the conditions of separation and observes the impact of these changes on the quantification. Here, we made variations in the mobile phase flow rate and the oleuropein detection wavelength. Changes in the retention time and area under the peaks were monitored (Table 4).

**Limit of detection and quantification.** Limit of detection (LOD) and limit of quantification (LOQ) were obtained following the standard deviation (SD) method. The slope of the calibration (S) curve and SD of the blank sample were used according to the following equations: LOD = 3.3 × SD/S. LOQ = 10 × SD/S.

**Specificity.** Specificity of the established quantification was assured by comparison of the RT values of the peaks for oleuropein in the samples with that of the standards.

**Quantification of oleuropein in O. europaea L. samples**

The test samples were subjected to HPLC analysis under similar chromatographic conditions for the quantification of oleuropein standard. The areas under the peaks with same RT value of oleuropein standards at 10.27 ± 0.01 were determined and the concentrations were calculated utilizing the obtained regression equation from the calibration curve (Table 5).

**Data analysis**

The software Chromelelon 7.2.8 (Dionex, Thermo Fisher Scientific®) was used for data analysis and processing. Obtained data were statistically treated using least square linear regression analysis.

**RESULTS AND DISCUSSION**

**Method development and validation**

The mobile phase used in the quantification composed of 20% acetonitrile in ultrapure water containing 1% acetic acid. A well resolved peak for oleuropein standard was obtained at RT of 10.27 ± 0.01 (Fig. 2). The calibration plot of the areas under the peaks against oleuropein concentrations (Fig. 3) was linear in the range 5–500 µg/mL. Linear regression data (Table 1) along with highly significant \( P < 0.0001 \) correlation coefficient (\( R^2 \)) of 0.9992 for the

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**Table 1.** Linear regression data for the calibration curve of oleuropein (n = 6)

| Parameters                  | Value        |
|-----------------------------|--------------|
| Linearity range (µg/ml)     | 5–500        |
| Regression equation         | \( Y = 0.148x + 0.5505 \) |
| Correlation coefficient     | 0.9992       |
| Slope ± SD                  | 0.148 ± 0.5473 |
| Intercept ± SD              | 0.5505 ± 23.12 |
| Standard error of slope     | 0.1432       |
| Standard error of intercept | 9.47         |
| 95% confidence interval of slope | 10.124–12.685 |
| 95% confidence interval of intercept | 254.1–414.2 |
| \( P \) value               | < 0.0001     |
obtained curve confirmed the good linear relationship. The obtained linear regression equation was \( Y = 0.148x + 0.5505 \), where \( Y \) is response and \( X \) is amount of reference standards.

The recovery after spiking of pre-analyzed sample with standard oleuropein ranged from 98.40 to 101.09%. Low values of % RSD (0.27–0.51) indicated that the established method is accurate. Data from the recovery experiments is presented in Table 2. To prove the method precision both repeatability and intermediate precision were explored and data is expressed as SD (%) (Table 3). Both experiments showed low RSD% values in the range of 0.09–0.51 and 0.39–0.53 for intraday and interday precision, respectively, indicating that the method is precise. The robustness of the quantification was checked by making small changes in both wavelength of detection and flow rate of the mobile phase as shown in Table 4 and Figs 4 and 5. RSD % for wavelength changes were 0.19–0.57 and 0.27–0.39 for flow

Table 2. Accuracy of the proposed method (\( n = 6 \))

| Excess drug added to analyte (%) | Theoretical content (ng) | Conc. found (ng ± SD) | % Recovery | % RSD |
|----------------------------------|--------------------------|----------------------|------------|-------|
| 0                                | 100                      | 99.28 ± 1.75         | 99.28      | 0.51  |
| 50                               | 150                      | 151.64 ± 2.38        | 101.09     | 0.43  |
| 100                              | 200                      | 197.97 ± 2.88        | 98.98      | 0.35  |
| 150                              | 250                      | 246.33 ± 3.04        | 98.40      | 0.27  |

Table 3. Precision of the proposed method

| Concentration (\( \mu g/ml \)) | Repeatability (Intraday precision) | Intermediate precision (Interday) |
|--------------------------------|-----------------------------------|---------------------------------|
|                                | Area ± SD (\( n = 6 \)) | Standard error | % RSD | Area ± SD (\( n = 6 \)) | Standard error | % RSD |
| 100                            | 15.74 ± 0.03                | 0.02 | 0.21 | 15.12 ± 0.81                | 0.33 | 0.53 |
| 250                            | 37.86 ± 0.03                | 0.02 | 0.09 | 37.73 ± 0.17                | 0.07 | 0.44 |
| 500                            | 71.63 ± 0.36                | 0.21 | 0.51 | 71.65 ± 2.83                | 1.16 | 0.39 |

Table 4. Robustness of the proposed HPLC method

| Concentration (\( \mu g/mL \)) | Detection wavelength | Original | Used | Area ± SD (\( n = 3 \)) | % RSD | RT |
|--------------------------------|----------------------|----------|------|-------------------------|-------|----|
| 250                            | 252 nm               | −2.0     | 40.53 ± 0.08 | 0.57 | 10.27 |
|                                | 254 nm               | 0.0      | 37.66 ± 0.21 | 0.19 | 10.27 |
|                                | 256 nm               | +2.0     | 36.51 ± 1.19 | 0.26 | 10.27 |

Flow rate (mL/min)

| Concentration (\( \mu g/mL \)) | Flow rate (mL/min) | Area ± SD (\( n = 3 \)) | % RSD | RT |
|--------------------------------|-------------------|-------------------------|-------|----|
| 250                            | 0.8               | −0.2                    | 37.73 ± 0.17 | 0.27 | 13.07 ± 0.01 |
|                                | 1                 | 0.0                     | 37.66 ± 0.21 | 0.17 | 10.27 ± 0.01 |
|                                | 1.2               | +0.2                    | 37.89 ± 0.29 | 0.39 | 8.47 ± 0.01  |
rate modification indicating the method is robust. LOD and LOQ of oleuropein as obtained from the analysis were 0.42 and 1.26 μg/mL respectively. The method based on these numbers can be used for quantification of small concentrations oleuropein effectively. The method is specific as the RT of the oleuropein peak in the samples and standard were identical and well resolved (Figs 2 and 3).

Quantification of oleuropein in Olive leaves

The major aim of the current study is to monitor the level of oleuropein in Olive leaves and try to correlate the content with the different factors surrounding the plants. To achieve this goal Olive leaves were collected from the same farm on monthly bases from Alshmly region, Hail province, North-Western Saudi Arabia. All samples were extracted and analyzed by the developed HPLC method. The obtained results indicated that the highest content of oleuropein was detected in August sample (2.410%) followed by February (2.170%) and July samples (1.862%). Observation of the average temperature in the region and trying to correlate it with the oleuropein content did not lead to any logical relation. The average temperature in January and December with low oleuropein content were very close to the average temperature during February (Table 5) [31]. The area lacks rainfall throughout the year eliminating the possible effect of the rainy season. Close monitoring of the plant growth stages revealed that July and August represent the stage where unripe fruits start the process of ripening. These two months showed high content of oleuropein. The month of February, with the second highest contents of oleuropein, represent the growth stage where the plants physiological system is initiated to reach the flowering stage. This numbers clearly indicate that the level of oleuropein is affected mainly by the growth stages of the plant. Previous study demonstrated that the levels of iridoids and secoiridoids in *Gentiana scabra* were increased in the presence of growth regulators supporting our finding [32]. It is also interesting to demonstrate the function of oleuropein role in plant protection. Destruction of plant tissues by herbivores initiate the enzymatic conversion of oleuropein into a very strong protein denaturant that has protein-crosslinking and lysine-decreasing activities. Oleuropein-specific β-glucosidase in organelles convert the glucoside moiety of oleuropein into a glutaraldehyde-like structure with strong

| Month     | Oleuropein Contents (% w/w) | Growth Stage | Average Temperature (°C) |
|-----------|------------------------------|--------------|--------------------------|
| January   | 0.134                        | -            | 9.6                      |
| February  | 2.170                        | -            | 11.8                     |
| March     | 0.046                        | Flowering    | 15.8                     |
| April     | 0.052                        | Flowering-Unripe Fruits | 20.3 |
| May       | 0.046                        | Flowering-Unripe Fruits | 25.6 |
| June      | 0.153                        | Flowering-Unripe Fruits | 29.2 |
| July      | 1.862                        | Unripe Fruits | 30.1                     |
| August    | 2.410                        | Unripe Fruits | 30                      |
| September | 0.318                        | Ripe fruits  | 27.8                     |
| October   | 0.079                        | Ripe fruits  | 23.1                     |
| November  | 0.027                        | Ripe fruits  | 16.9                     |
| December  | 0.063                        | -            | 11                       |

*Data obtained from https://en.climate*

Fig. 3. Chromatograms of standard Oleuropein detected at 252, 254, 256 nm
protein denaturing, protein-crosslinking, and lysine-alkylating activities. These activities have adverse effects against herbivores due to decreasing the nutritive value of dietary protein completely [33]. The increase in oleuropein contents during these stages may be linked to the protective role of the secoiridide when the plants become more attractive to predators. The obtained results clearly correlate the level of oleuropein to the level of plant growth regulator that increased prior to these stages of growth. After ripening of the fruits the plants enter some kind of resting stage where the levels of growth regulators and consequently, oleuropein are minimal.

CONCLUSION

Simple, accurate, precise and sensitive HPLC method was developed for the quantification of oleuropein in Olive leaves. Monitoring the level throughout the year revealed two peaks for high level of oleuropein. Observation of the environmental condition at the collection area led to the conclusion that the variation is not related to such conditions. The two maximum concentrations could be correlated to the growth stages of the plant. The two high levels of oleuropein were observed in the pre-flowering and pre-
ripening of the fruits. Consequently, the production of oleuropein could be accelerated by the growth regulators that expected to be in maximum activity at these stages. It is recommended to collect Olive leaves just before fruit ripening to obtain the maximum content of the bioactive oleuropein.

ACKNOWLEDGEMENTS

This publication was supported by the Deanship of Scientific Research at Prince Sattam Bin Abdulaziz University, Al kharj, Saudi Arabia.

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