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Determination of the Antioxidant Properties of the Syrian Olive Leaves Extracts and Isolation Oleuropein by HPLC Techniques

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The phenolics of Syrian olive leaves were determined in alcoholic and aqueous extracts by the ultrasonic bath. The total phenolic and flavonoids contents were compared, and the IC50 values of olive leaves extracts were calculated for the inhibition of the free radical of 2,2-diphenyl-1 picrylhydrazyl ($DPPH^{\cdot}$), and free monocation radical of 2.2,-azino-bis-[3-ethyl benzothiazoline-6-Sulfonic Acid] ($ABTS^{+\cdot}$) and the results were compared with vitamin C and oleuropein standard values. Oleuropein content was quantified in extracts using high-performance liquid chromatography (HPLC), and isolated by TLC, then it was detected by HPLC-MS. The results showed that the total phenolic content and total flavonoids content for alcoholic extracts were higher than those in aqueous extract contents, with significant differences in the statistical study. There were no significant differences in their ability to inhibit $DPPH^{\cdot}$, opposed to the result of the monocation radical ($ABTS^{+\cdot}$), where the inhibition capacity of the ethanolic extract was greater than that in the aqueous medium. The study also showed that the alcoholic extract contains a higher concentration of oleuropein (88.50 ± 9.67 mg g^{-1}) compared to the aqueous extract (37.60 ± 6.84 mg g^{-1}), allowing the use of Syrian olive leaves extracts as natural antioxidants. The isolated oleuropein yield by TLC was 0.43-0.1 mg g^{-1} in ethanolic and aqueous extracts as well.

Keywords: Total phenols, Total flavonoids, Antioxidant activity, IC_{50}, Oleuropein

INTRODUCTION

The olive tree, botanically-classified as *Oleaeuropaea* L., is one of the most important fruit trees in Mediterranean region [1], considered as a source of food, hygiene and curative properties [2]. Olive tree leaves and fruits have been widely used in traditional remedies in European and Mediterranean countries such as Greece, Spain, Italy, France, Turkey, Morocco, and Tunisia [3], because of its medical effects on lowering blood cholesterol [4], lipid [5], hypertensive [6] and hyperglycemia [7]. It also has anti-tumor growth effects [8], and anti-fungal growth and arteriosclerosis [9]. Olive tree is considered as the most important rain-fed crops in Syria, and has a distinct economic importance for the possibility of cultivation in lands that are less fertile and not suitable for other crops. Olive leaf is a low-cost, rich renewable source of natural phenolic antioxidants [10]. The percentage of leaves is often ten percent of the harvest weight. Recently, phenolic compounds in these leaves have attracted considerable interest around the world because of their health and medical benefits [11,12]. Oleuropein and its derivatives are the most important phenolic compounds in olive leaves [13]. Figure 1 shows the structure of oleuropein, the main phenolic compound in olive leaves [1]. Their percentage in leaves is very large compared to their percentage of oil [12], whereas simple phenols and acids are low in contrast to flavonoids [14]. According to the references, there is a possibility of the breakdown of the oleuropein into other compounds such as hydroxytyrosol, oleanolic acid, the

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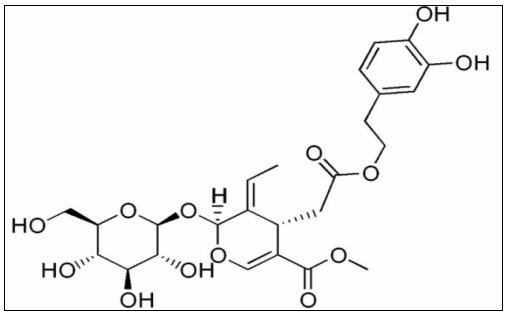

Fig. 1. Structure of oleuropein as the main phenolic compound in olive leaves.

oleuropein aglycone and glucose [15,16]. Some factors influencing on the phenolic composition are the collection time [17], drying conditions [18], geographical area [19], tree type [20,21], and the extraction method [19,21]. The aim of this research is to determine the total phenolic and flavonoid contents and also to study the ability of inhibitition the free radicals in order to use these extracts as natural antioxidants. To this end, the amount of the most important compound in olive leaves (oleuropein) in aqueous and alcoholic extracts is determined and subsequently isolated by TLC and HPLC-MS techniques

EXPERIMENTAL

Chemicals

Folin-Ciocalteu reagent, gallic acid (97.5%), quercetin (98.0%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), HPLC grade acetonitrile (99.9%) and oleuropein used as a standard (98.0%) were purchased from Sigma-Aldrich. Ethanol (99.8%), aluminum chloride (99.0%), potassium acetate (99.0%) and sodium persulfate were provided from Riedel-de Haen. 2,2'-Azino-bis(3-ethyl benzothiazoline-6-sulphonic acid (ABTS) (99.0%) was purchased from Fluka. Acetic acid (99.5%) was supplied from Panreac, sodium carbonate (99.5%) from Qualikens, and chloroform from AVONCHEM (99.5%). Deionised water, provided from a Millipore Milli-Q water purification system, was used to prepare all mixture analyses.

Instruments

Vortex MS1 mini shaker (KAI), ultrasonic water bath model transonic 460/H (Elma), UV-Vos spectrometer (JASCO). Humidity meter (Sartorius/MA35). High performance liquid chromatography (HPLC) model (KNOUER). TLC plates silica gel 60 F_{254} pre-coated(5*20 cm / layer thickness 0.25 mm) from MERCK. HPLC-MS (Shimadzu, Japan).

Plant Material

Olive leaves were collected in June 2017 from khan Arnabeh (Quneitra Governorate), Syria. The plant was identified by Prof. Jurjet Babojian (Department of Plant Biology, Faculty of science, Damascus University, Syria). The olive leaves were dried in the shade away from the sunlight for 10 days, ground with an electric mill, then stored in a dark, dry, tightly sealed place at room temperature until use. The percentage of moisture M% was determined using the Sartorius/MA35 device, with a certain weight of newly harvested fresh olive leaves under 105 °C. The extraction yield was calculated by drying the plant extracts with the dryer device until the weight was stable, and then the weight was calculated. All the tests were repeated 3 times.

Extraction Procedures

1.00 g of olive leaves powder was added to 20 ml ethanol (70%) for ethanolic extracts, or to 20 ml distilled water for aqueous ones, stirred well by piping for homogenization for one minute, then placed in the ultrasonic bath at (75 ± 2) °C, for 30 min, filtered with 0.45 µm filters and kept in refrigerator until use. The extraction was repeated for 3 times. The ultrasonic extraction method was used for its ability to improve extraction by accelerating the release of bioactive substances from cell walls and

facilitating their transmission [22].

Antioxidant Activity Determination of Olive Leaves Powder

Determination total phenolic content. The total phenolic content (TP) was determined using the Folin-Ciocalteu reagent method [23] with little modifications. To 1 ml of diluted extract, 4.8 ml of distilled water, 4 ml of Na_2CO_3 (2% w/v), and 200 µl of Folin-Ciocalteu reagent were added and allowed to stay at room temperature for 60 min. The absorbance was measured at 760 nm. A calibration curve of gallic acid solutions were prepared in (70%) ethanol (0-150 mg l^{-1}); slope = 0.0041, and $R^2 = 0.9991$) (Fig. 2). Total phenolic compounds were determined according to the following equation (Eq. (1)) obtained from the standard gallic acid graph, where Y is the absorbance at 760 nm and X is total phenolic content in extracts.

$$y = 0.004x - 0.0012 \quad (1)$$

Results were expressed as mg of gallic acid equivalents per g of dry leaves (DL).

Determination of Total Flavonoid Content

Estimation of the total flavonoids content (TF) was performed according to the procedure described in reference [24] and modified by reference [25]. In summary, 1 ml of diluted extract was mixed with 3 ml ethanol in a test tube followed by addition of 200 µl of $AlCl_3$ (10%), and 200 µl potassium acetate solution (1 M). Then, 5.6 ml distilled water was added into the solution and mixed. The mixture was allowed to be at room temperature for 40 min. The absorbance was measured against the blank solution at 440 nm. A calibration curve of quercetin solutions was prepared in ethanol (70 %) (0-100 mg l^{-1}; slope = 0.0082, and $R^2 = 0.9998$) (Fig. 3). Total flavonoids compounds were determined according to the following equation (Eq. (2)) obtained from the standard quercetin graph, where Y is absorbance at 440 nm and X is the total flavonoids content in extracts

$$y = 0.008x - 0.00001 \quad (2)$$

Samples were analyzed in triplicates. Results were expressed as mg of quercetin equivalents per g of dry leaves (DL).

Radical Scavenging Activity Assay (DPPH· Assay)

One of the quick methods to evaluate antioxidant activity is the scavenging activity on DPPH·. The effect of the olive leaves extract on DPPH· radical was estimated according to the literature [26]. 3 ml of freshly prepared ethanolic DPPH· solution (45 µg l^{-1}) was mixed with 300 µl of the extract samples at varying concentrations ranging 0.2-1 mg l^{-1}. The mixture was shaken vigorously and allowed to stay for 30 min in the dark at room temperature. The decrease in absorbance (A) was measured at 515 nm with a spectrophotometer. The inhibition percentage of the radicals (I%) was calculated according to the following Eq. (3):

$$I_{DPPH}\% = [(A_b - A_a)/A_b] \times 100 \quad (3)$$

where A_b is the absorbance of the control reaction (containing all reagents except the sample) and A_a is the absorbance of the sample. A calibration curve of vitamin C solutions was prepared in ethanol (70%) (0.02-0.1 mg l^{-1}; $R^2 = 0.9977$) (Fig. 4) that was used as a control, treated as the sample and at the same condition. The IC_{50} values were calculated by the linear regression method through plots of the antiradical activity percentage against the concentration of the tested compounds.

$ABTS^{+\cdot}$ Radical Scavenging Assay

The total antioxidant activity by radical cation ($ABTS^{+\cdot}$) assay was determined according to the method in reference [27] with little modifications. The $ABTS^{+\cdot}$ cation radical solution was prepared by reacting similar quantities of 7 mM of ABTS and 2.45 mM of sodium persulphate ($Na_2S_2O_8$) solutions for 16 hours at (2-3 °C) in the dark. Before using the solution, it was diluted with distilled water to obtain an absorbance of (0.75 ± 0.02) at 734 nm. The reaction mixtures were composed of 3ml of $ABTS^{+\cdot}$ solution and 200 µl of extracts at different concentrations range (0.2-1.0) mg l^{-1}. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer. The blank was run in each assay and all measurements were taken after 10 min.

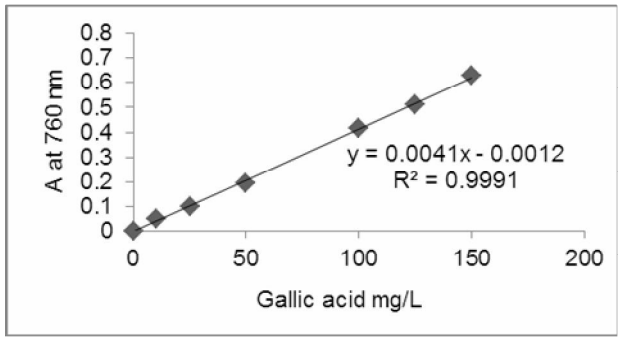

Fig. 2. Standard curve of gallic acid to determine the total content of phenols TP.

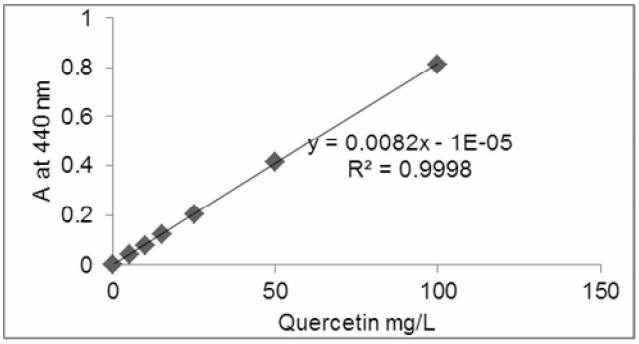

Fig. 3. Standard curve of quercetin to determine the total content of flavonoids TF.

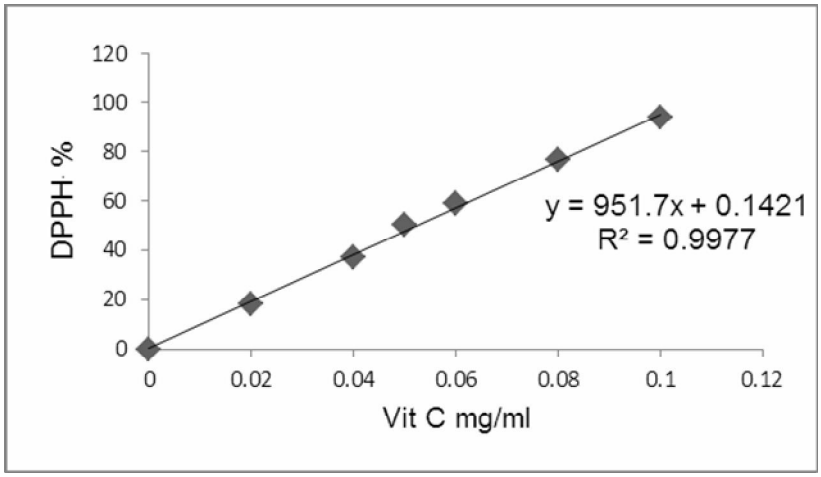

Fig. 4. Standard curve of Vit C for DPPH· Assay.

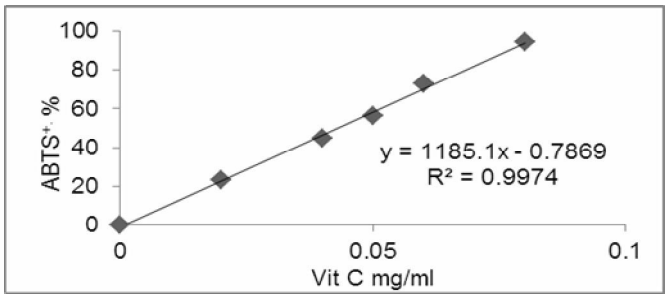

Fig. 5. Standard curve of Vit C for $ABTS^{+\cdot}$ Assay.

Table 1. Time Schedule for the Oleuropein Separation with HPLC

Time (min)	A	B
0	95	5
30	80	20
40	75	25
42	70	30
47	0	100
50	0	100
53	95	5
60	95	5

Table 2. Time Schedule for the Oleuropein Separation with HPLC-MS

Time (min)	A	B
0	90	10
10	80	20
35	60	40
40	0	100
45	0	100
46	90	10
50	90	10

The $ABTS^{+\cdot}$ scavenging capacity of the extract was compared with that of ascorbic acid (Fig. 5), and percentage inhibition was calculated as Equation (4):

$$I_{ABTS}\% = [(A_b - A_a)/A_b] \times 100 \quad (4)$$

where A_b is the absorbance of the blank and A_a is the absorbance in the presence of extract. The IC_{50} values were calculated by the linear regression method through plots of the antiradical activity percentage against the concentration of the tested compounds.

Determination of Oleuropein in Olive Leaves Extracts by HPLC-PDA

For determination of oleuropein from Syrian olive leaves extracts, HPLC system (Shimadzu, Japan) with an LC-20ADXR pump, CTO-20A column oven, and SPD-20A UV detector was employed. For HPLC determination of oleuropein in different extracts, the C18 column (C18, 250 mm × 4.5 mm ID, 5 µm) was used with gradient mobile phase program; A: 2.5% acetic acid and B: acetonitrile (Table 1), at a flow rate of 1.0 ml min^{-1}, and 25 °C. PDA detector at 254 nm was used for oleuropein determination. The injection volume was 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 quantization was carried out using the external standard method. The concentration of oleuropein in the extracts was calculated using peak area and the calibration curves were obtained from oleuropein standard solution.

Thin Layer Chromatography (TLC) Procedure

To isolate and identify the oleuropein from the extracts, thin layer chromatography was performed. Oleuropein standard and olive leaves extracts were run on glass-backed silica gel TLC GF_{254} pre-coated HPTLC Plate.

Identification of Isolated Oleuropein in Extracts by HPLC-MS

For identification of isolated oleuropein from Syrian olive leaves extracts, HPLC system (Shimadzu, Japan) with an LC-20ADXR pump, CTO-20A column oven, and SPD-20A UV detector were employed. The HPLC method used C_{18} column (C_{18}, 250 mm × 4.5 mm ID, 5 µm) with mobile phase gradient program, according to reference [28], consisting of A: 2.5% acetic acid and B: acetonitrile, (Table 2), at a flow rate of 1.0 ml min^{-1}, and 25 °C. PDA detector at 280 nm was used for oleuropein determination. The injection volume was 20.0 µl for both standard and sample solutions. The ESI source was operated in the negative mode to generate $[M-H]^-$ ions under the following conditions: a vaporizer temperature 450 °C; dry gas (nitrogen) and nebulizing gas flow was 1.5 l min^{-1}. The MS data were acquired as full scan mass spectra at 50-1100 m/z using 200 ms for the collection of the ions in the trap.

Statistical Analysis

Statistical package for the social science (SPSS, 20) was used for statistical analysis. Data were expressed as mean ± SD of three different experiences. Comparison of the antioxidant activity and IC_{50} values was performed by one-way and tow-way ANOVA (univariate analysis of variance) with post hoc test (Bonferroni test), respectively; the significance level was $P < 0.05$.

RESULTS AND DISCUSSIONS

Determination of the Moisture Ratio in the Plant

The percentage of moisture M% was determined, M% = 47.00 ± 1.68. The previous value shows that the studied leaves have a large content of water, about half of their weight, indicating their great ability to reservation water.

Determination of the Yield of Olive Leaves Extracts

The yield of ethanolic (70%) plant extracts was calculated. Table 3 shows the percentage of dry weight of olive leaves in the extracts studied. There are significant differences in the percentage yield results of the ethanolic and aquatic extracts, with the highest percentage in the ethanolic extract indicating the nature of the extracted materials.

Antioxidant Properties of Olive Leaves Extracts

The antioxidant properties of two types of aqueous and ethanolic extracts determined are subsequently compared.

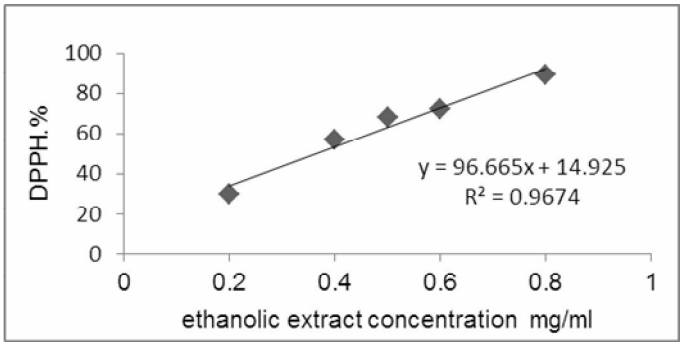

Fig. 6. Increase the inhibition capacity of DPPH% by increasing the concentration of ethanolic olive leaf extract.

Table 3. Mean Percentage Yield of Olive Leaves Extracts

Extract type	Yield (%)
Ethanolic extract	34.07 ± 0.01[a]
Aqueous extract	32.97 ± 1.79[b]

[a,b]Values are differ significantly from each other According to the Independent-sample T test ($P < 0.05$).

Table 4. The Values of the Total Phenolic and Flavonoid Contents for Olive Leaves Extracts

Extract type	TP ($mgGa_{Es}/g_{DL}$)	TF (mgQ_{Es}/g_{DL})
Ethanolic extract	96.89 ± 5.88[a]	4.19 ± 0.61[c]
Aqueous extract	73.17 ± 2.03[b]	2.18 ± 0.14[d]

[a,b,c,d]Values are differ significantly from each other according to the Independent-sample T test ($P < 0.05$), Ga_{Es}: gallic acid equivalents, Q_{Es}: Quercetin equivalents, DL: dry leaves.

Determination of the Total Phenolic and Flavonoids Contents

Table 4 shows the values of the total phenolic and flavonoids contents for both of the aqueous and ethanolic olive leaves extracts. The total content of phenols in ethanolic extracts was higher than that in aqueous extracts, with significant differences between TP and TF values for both extracts, indicating the phenolic compound properties that found in olive leaves, which show greater tendency to extract in ethanolic extracts than aqueous ones. The results showed that the percentage of total phenols is much higher than that in the total flavonoids, showing that these extracts

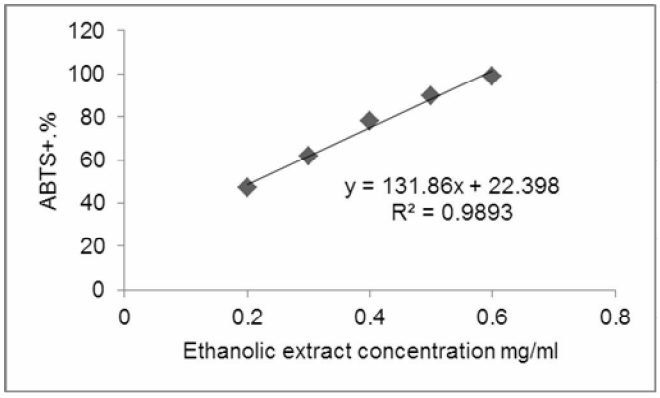

Fig. 7. Increase the inhibition capacity of $ABTS^{+\cdot}$ by increasing the concentration of ethanolic olive leaf extract.

Table 5. The Values of IC_{50} of Vit C and the Olive Leaves Extracts on $DPPH^{\cdot}$ Assay

Sample	IC_{50} (mg ml^{-1})
Vit C	0.05 ± 0.01^{a}
Ethanolic extract	0.39 ± 0.04^{b}
Aqueous extract	0.47 ± 0.01^{b}

Similar letters b indicate no significant differences in IC_{50} values for both aqueous and ethanolic extracts in the $DPPH^{\cdot}$ test.According to SPSS.

Table 6. The Values of IC_{50} of Vit C, Oleuropein, and the Olive Leaves Extracts on $ABTS^{+\cdot}$ Assay

Sample	IC_{50} (mg ml^{-1})
Vit C	0.04 ± 0.01^{a}
Oleuropein	0.02 ± 0.01^{a}
Ethanolic extract	0.19 ± 0.03^{b}
Aqueous extract	0.34 ± 0.04^{c}

^{a,b,c}Values are differ significantly from each other according to spss).

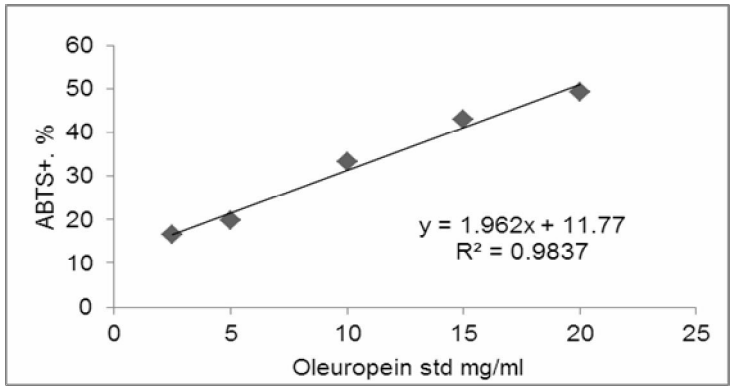

Fig. 8. Standard curve of oleuropein for ABTS $^{+\cdot}$ Assay.

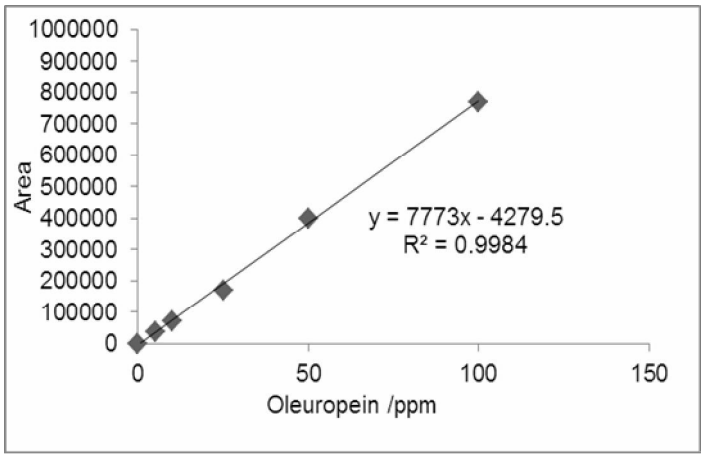

Fig. 9. Standard curve of oleuropein for HPLC separation.

contain a high percentage of non-flavonoids phenols.

Scavenging Activity of DPPH$^{\cdot}$ Radicals

The scavenging effect of olive leaves extracts on DPPH$^{\cdot}$ radical was increased with concentration, as shown in Fig. 6. The scavenging activity of the ethanolic extract was equally effective to the aqueous one according to the statically study. However, in the current study, none of the evaluated samples showed activity as strong as vitamin C; that is known for its radical scavenging activity. The estimation of the antiradical capacity of the different extracts was performed by determining the value of IC_{50} (Table 5).

Scavenging Activity of ABTS$^{+\cdot}$ Radicals

The scavenging effect of olive leaves extracts on ABTS$^{+\cdot}$ radical was increased with concentration as shown in Fig. 7. The estimation of the antiradical capacity of Vit C, oleuropein standards, and different extracts were performed by determining the value of IC_{50}, Table 6 shows that the scavenging activity of the ethanolic extract is more effective than the aqueous one, in accordance to the statically study. There is no significant differences in IC_{50} values for both of Vit C and oleuropein standards in the ABTS$^{+\cdot}$ test.

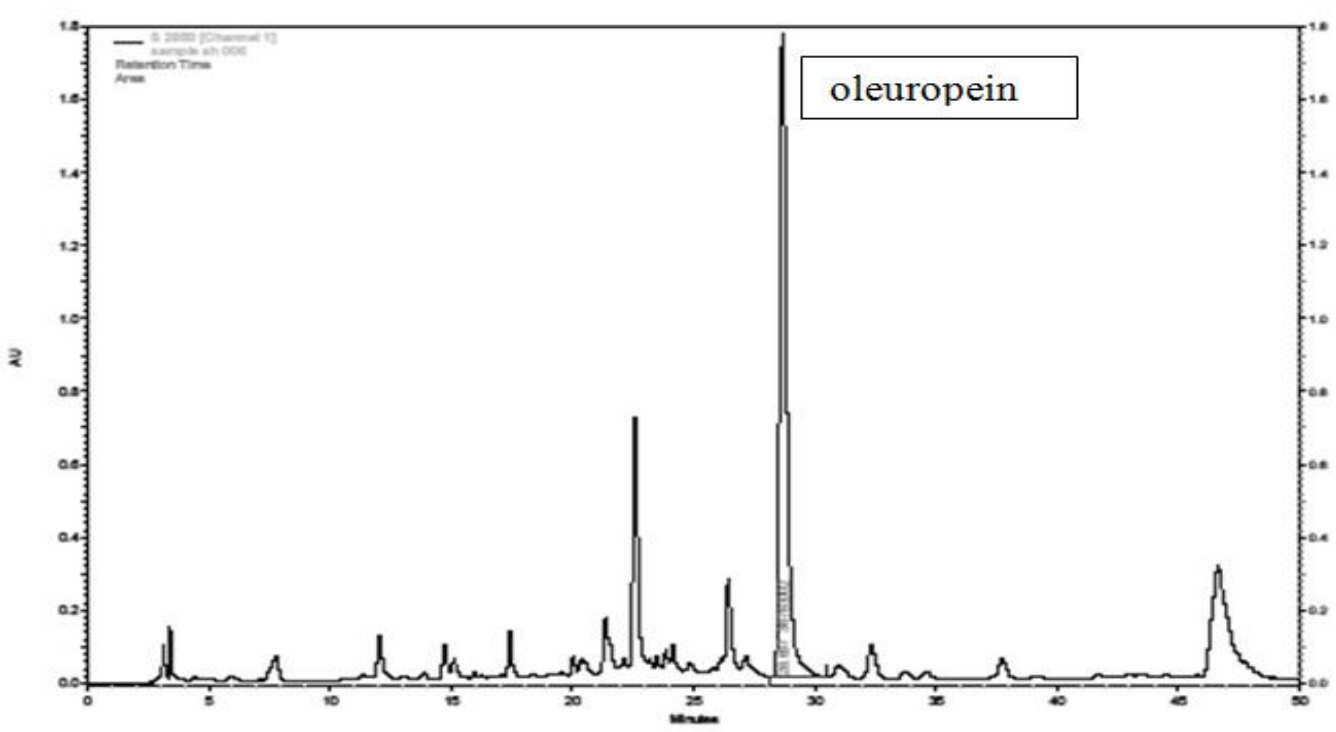

Fig. 10. Chromatogram of olive leaves ethanolic extract.

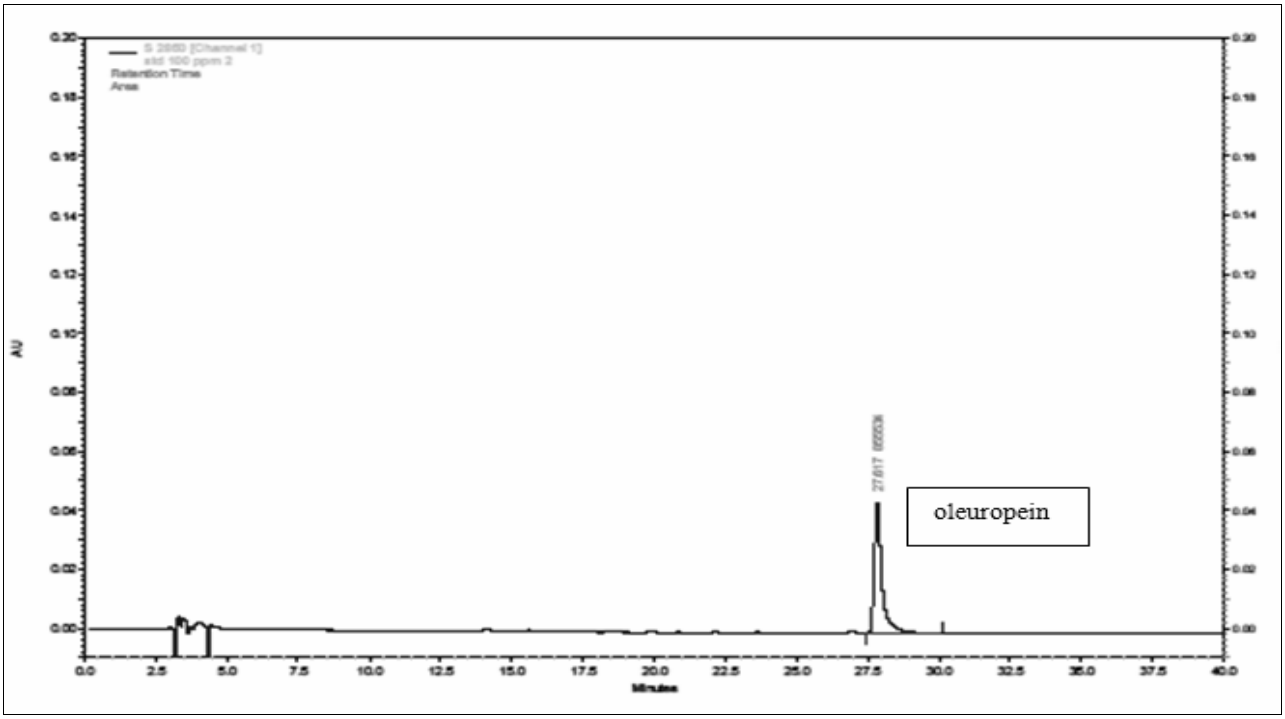

Fig. 11. Chromatogram of standard oleuropein (100 ppm).

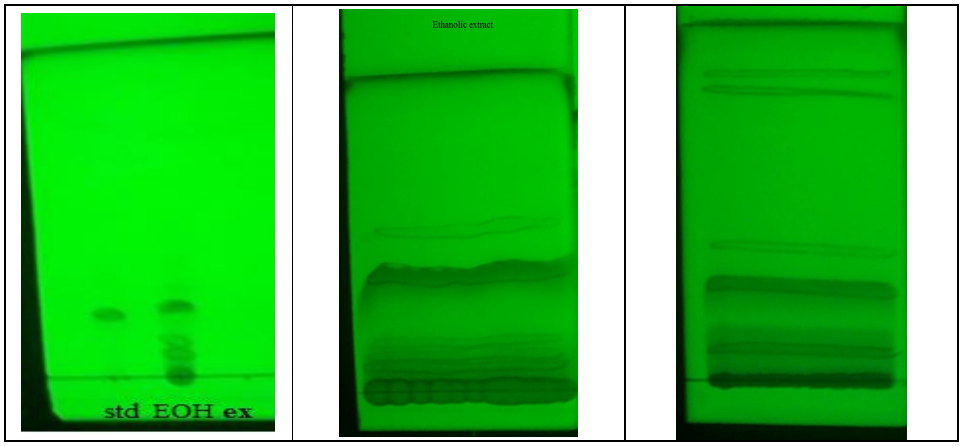

Fig. 12. TLC of oleuropein standard, ethanolic and aqueous olive leaves extracts.

Table 7. Oleuropein Content in Olive Leaves Extracts

Extraction type	Oleuropein conc. (mg g^{-1})	Oleuropein conc. (%)
Ethanolic	88.50 ± 9.67^{a}	8.85 ± 0.96^{c}
Aqueous	37.60 ± 6.84^{b}	3.76 ± 0.69^{d}

The letters a, b, c, d indicate that there are significant differences in the extraction ratio of Oleuropein in the ethanolic and aqueous extracts according to SPSS.

Table 8. Identification of Oleuropein in Standard and Olive Leaf Extracts by HPLC-MS

Oleuropein	$[M-H]^-$ *m/z*
Std	539
Ethanolic extract (TLC)	539
Aqueous extract (TLC)	539

Table 9. TLC Oleuropein Content Yield in Olive Leaves Extracts

Extraction type	Oleuropein conc. (mg g^{-1})	Oleuropein conc. (%)
Ethanolic	0.43 ± 0.00^{a}	0.04 ± 0.00^{c}
Aqueous	0.10 ± 0.00^{b}	0.01 ± 0.00^{d}

The letters a, b, c, d indicate that there are significant differences in the extraction ratio of oleuropein in the ethanolic and aqueous extracts according to SPSS.

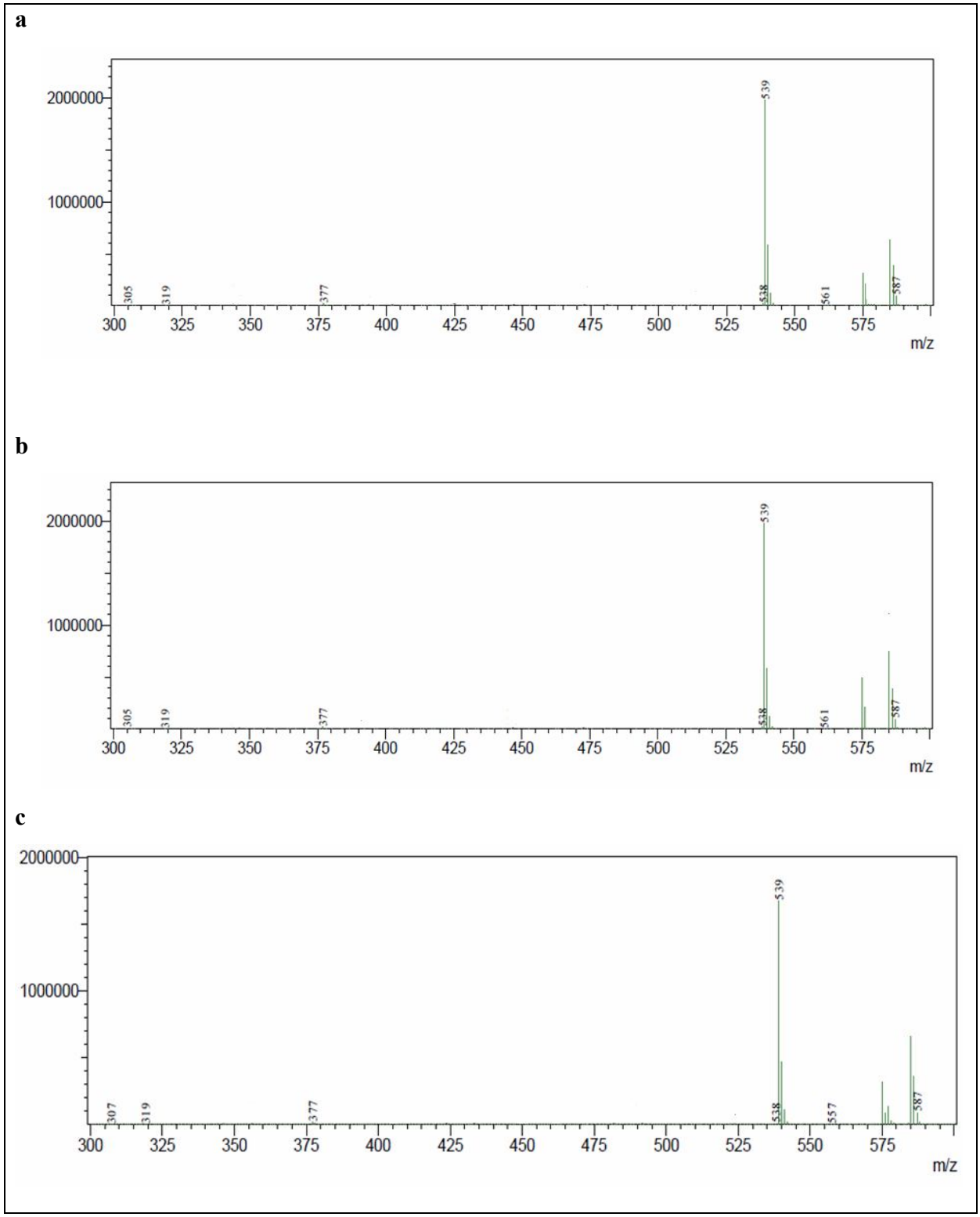

Fig. 13. The MS spectrums of the olueropein standard (a), oleuropein TLC isolated from ethanolic (b), and aqueous (c) olive leaves extracts.

Determination of Oleuropein in Olive Leaves Extracts by HPLC-PDA

Figure 9 shows the oleuropein standard calibration curve. The amount of oleuropein was expressed as milligram per gram of olive leaf powder.

Figures 10 and 11 show chromatograms for olive leaves ethanolic extract sample and the oleuropein standard respectively. Table 7 shows the concentration and percentage of the oleuropein concentration in both ethanolic and aqueous extracts. The highest percentage of the ethanolic extract is observed, as documented by reference [1]. According to the reference [29], the approximate value of the oleuropein concentration was 7.08 ± 0.05% in the ethanolic extract. This value was calculated by extracting olive leaves with an ethanolic mixture (75%) in an ultrasonic bath (with some differences in the extraction conditions). It is noted that the value obtained in this study - using ultrasonic extraction-exceeds those reported in other references using traditional extraction methods, such as simple extraction (soaking method) (9.2 ± 0.3 mg g^{-1}) [30], or Soxhlet extraction (10.0 ± 0.26 mg g^{-1}) [1], (6.85 ± 0.17 mg g^{-1}) [31].

Thin Layer Chromatography (TLC) Procedure

The optimal solvent for the identification of compounds was determined by varying the solvents and its ratios for developing the solvent system, and the best was: chloroform/ methanol (9/1) for one hour, then the plates were dried at room temperature for 15 min, and visualized by exposure to UV light (254 nm) (Fig. 12). The band of the desired compound in both of extracts was collected, dissolved in 0.5 ml 70% EOH, centrifuged, and then prepared for HPLC-MS analysis.

Identification of Oleuropein in Olive Leaves Extracts by HPLC-MS

Figure 13 Shows the MS spectrums of the oleuropein standard, and oleuropein isolated by TLC from ethanolic and aqueous olive leaves extracts, respectively. The result shows the oleuropein ion scan modes of *m/z* 539 $[M-H]^-$ by comparison the MS spectrums. The perfect match was observed for oleuropein standard and oleuropein isolated from the olive leaves extracts in this study. Table 8 shows the deprotonated molecule $[M-H]^-$ in full-scan mode in standard and olive leaves extracts. Table 9 shows the concentration and the concentration percentage of oleuropein yield isolated by TLC in both of the ethanolic and aqueous extracts.

CONCLUSIONS

This study showed the importance of Syrian olive leaves as a renewable cheap natural antioxidant source, because of the high total amounts of phenol and flavonoid contents, in addition to its ability to inhibit $DPPH^{\cdot}$ and $ABTS^{+\cdot}$. Oleuropein (the major compound in olive leaves) was also detected and isolated from the extracts by TLC, HPLC-PDA, and HPLC-MS techniques.

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