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Ultrasonic-Assisted Extraction and Determination of Oleuropein from Fresh Olive Leaves by HPLC-DAD and its Effects on Human Health

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ABSTRACT: Total polyphenols were studied in the olive leaves collected from Lattakia (Qurdaha area). Phenolic compounds were extracted from fresh olive leaves using two extraction methods, maceration and ultrasound assisted extraction. The highest polyphenolic content was found in fresh olive leaves, which were carried out from the ultrasound extracted with ethanol/water (80%) at the temperature (40°C) and at the time of extraction (20 min). The total polyphenolic content extracted was estimated in milligram equivalent Gallic acid per gram of olive leaves (157.2539 mgGAE / gDM).

Oleuropein was separated and determined in samples of fresh olive leaves obtained in optimum extraction conditions in both extraction methods (maceration, ultrasound) using HPLC-DAD technology. The C18 chromatography column was used (250 x 4.6 mm, 5 µm) with injection volume (20 µL), detector wavelength (254 nm), using a mobile phase of solution (A): Acetic acid-acidized water (0.1%), solution (B): Acetonitrile. The separation was carried out with a gradient system.

The results showed that the highest concentration of Oleuropein was obtained from the extract using ultrasound apparatus. The amount of Oleuropein identified using HPLC-DAD technology was (25.06 mgOLE/gDM).

Keywords: Polyphenols; Oleuropein; Extraction; Ultrasonic; Olive Leaves; HPLC.

I. INTRODUCTION

Olive trees (Olea europaea, Oleaceae) are widely distributed in China, Spain, Italy and Greece [1]. It is one of the most important crops in the Mediterranean countries, with more than eight million hectares of olive trees spread throughout the world, including the Mediterranean basinof which is (98%) [2] [3]. Syria is one of the countries that cultivate olive. The estimated cultured area is 684490 hectares with annual production of about 1000,000 tons of olive fruit [4].

Olive industry wastes that have not been properly treated are a major ecological issue for olive-producing countries, and therefore approaches aiming at valorising olive wastes, primarily those allowing a sustainable recovery of valuable natural components, are gaining acceptance [5].

The products derived from olive tree such as olive oil, fruit, and leaves have beneficial compounds such as flavonoids and polyphenol used by native people of the Mediterranean region as a folk medicine to treat many diseases and thereby very interesting worldwide [6].

Olive leaf extract (OLE) has been used traditionally as a herbal supplement since it contains polyphenolic compounds with beneficial properties ranging from increasing energy levels, lowering blood pressure, and supporting the cardiovascular and immune systems [7], in addition to its potential to reduce lipid accumulation in hypertrophic and insulin resistant adipocytes [8].

The importance of olive leaves is characterized by the presence of phenolic compounds and polyphenols, which are very important as raw materials in the pharmaceutical industry and as food stuffs, the most important of which is the oleorubin, which will be used in this research to determine its concentration in the olive leaves studied.

Phenolic compounds are secondary metabolites of plants [9], and are class of compounds consisting of a hydroxyl group - (OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is the phenol which is also called carbolic acid C6H5OH. Phenolic compounds are classified as simple phenols or polyphenols base on the number of phenol units in the molecules [11] [10].

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Oleuropein, which is a secoiridoid, is the major and most abundant phenolic compound in olive leaves and fruits and is responsible for the characteristic bitterness of the olive fruit [12], its concentrations can reach up to 140 mg.g-1 (DM) levels in some olive species; and the content ranged between 60-90 mg.g-1 (DM) of dry matter in young olive cultivars [13].

II. MATERIALS AND METHODS

2.1. Samples Preparation:

Olive leaves samples were obtained from trees localized in Lattakia-Syria. The collection was directly from the trees in the middle of November 2016.

Fresh olive leaves were grinded to obtain olive powder which was stored at room temperature in dark until extraction.

2.2. Chemicals and Reagent:

Oleuropein (≥98.0%) which used as a standard was obtained from Sigma-Aldrich. Chromatographic grade-double distilled water, HPLC grade acetonitrile (Merck), analytical grade acetic acid, ethanol, methanol were obtained from Sigma-Aldrich company.

Standards of gallic acid was purchased form Sigma-Aldrich (St Louis, USA).Folin-Ciocalteu reagent and ethanol (analytical grade) were provided by Scharlau (Barcelona, Spain).

2.3. Extraction Method:

2.3.1. Simple Extraction (Maceration) Method:

5 grams of olive leaves powder were macerated in 100 mL of 80% ETOH for 24 h at 40ºC. The extracts were then filtered through a Whatman No.1 filter (Whatman, UK). The filtered extracts were then evaporated in rotary evaporator at room temperature under vacuum, and the concentrated extracts were stored in a refrigerator at 2-8°C until used.

2.3.2. Ultrasonic-Assisted Extraction Method:

5 grams of olive leaves powder were macerated in 100 ml of 80% ETOH and placed in a bath of ultrasonic for 20 min at 40ºC. The extracts were then filtered through a Whatman No.1 filter (Whatman, UK). The filtered extracts were then evaporated in rotary evaporator at room temperature under vacuum, and the concentrated extracts were stored in a refrigerator at 2-8°C until used.

2.4. Determination of total polyphenol contents:

Total quantity of polyphenols of the olive leaf extract was determined using the Folin–Ciocalteu procedure [14]. 1 ml of diluted extract (at a dilution of 1:50 in distillated water) was mixed with 0.5 ml of Folin–Ciocalteu reagent. 1.25 ml of 200 g.kg-1 aqueous sodium carbonate solution was added to the mixture after 5 minutes. Samples were then shaken in a vortex mixture and incubated at 30°C for 90 min. The absorbance of blue colored mixtures was recorded at 760 nm against a blank containing 1 ml of water, 0.5 ml of Folin–Ciocalteu reagent and 1.25 ml of aqueous sodium carbonate solution (200 g.kg-1). The amount of total polyphenols was expressed as mg Equivalent Gallic Acid.g-1 dry powder. For the gallic acid, the curve absorbance versus concentrations is described by the equation $y=1.333x + 0.0014$ (R^2 = 0,9985). All measurements were done in triplicate.

2.5. Determination of Oleuropein in Olive Leaf Extracts by HPLC:

For determination of oleuropein from olive leaf extract, reversed phase HPLC method was used with silca-based C18 bonded phase column (C18, 250mm × 4.6 ID) with mobile phase consisting of a mixture of water and acetonitrile (80/20 volume ratio) containing 1% acetic acid at a flow rate of 1.0 mL/min. Diod Array Detection at 254 nm was used for oleuropein determination.

The injection volume used is 20.0 µl for both standard and sample solutions. Identification of oleuropein in olive leaves extracts was based on retention times in comparison with standard of oleuropein. The quantitation was carried out using external standard method. The concentration of oleuropein was calculated using peak area and the calibration curves obtained from oleuropein standard solution. The amount of oleuropein was expressed as milligram per gram of olive leaf powder. The samples were eluted according to the following gradient:

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Table 1. Gradient elution program used for HPLC-DAD:

TIME	Flow Rate ml/min	H2O with 0.1% Acetic Acid	Acetonitril
0	1	95	5
20	1	80	20
25	1	80	20
30	1	80	20
40	1	75	25
42	1	70	30
47	1	0	100

III. RESULTS AND DISCUSSION

3.1. Determination of total polyphenol contents:

Total phenol compounds which determined by Folin-Ciocalteu method, are reported as Gallic acid equivalents by reference to standard curve (y=1.333x + 0.0014, R^2 = 0,9985). The total phenolic content of extracts was highest in ultrasonic-assisted extraction sample (157.2539 mgGAE/gFM) and lowest in leaves of maceration sample extract (99.0025 mgGAE/gFM). Results showed that the total amount of phenols was higher in the ultrasonic-assisted extraction against of maceration (Figure 1).

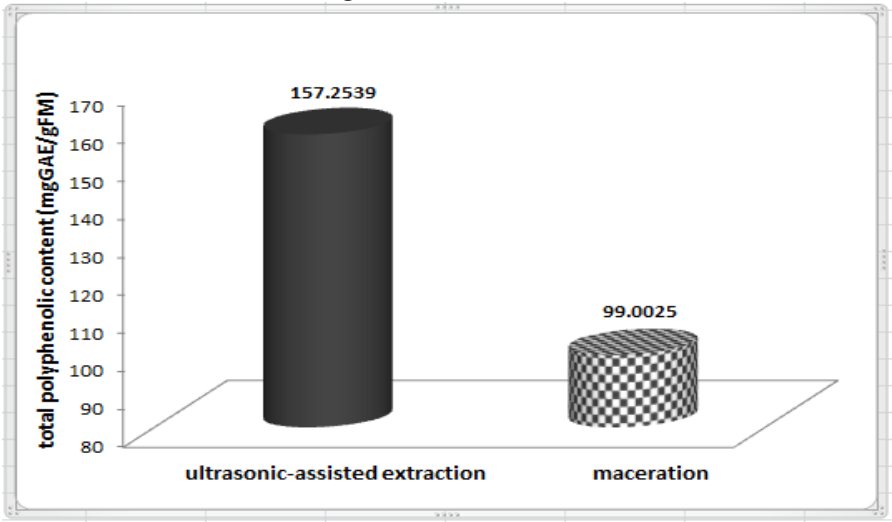

Figure 1. Total polyphenolic content extracted by ultrasonic and maceration method expressed as mg Equivalent Gallic Acid per gram Fresh olive leaves

The high total content of polyphenols extracted by ultrasound is due to the fact that ultrasound interacts with plant material and changes physical and chemical properties. The ultrasonic extraction method can be used at a moderate temperature suitable for heat-sensitive polyphenols compounds. The cavity effect facilitates the release of extractable compounds and promotes the transfer of mass extracted from the plant material to the solvent by disrupting plant cell walls [15].

Studies have confirmed that ultrasound-assisted extraction is useful for extraction of polyphenols due to process simplicity and low temperatures (40-60°C, high recovery rate, high energy efficiency and high extraction output). The ultrasound-assisted extraction mechanism includes mechanical effects that disrupt the cell wall, reduce particle sizes, and enhance the mass for transport across the cell membrane [16].

3.2. Determination of Oleuropein in Olive Leaf Extracts by HPLC:

Each of the standard Oleuropein solutions was injected five times according to the chromatographic conditions.

The standard curve of the chromatographic peaks of the standard solutions was plotted as shown in Figure (2). The graph representation shows a linear relationship within

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the studied field and the correlation coefficient is equal to R2 = 0.9983.

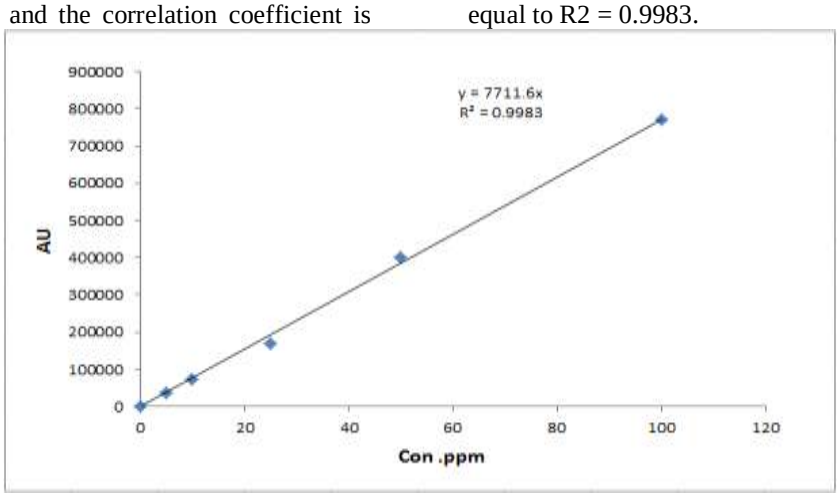

Figure 2. Standard Curve of Oleuropein

3.3. Determination of Oleuropein in Fresh Olive Leaves Samples:

(20 µL) of olive leaf extracts were injected into the HPLC-DAD after adjusting the chromatographic conditions. The corresponding chromatograms were obtained. The retention time of Oleuropein was measured in each chromatogram with the retention time of the standard chromatograms. Oleuropein concentration was determined as (ppm).

Sample 1: Fresh olive leaves extracted using 80% ethanol/water at 40°C in a water bath in the ultrasound apparatus for 20 min.

Sample 2: Fresh olive leaves extracted using 80% ethanol/water at 40°C by maceration for 24 h.

The measurement was repeated three times per sample and the results were as shown in Table (2):

Table 2. Results of Oleuropein Determination Tests in Olive Leaf samples

Sample	Average of peak area (AU)	RSD%	Retention Time (min)	Oleuropien Concentration (ppm)
1	9663002	0.00517	26.413	1253.048
2	2510879	0.001991	26.535	325.598

Results in table (2) showed that the concentration of Oleuropein in the fresh olive leaf sample extracted in (80% ETOH) at (40°C) for (20 min) using ultrasonic-assisted extraction is 1253.048 ppm. This value is equal to (25.06 mgOLE / g DM), which is the highest amount of Oleuropein in olive leaf samples, while Oleuropein concentration in olive leaves sample which extracted by maceration method using (80% ETOH) at (40°C) for (24 h) was (325.598 ppm), which is equivalent to (6.512 mgOLE / g FM).

Taking into consideration that the detection limit of the device used is 0.100 mAU. The table shows the values of the relative percentage deviation (RSD%) which does not exceed (2%). This confirms the measurement accuracy in the analysis using HPLC. The chromatograms of test samples show the accuracy of the separation process, As well as retention times significantly match compared to standard solutions.

Figures (3,4) are chromatograms of olive leaf samples which are represented in the chromatographic conditions described above.

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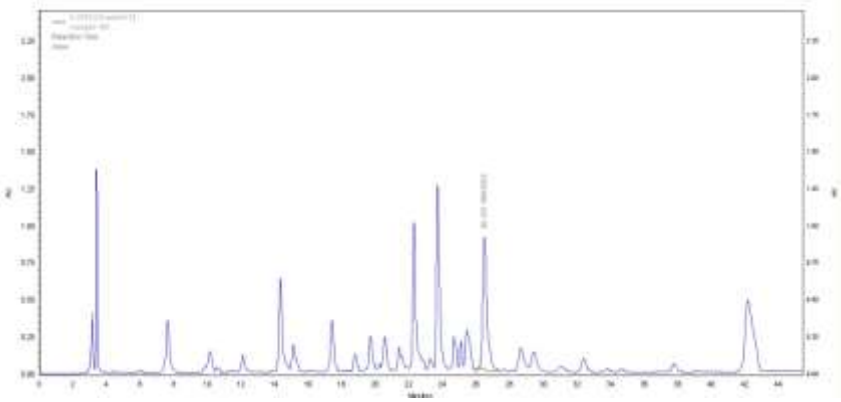


Figure 3. Sample 1: Chromatogram of Fresh olive leaves extracted using 80% ethanol/water at 40°C in a water bath in the ultrasound apparatus for 20 min.

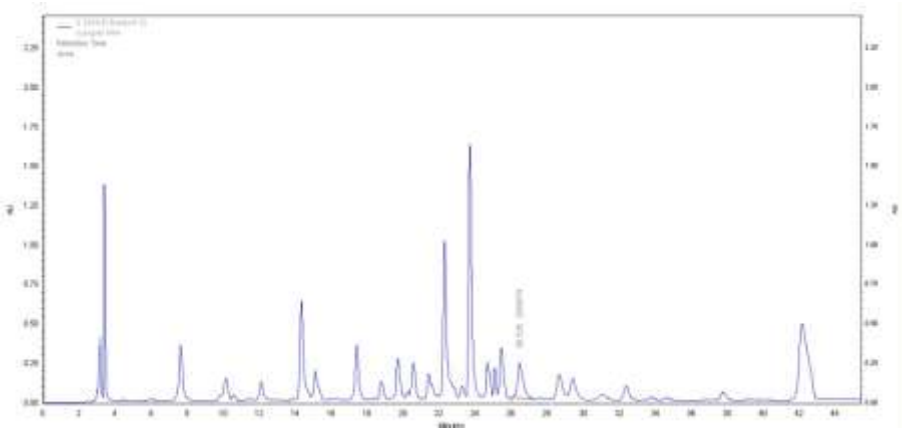
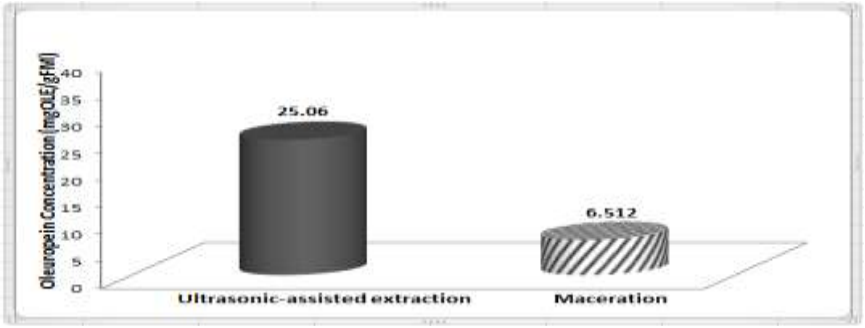


Figure 4. Sample 2: Chromatogram of Fresh olive leaves extracted using 80% ethanol/water at 40°C by maceration for 24 h.

Figure (5) shows the concentration of Oleuropein in fresh olive leaves samples extracted using the ultrasound device is higher than that obtained by the method of maceration, which is consistent with the results mentioned in reference studies [1,5,17,18]. The high concentration of Oleuropein in fresh olive leaves samples extracted using the ultrasound device is due to the fact of the application of ultrasound disrupts the structure of the plant cell wall and speeds up the diffusion of solvents through the membranes, thus facilitating the release of their contents [11,19,20].



Fig(5): Oleuropein concentration in Fresh Olive Leaves Samples

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3.4. The Role and Effect of Oleuropein on Human Health:

Oleuropein has antioxidant, antihypertensive and hyperglycemic properties, as well as its ability to adjust cholesterol levels, and its anti-inflammatory effect and its role in the treatment of obesity [21], in addition to its anti-cancer, antiviral, and antimicrobial effects [12,22]. It was reported that the application a dose of 400µM Oleuropein and Hydroxytyrosol caused a significant reduction in the proliferation of colon cancer cells 24 hours after treatment. The Oleuropein extracted from olive leaves showed anti-cancer effects in prostate, breast and liver cancer, depending on the dose, after 72 - 24 hours of application [13].

IV. CONCLUSIONS

In the previous study, the following conclusions were reached:

1. Fresh olive leaves are a natural source of polyphenols, which are a natural source of antioxidants.
2. Ultrasound extraction is one of the best ways to extract polyphenols compounds from fresh olive leaves compared to the way of maceration in terms of saving time and economic cost.
3. The highest amount of Oleuropein, the most abundant polyphenolic compound in olive leaves, can be extracted using the ultrasonic device from fresh olive leaves using the ethanol/water solvent (80%) at (40°C) for (20 min).
4. Oleuropein can be separated and identified in olive leaves using HPLC technology that is accurate and highly sensitive.

Conflicts of Interest

The authors do not have any conflicts of interest regarding the content of the present work.

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