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Wild versus Cultivated Olive Leaves Extracts: Antioxidant Activity, Analysis of Total Phenolics and Oleoropein Content

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Abstract

Olive leaves are being used in the last decades as a potential source of beneficial compounds in medicines, cosmetics, and food industry. In this work a comparison between Palestinian wild and cultivated olive leaves extracts obtained using different solvent systems under different conditions was performed. The crude extracts were studied for their total phenol and oleuropein content, in addition to their antioxidant activity which was evaluated using DPPH free radical scavenging method and by studying their effect in stabilization of olive oil samples towards oxidation. The stability of olive oil samples enriched with extract additives was estimated by measuring peroxide values, absorption coefficients K_{270} and K_{232}. The results showed that olive oil stabilizing effect of crude extract was higher than that of commonly used synthetic antioxidants such as BHT. The wild leaves extracts exhibited higher levels of DPPH inhibition than cultivated. The metabolic extract of cultivated olive leaves at pH 7 has higher total phenol content and exhibits better oil stabilizing effect in comparison to other extracts. The identification and quantization of the major phenol component of these extracts (oleuropein) was performed using TLC and HPLC. It was shown that wild olive leaves have higher oleuropein content (23.9%) than cultivated which have oleuropein content of (6.8%). The highest oleuropein content was found in wild olive leaves extract by using methanol-water 80:20 at pH = 3 and 48 h.

Keywords: Solvent extraction, olive leaves, phenolic compounds, oleoropien, antioxidants.

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1. Introduction

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances traditionally referred to as secondary metabolites. These components possess high ant oxidative, antimicrobial, antiviral and an inflammatory properties[1] and thus they are being intensively studied and used as natural alternatives for synthetic drugs and food additives [2] .

In Palestine, one of the most common and popularly used is the products obtained from olive tree (fruit, oil, leaves). Olive fruit and oil are rich in phenol compounds with bioactive properties providing, among other things, antiviral, antitumor and antioxidant activity [3-7]. However, olive leaves gather the interest of the scientific community and the industries worldwide as their health promoting benefits are constantly being shown by an ever-increasing number of scientific data [8]. They are considered as byproducts of olive farming, one of the most important activities in the Mediterranean region, representing almost 10% of the total weight of materials arriving to the olive mill. Historically, olive leaf was used for the treatment of malaria and associated fever [9].

Olive leaf offered a capacity to lower blood pressure and increase blood flow in the coronary arteries [10], relieved arrhythmia and prevented intestinal muscle spasms [11]. In addition, olive leaves could be used not only in medicines, cosmetics, and pharmaceuticals, but they can also be used to improve the shelf life of foods. In fact, olive leaves have been mixed with overripe olives before processing to produce oils with a more marked flavor and a higher resistance to oxidation, or they have been used directly as supplements for oils, food materials, and food additives. Also, phenol extracts of olive leaves have been obtained to perform tablets which are commercially available as dietetic products and/or food integrators [12].

The adult leaves may be regarded as good source of powerful antioxidants. It has been reported that free radical-scavenging activity of leaves extracts is influenced by climatic factors, variety and harvesting stage [4-5]. The main constituents of olive leaves are carotenoids and phenolic compounds, which are both lipophilic and hydrophilic. The lipophilics include tocopherols, while the hydrophilic include flavonoids, phenol alcohols and acids, secoiridoids and their metabolites. The secoiridoids, which are glycosidated compounds, are produced from the secondary metabolism of terpenes as precursors of several indole alkaloids. Tocopherols, phenolic acids, phenolic alcohols and flavonoids are present in many fruits and vegetables belonging to several botanical families, but secoiridoids are present exclusively in plants of the family of Olearaceae [13]. Major phenols include hydroxytyrosol, tyrosol, oleuropein [14], and ligstroside [5].

It has been proven that the free radical scavenging activity of plant extracts is mainly ascribed to the concentration of phenolic compounds present in the plants [15]. And thus they are considered as a source of powerful antioxidants [10]. However, a recent study demonstrated that the phenolic extracts of olive leaves acted as antioxidants in the range of 50 to 200 ppm [16].

Oleuropein, which is a secoiridoid, is a 3,4-dihydroxy-phenylethanol (hydroxytyrosol) ester with a β-glucosylated elenolic acid. It is the major and the most abundant phenolic compound in olive leaves and fruits of *Oleaceae* [4,17], and is responsible for the characteristic bitterness of the olive fruit. It has been reported that oleuropein possesses many beneficial effects on human health, such as antioxidative [18], antimicrobial [19], antiviral [20], anti-inflammatory [21] and hypolipidemic [22] properties. In vitro studies have demonstrated that oleuropein acts as inhibitor of platelet-ctivating factor activity [23] and might be a modulator of metabolism. It improves lipid metabolism to protect against obesity problems [24].

Furthermore, oleuropein intervenes in the developmental processes of olive fruits and tree and defends olive tree against the attack of pathogens and insects [25] . The concentration of oleuropein can reach up to 140 mg/g (14%) on a dry matter basis in young olive fruits and 60-90 mg/g of dry matter in the leaves. The concentration of oleuropein in olive leaves vary from season to season [10,26,27]. It is easily extracted as part of the phenolic fraction of olive fruits, leaves and seeds, but it has not been reported in virgin olive oils [12]. For the extraction of oleuropein innovative methods have emerged that are mainly based on the usage of polar solvents and accelerated energies [28]**.**

Recently several studies focused on contents of the olive leaves and extraction of these high-added value compounds from olive leaves. Solvent extraction is a favorable process since heat sensitive materials can be recovered at low temperatures. For this reason it is preferred for the manufacturing of polyphenol-rich products from plants for their further use in pharmacological, food and cosmetic industry [29-32].

Researchers are continuously seeking those natural antioxidants that will sufficiently protect fats and oils from oxidation. Synthetic antioxidants are effective and inexpensive, but due to their toxicity recent research has focused on antioxidant compounds derived from leaves and fruit of olive trees, numerous fruits and vegetables, as well as aromatic plants and spices. Enriched commercially available oils (olive oil, sunflower oil, palm oil and a vegetable shortening) with polyphenols by adding olive leaf extract to the product has shown higher stability [16,33-34]. Although many researches were performed on the extraction of olive leaves, determination of phenolic compounds and their antioxidant activity [16,28-31,35], this study aims at providing a detailed comparison between wild and cultivated Palestinian olive leaves extracts as a potential source of phenolic natural antioxidants. Also the effects of extraction conditions (pH, solvent, and time) on the crude extract quality for both leaves types is demonstrated.

2. Experimental

2.1. Materials and Equipment

Solvents and reagents for extraction, TLC analysis, olive oil tests and total phenolic content determination (ethanol, methanol, acetic acid, chloroform, isooctane, cyclohexane, potassium iodide, sodium thiosulfate, starch, sodium bicarbonate, butylated hydrxytoluene (BHT), gallic acid, Folin-Ciocalteu's reagent were of analytical grade, but those for HPLC analysis (acetic acid 100% glacial, methanol, acetonitrile and water) were of HPLC grade and all were purchased from Sigma Chemical Co. (Sigma-Aldrich Company Ltd., Dorset, Great Britain). Oleuropein standard was obtained as HPLC grade and purchased from Merck (Darmstadt, Germany). TLC-plated used were silica gel A 60 from Merck company.

The spectrophotometric analysis was performed using (Shimadzu UV-2450 UV-Visible Spectrophotometer, Japan), and HPLC analysis using an HPLC system (SHIMADZU Prominence-I LC-2030C, Tokyo, Japan) with shim-pack VP-ODS column, and UV detector was used.

2.2. Olive Leaves Collection And Preparation

Two types of fresh green olive leaves Cultivated (C) and wild (W) were collected from Beit Rima, Ramallah, West Bank in July, 2015. All samples were dried at ambient temperature [36] in good ventilated area away from direct sunlight for 3 weeks. Air-dried plant materials were ground into a fine powder under law temperature and stored at room temperature in dark for extraction.

2.3. Methods

2.3.1. Extraction

Samples of powdered material were extracted with two solvent systems (80% aqueous ethanol, and 80% aqueous methanol), with the volume to weight ratio of 50:1, at room temperature for different time periods under different pH values. Extractions were carried out under intensive stirring with magnetic stirrer. The supernatant was separated from the solid residue by filtering and centrifuging for 10 min at 5000 rpm. The solvent was removed and the solid extract was totally dried under reduced pressure at 45 °C, using a rotary evaporator. The dry extracts stored at - 20 °C until used for further analyses.

2.3.2. Determination of Total Phenolic Content (TPC)

Analysis of total phenolic content in olive leaves extracts was performed using Folin- Ciocalteu reagent according to the procedure described in literature [37]. Briefly, 0.50 ml of solution of extract (0.20 mg/ml) was mixed with 2.00 ml of Folin-Ciocalteu reagent and then 2.50 ml of 7.5 % sodium bicarbonate solution is added. The solution was incubated room temperature for 40min. The absorbance was measured at 760 nm. Gallic acid was used as standard. Total phenolic content was expressed as mg gallic acid per gram of extract (mg/g).

2.3.3. The Free Radical Scavenging Activity of Extracts

The free radical scavenging activity for all obtained extracts was determined using DPPH (2,2-diphenyl-1-picryl-hydrazyl) according to the method described in literature [1]. Briefly, 0.020 M solution of DPPH in methanol and series of solutions of each extract with concentrations 10, 20, 40, 100, and 200 ppm were prepared.

Then to 4.00 ml of DPPH solution 1.00 ml of extract solution was added, left at room temperature in for 30 min and the absorbance was measured at 517 nm. The percentage inhibition of DPPH for each sample was determined using the following formula: % s DPPH scavenging activity = $[(B_{lank} - A_{sample}) / B_{lank}] \times 100$, Where B_{lank} is the absorbance of DPPH control solution and A_{sample} is the absorbance in the presence of extract. The concentration of extract that resulted in 50% inhibition (IC_{50}) in $\mu g/ml$ was determined from the curve showing the dependence of % activity on the concentration.

2.3.4. TLC and HPLC analysis of extracts

TLC analysis of standard oleurope in and obtained extract was performed on TLC plates (10 cm length) using different mobile phases. Visualization was performed by UV-light and staining by I_2. The quantitative determination of oleurope in was performed using HPLC according to the method developed and validated by Al-Rimawi. F. [26, 36], where two solvent gradient methods was used: 0.1% acetic acid in water/acetonitrile (80/ 20) with UV detection at 280 nm and isocratic elution at a flow rate of 1.0 mL/min, and injection volume was set to 10 µL.

2.3.5. Evaluation of the Extracts Efficiency in Stabilizing Olive Oil Towards Oxidation

The crude extracts in certain concentrations were separately added to different olive oil samples. After adding the crude extract, the stability of oil samples towards oxidation was determined. The oxidative deterioration level was assessed by the measurement of: peroxide value (PV), conjugated dienes (CD - K_{232}) and conjugated trienes (CT - K_{270}). The oxidation stability of these samples was compared with two control samples: one contains synthetic antioxidant (BHT) the second contains no additives (blank).

2.3.5.1. Determination of Peroxide Value (PV)

Determination of PV of olive oil samples was conducted according to the analytical method described in European Official Method of Analysis (Commission Regulation EEC N-2568/91- Determination of peroxide value). Chloroform, acetic acid and saturated potassium iodide solution were added to 1.0000 g of an olive oil sample and were kept in dark at room temperature for exactly 1 minute, and then deionized water was added. Titration of free iodine was carried out with 0.010 M sodium thiosulfate solution. The PV was expressed in mill equivalents of oxygen per kg of oil (meq of O_2 / kg).

2.3.5.2. Specific Absorption Coefficient at 232 nm And 270 nm (K_{232} and K_{270})

European Official Method of Analysis (Commision Regulation EEC N-2568/91) was used for the determination of specific absorption coefficients of the olive oil samples. 0.2500 g of olive oil was weighed, placed into a 25.0-mL volumetric flask and diluted with cyclohexane. The sample was homogenized using vortex. Then the absorbance of resulting solution was measured at 232 nm and 270 nm using cyclohexane as blank.

3. Results and Discussion

3.1. Extraction

In order to achieve the optimum extraction conditions, that enable to get high extract recovery, high total phenolic content and high antioxidant activity, three variables were examined: type of olive leaves (cultivated and wild), solvents and pH. For each type of olive leaves exactly weighed three grams of dried and milled leaves were taken and 150.0 ml of different solvents mixture added at different pH as shown in Table 3.1. The extraction was carried out at ambient temperature for 48h.

To determine the effects of the mentioned variables (type of leaves (wild or cultivated), pH, and solvent system), we performed all extractions at fixed time of 48 h. The data presented in the Table 3.1 definitely confirm that the yield of solid extract from wild olive leaves was significantly higher than that from cultivated leaves. Furthermore, the percent of solid extract from different types of olive leaves varied over a wide range (25- 40)%. The maximum recovery of solid extract was obtained from wild leaves using ethanolic system at pH 1. At pH 7 the methanolic extracts from both types leaves have higher recovery than ethanolic, but at lower pH vice versa.

Table 3.1. The yield of solid extract and total phenolic content (TPC) of extracts from wild and cultivated olive leaves.

Solvent system	pH	Wild leaves	Cultivated leaves	Wild leaves	Cultivated leaves
Methanol: water 80:20	7	34.8 %	29.5 %	104.5	132.6
Methanol: water 80:20	3	24.3 %	28.8%	96.0	101.4
Methanol: water 80:20	1	35.6 %	30.4 %	89.6	81.4
Ethanol: water 80:20	7	32.5%	25.6 %	99.3	90.8
Ethanol: water 80:20	3	39.8%	27.0%	87.5	82.7
Ethanol: water 80:20	1	40.0 %	34.9 %	70.8	49.5

The recovery of solid extract at low values of pH was more than at higher ones. This can be explained by the fact that acidification of olive leaves increases phenolic compounds' diffusion from the plant material through cell wall disintegration.

3.2. Determination of Total Phenolic Content (TPC) of Extracts

The total phenolic content was determined using the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as milligrams gallic acid per gram extract (mg GA/g extract).

The calculations of total phenolic content were based on the obtained calibration curve from analyses of gallic acid standard solutions. For the used calibration curve: $R^2 = 0.9998$, equation: $y = 0.0091x + 0.0038$. The results of analyses and calculations are shown in Table 3.1. The phenolic content of extracts varied in response to different types of olive leaves, solvents and pH, and ranged from 49.5 to 132.6 mg GA/g of extract. However, the maximum phenolic content was determined in the methanolic extract of cultivated (132.6 mg GA/g of extract) at pH 7.

According to the data in the Table 3.1 above, it is notable that the ethanolic wild olive leaves extracts have higher TPC than cultivated, but the methanolic extracts of cultivated leaves pH 3 and 7 contain higher levels of phenolics. Furthermore, the use of methanol resulted in higher content of phenolic compounds than ethanol at the same values of pH. This can be explained by the fact that solvents could significantly affect total phenolics due to differences in their polarities, which might influence the solubility of various constituents present in olive leaves. Hence, the selection of the appropriate solvent is one of the most importanr steps in optimizing the recovery of plant phenolics. The effect of pH on total phenolics in different extracts appears clearly so that extraction at pH 7 results in higher recovery of phenolic compounds than at pH 3 and 1.

3.3. Analysis of Olive Leaves Extracts for Oleuropein Content

3.3.1. TLC Analysis of Oleoropein

At this stage of work we aimed at qualitative analysis and identification of oleuropein using TLC. So we first tried to develop TLCs of standard oleuropein using the eluent systems reported in the literature [38] that are (methanol: ethyl acetate: benzene 20:40:40) and (methanol: ethyl acetate: benzene 50:20:30), but they gave R_f equal to zero, so we were to find an optimal eluent system for TLC analysis of oleuropein , which gives good separation and retention factor (R_f).

To do this, we studied more than 20 eluent systems on standard commercial oleuropein and extracts. According to R_f values for oleuropein, we found several acceptable systems: (1) methanol : ethyl acetate: benzene, 50:30:20 (2) methanol : ethyl acetate : benzene, 60:20:20 (3) methanol : ethyl acetate : benzene, 50:40:10 that gave R_f 0.61-0.71. But the optimal eluent system according to both R_f values and separation of the extract components was found to be methanol: ethyl acetate : benzene, 40:40:20 which enabled to separate oleuropein with R_f of 0.76-0.78. Other systems like (Methanol : acetonitrile : benzene, 70:20:10, 60:20:20 or Methanol : acetonitrile, 80:20 or water : acetontrile, 80:20) give R_f value ranged from 0.84 -0.92. So the system methanol : ethyl acetate : benzene, 40:40:20 was used for detection of oleuropein in all obtained extracts (Fig . 3.1)

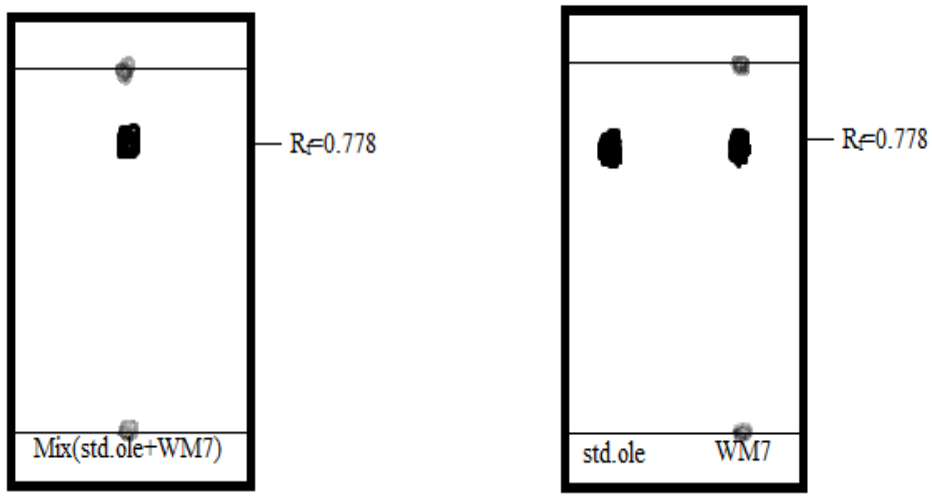


Fig. 3.1. TLC chromatograms of standard oleuropein (a) and metanolic wild olive leaf crude extracts at pH 7 (b) . Where WMC: wild metanolic extract at pH 7, std. Ole: standard oleoropein.

TLC analyses confirmed the presence of oleuropein in all extracts except those obtained at pH of 1 were no oleuropein was detected. This fact can be explained by the degradation of oleuropein at extremely low pH .

3.3.2. Determination of Oleuropein Content in Olive Leaves Extracts by HPLC

For quantitative determination oleeuropein in the obtained extracts we used the HPLC method developed and validated by Fuad Al-Rimawi [26,36]. First a sample of standard solution of oleuropein with appropriate concentration was subjected to analysis. From the chromatogram obtained (Fig. 3.2), it is evident that oleuropein was eluted with a retention time of 20.8 minutes.

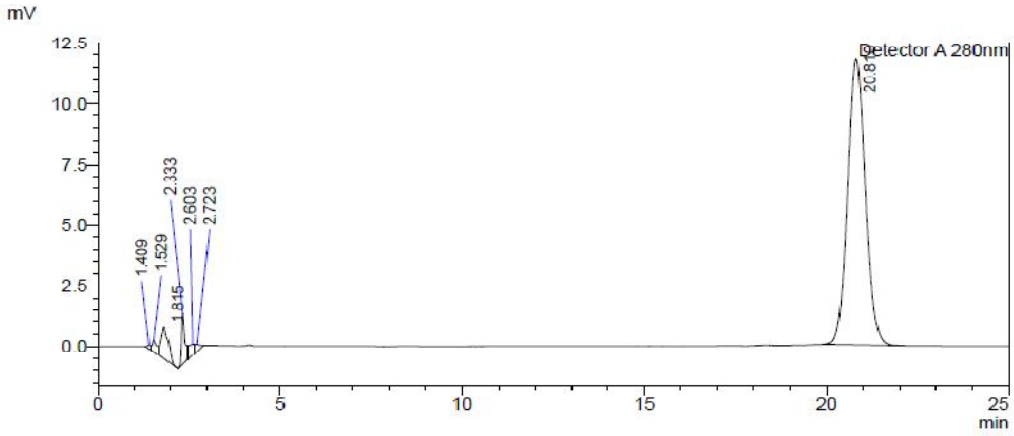


Fig.3.2. HPLC chromatogram of standard oleuropein .

HPLC analyses of dried olive leaf extracts were then performed. Two of the obtained chromatograms from the analysis of crude extracts solutions (methanolic extracts of wild leaves at pH 7 and 3) are presented in Fig. 3.3 and Fig 3.4 respctively. It is obvious that the major polyphenol in the extracts is oleuropein, with retention time 20.7 and 20.8 which completely agrees with the analysis of standard oleuropein (Figure 3.2).

In addition, the other peaks that appear in the chromatogram correspond to other phenolic compounds that are present in the olive leaves in much less levels than oleurppe in. The HPLC profile shows also several peaks with retention time 1.5-2.1 that can be related to the solvent used.

To evaluate the linearity range of the method, different calibration standards of oleurope in were analyzed by HPLC, and the responses are recorded [26]. As a result, the calibration curve obtained was linear with R^2 =0.9996 in the range from 25 – 200 ppm. This result demonstrates the linearity of this method over a wide dynamic concentration range [26]. The least-square regression line equation was: y = 2024X + 7214, where Y is the area of corresponding peak and X is the concentration of the oleuropein standard (ppm). This calibration curve was then used for quantitative determination of oleurope in the analyzed extracts. The effects of solvent, time and pH of extraction on oleurope in content were also studied.

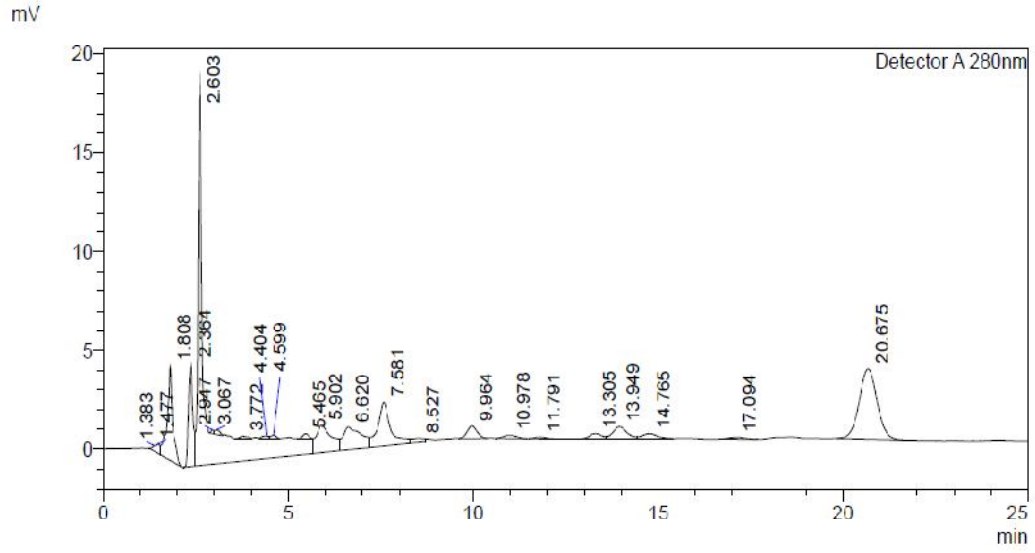


Fig. 3.3: HPLC chromatogram of wild olive leaf crude extracts with methanol at pH 7.

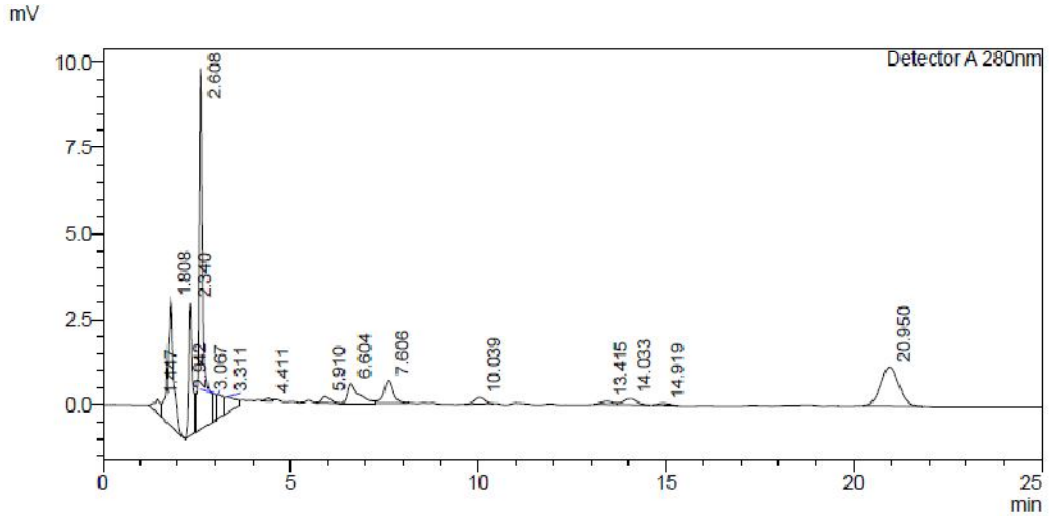


Fig.3.4: HPLC chromatogram of wild olive leaf crude extracts with methanol at pH 3.

3.3.2.1. The Effect of Extraction Solvents and pH on Oleurope in Content

As reported in literature [28], highly polar solvent systems are suggested to be more effective in oleuropein extraction from olive species. Thus two types of solvent systems (methanol-water, 80:20 and ethanol-water, 80:20) were used to extract oleuropein from olive leaves. However the results presented in Table 3.2 indicate that higher oleuropein levels were observed using the methanolic system for both types of leaves. It can also be confirmed that wild leaves extraction yield is significantly higher than that of cultivated using both systems.

The influence of the extractant pH on oleuropein content obtained under optimal working conditions of the other variables was also studied (Table 3.2) Diluted aqueous solution of hydrochloric acid was used to adjust the extractant pH values as required.

Table 3.2. Oleuropein content in extracts obtained after 48 hours .

		Wild leaves	Cultivated leaves
Methanol: water 80:20	7	18.4	6.7
Methanol: water 80:20	3	23.9	6.8
Methanol: water 80:20	1	0	0
Ethanol: water 80:20	7	14.5	6.6
Ethanol: water 80:20	3	15.3	4.6
Ethanol: water 80:20	1	0	0

As shown in Table 3.2, higher yields of oleuropein were observed at an optimum pH of 3, while the use of higher or lower pH values caused a significant decrease in the yields of oleuropein. This may be related to higher extraction efficiency at pH 3 compared with pH 7, and lower degradation of oleurope in than pH 1. In addition, the most significant increase in oleurope in content in extract at pH 3 (23.9%) compared with that obtained at pH 7 (18.4%) was that of wild leaves using methanol. No oleuropein is detected in extracts obtained at pH 1. It can be explained by the degradation of oleurope in at this extremely low pH and that confirms the results obtained by TLC analyses. However, our results regarding the effect of pH in general agree with those of the work in the literature [39], but we obtained significantly higher yields of oleorope in.

3.3.2.2. Effect of Extraction Time on Oleurope in Content

Table 3.3 represents data about the oleurope in content of some extracts from wild and cultivated leaves determined as a function of time.

These data indicate that the content of oleurope in in olive leaves obtained from both types (cultivated and wild) vary over a range from 1.8% -23.9% of olive leave dried extract. It is obvious that the oleuropein content increases with time till 48 hours, after what it decreases. This can be due to the degradation of oleuropein in the solution after this long period of time. If compare the results for extracts at different pH it can be seen that a there is an increase in oleuropein content with the time continued till 48 hours for pH 7, but extracts at pH 3 has shown no significant change in oleuropein content after 24 h. It can be concluded that the optimum extraction time at room temperature is 24-48 hours. The highest amount of oleuropein content (23.9%) was obtained from wild olive leaves using the methanolic extraction system (methanol : water, 80:20) at pH 3 and after 48 hours .

Table 3.3: Dependence of oleurope in content of extract on the extraction time.

	% oleurope in in dry extract					
sample	after 3 h	After 6 h	after 24 h	after 30 h	after 48 h	after 72 h
wild methanolic extract at pH 7	5.4	14.4	15.8	17.0	18.4	14.8
wild ethanolic extract at pH 7	-	-	12.0	-	14.5	-
cultivated methanolic extract at pH 7	-	-	6.1	-	6.7	-
cultivated ethanolic extract at pH 7	-	-	4.8	-	6.6	-
wild methanolic extract at pH 3	5.6	15.6	22.8	22.8	23.9	22.1
wild ethanolic extract at pH 3	-	-	12.6	-	12.3	-
cultivated methanolic extract at pH 3	1.8	-	5.8	-	6.7	-
cultivated ethanolic extract at pH 3	-	-	4.6	-	4.6	-

3.4. The Antioxidant Activity of Extracts

3.4.1. DPPH Radical Scavenging Activity

The results of determinantion of DPPH radical scavenging activity of the studied extracts are included in Table 3.4. They are presented as the concentration of extract that exhibits a 50% inhibition of DPPH in its 0.020 M solution (IC_{50}).

Table 3.4: DPPH IC_{50} of olive leaves extracts obtained after 48 hours.

		Wild leaves	Cultivated leaves
Methanol: water 80:20	7	27.3	27.8
Methanol: water 80:20	3	27.7	29.1
Methanol: water 80:20	1	28.8	36.0
Ethanol: water 80:20	7	29.0	29.5
Ethanol: water 80:20	3	28.2	34.1
Ethanol: water 80:20	1	27.1	34.0

The obtained data Indicate that wild olive leaves extracts has higher antioxidant activity than cultivated. While ethanolic extracts of both types of leaves shows lower activity than metahnolic except those obtained at pH 1. We can also note that the extraction at higher values of pH results in higher radical scavenging activity of extract. These results agrees with the total phenolic content data with the exception of methanolic extracts at pH 3 and 7, where the cultivated leaves extracts contain higher levels of total phenolics. However the highest DPPH inhibition was demonstrated by methanolic extracts from both wild and cultivated leaves at pH 7. The 50% DPPH inhibition was achieved using these extracts with concentration of 27.3 and 27.8 $\mu g/ml$ respectively.

3.4.2. The Efficiency of Extracts in Stabilizing Olive Oil Towards Oxidation

Two extracts with the higher total phenolic content and DPPH free radical scavenging activity (that are methanolic extracts of wild and cultivated leaves at pH 7) were chosen to be studied for their efficiency in increasing the oxidation stability of olive oil in comparison with one of the commercial synthetic antioxidants (butylated hydroxytoluene BHT).

For this olive oil samples enriched with these extracts (400 ppm) and other with pure BHT (200 ppm) were subjected to accelerated oxidation conditions (50°C) and incubated for 12 days. Then the oxidative deterioration level of extract and BHT stabilized samples and control one (without any additives) was assessed by measurement of peroxide value (PV), conjugated dienes (K_{232}) and trienes (K_{270}). The results are shown in Table 3.5.

Table 3.5 : Alteration in oxidation parameters (PV, K_{232}, K_{270}) for oil samples enriched with wild, cultivated leaves extracts at pH 7 and BHT and control through the period of study.

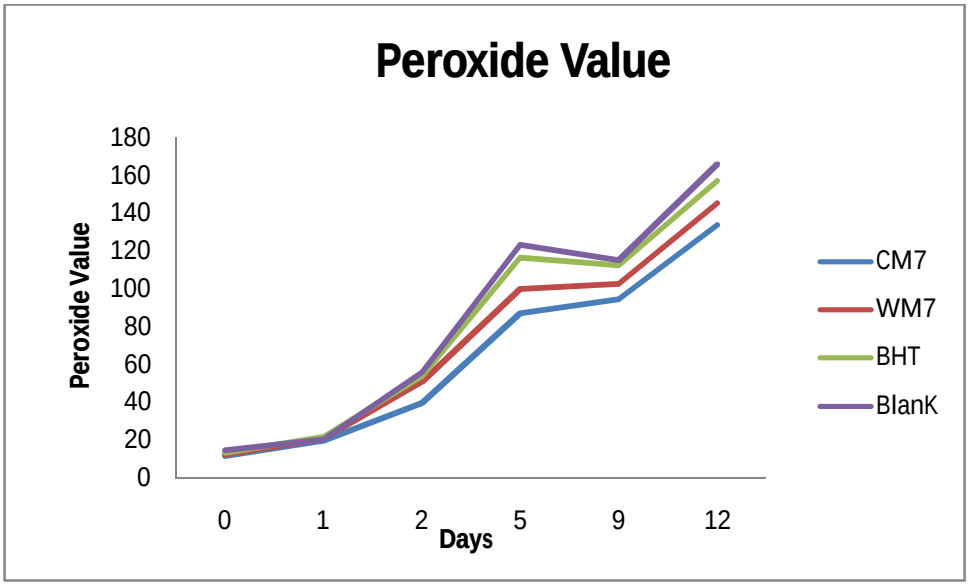
Days	(CM7)*			(WM7)*			(BHT)			(control)**		
	PV	K_{232}	K_{270}	PV	K_{232}	K_{270}	PV	K_{232}	K_{270}	PV	K_{232}	K_{270}
0	11.9	0.209	0.020	12.7	0.209	0.020	13.65	0.209	0.020	14.9	0.209	0.020
1	19.9	0.215	0.019	21	0.224	0.018	22.2	0.219	0.022	20.63	0.241	0.021
2	40.1	0.251	0.021	51.2	0.258	0.020	54.1	0.263	0.022	56.11	0.273	0.024
5	87.2	0.278	0.023	100.3	0.282	0.021	116.8	0.282	0.025	123.5	0.301	0.025
9	94.7	0.298	0.025	102.9	0.295	0.022	112.6	0.303	0.027	115.5	0.302	0.028
12	133.9	0.317	0.026	145.5	0.317	0.022	157.4	0.323	0.027	166.2	0.323	0.031

*** CM7: methanolic cultivated olive leaves extract at pH 7, WM7: methanolic wild olive leaves extract at pH 7 .**

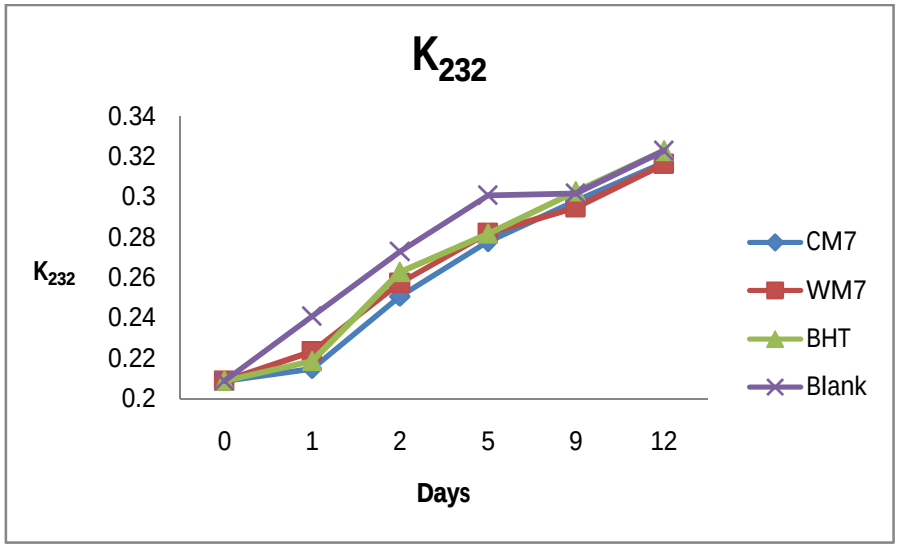
**** Control: oil samples without additives (blank)**.

It is evident from the results Table 3.5 that adding extracts with 400 ppm lowered the PV by 12-19 % with respect to blank. On the other hand, the addition of pure synthetic antioxidant (BHT) with a concentration of 200 ppm decreased the PV by 5% which is also shown in Figure 3.5 the rate of decline in PV was the highest in the oil sample stabilized with methanolic cultivated extract at pH 7, followed by wild extract.

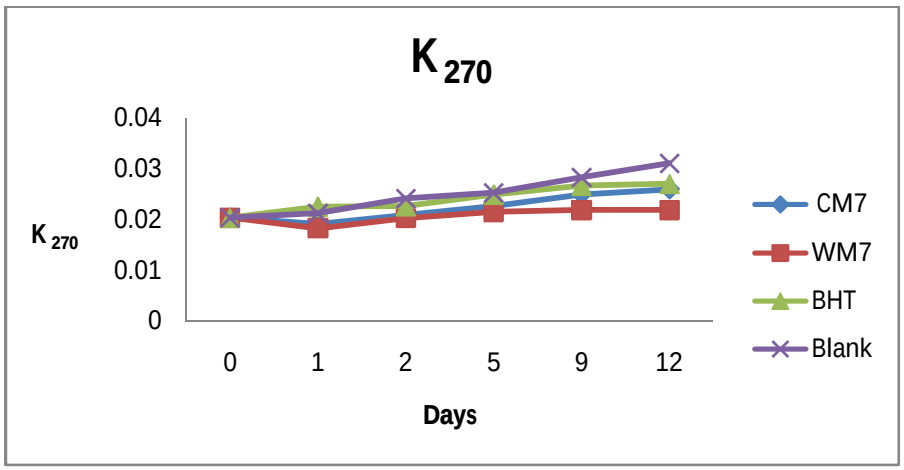
These results are compatible with the content of total phenolics, which are supposed to be responsible for preventing oxidation process. The data about conjugated dienes (CD) and trienes (CT) contents of olive oil samples stabilized with methanolic extracts and BHT from Table 3.5 are shown in Figures 3.6 and 3.7. The CD and CT contents increased during incubation. The oil samples which stabilized with extract showed lower levels of CD and CT compared to the control which have the highest level, this indicated the antioxidant potential of the extracts. A decline of CT in olive oil samples stabilized with extracts was 16 - 29 % compared with the blank, and 12 % for synthetic antioxidant (BHT).



Figurer 3.5: Peroxide Value (meq / kg oil) of stabilized and control oil.



Figurer 3.6. Conjugated diene values (K_{232}) of stabilized and control oil.



Figurer 3.7: Conjugated trienes (K_{270}) of stabilized and control oil.

In conclusion, the previous results have shown that the cultivated crude extract which has higher total phenolics is more effective in stabilizing olive oil than wild. However, both crude extracts have higher potent towards enhancing the oxidative stability of oil than synthetic antioxidant (BHT).

3.4.2.1. Effect of Concentration on Antioxidant Activity

In order to study the effect of the concentration of the extracts on their ability to protect olive oil samples from oxidation, four oil samples with cultivated methanolic extract at pH 7 (having the higher oil stabilizing effect) at different concentrations (50, 100, 300, 500) ppm were prepared. These samples were kept at 25°C for 25 days and the oxidative deterioration level was monitored by measurement of PV and K_{232} factors. The results are shown in Table 3.6.

Table 3.6: Alteration in oxidation parameters (PV, K_{232}) for oil samples with enriched methanolic cultivated leaves extract at pH 7 with different concentrations through the incubation time.

	50 ppm		100 ppm		300 ppm		500 ppm		Control	
Days	PV	K_{232}	PV	K_{232}	PV	K_{232}	PV	K_{232}	PV	k K_{232}
0	16.70	0.219	16.65	0.219	16.97	0.219	15.40	0.219	16.80	0.219
5	30.75	0.236	28.75	0.234	31.80	0.237	28.20	0.228	34.30	0.233
8	35.20	0.239	34.18	0.239	34.05	0.239	32.50	0.238	35.10	0.247
12	40.60	0.250	38.90	0.253	41.30	0.247	41.02	0.249	42.70	0.264
14	47.90	0.260	47.60	0.258	48.60	0.257	48.20	0.252	49.76	0.266
18	53.50	0.264	52.16	0.262	55.60	0.257	55.30	0.276	61.25	0.279
20	63.30	0.269	62.29	0.269	65.83	0.269	66.06	0.271	70.59	0.282
25	80.18	0.274	77.16	0.269	83.43	0.285	83.25	0.285	88.58	0.299

The data presented in Table 3.6 confirm that the most effective extract concentration was 100 ppm, since it has the higher ability of inhibiting oxidation (has the lowest peroxide value and K_{232}). Lowering in the ability of 300 and 500 ppm to stabilize oil samples is related to the fact that at high concentrations they act as pro-oxidants.

Our results agreed with a previous study which concludes that the olive leave extracts effectively act as antioxidants in the concentration range of 50 to 200 ppm [16].

3.4.2.2. The Effect of Extraction pH And Solvent on the Antioxidant Activity of Extracts

To study the effect of extraction solvent and pH on the efficiency of extracts in enhancing the oxidative stability of oil, samples of oils enriched with wild methanolic extracts at pH 7, and pH 3 and ethanolic extract at pH 3 were prepared. These samples were kept at 25°C to investigate their oxidation stability. Values of peroxide and K factors shown in Table 3.7.

Table 3.7 : Alteration in oxidation parameters (PV, K_{232}) for oil samples with methanol wild leaves extract at pH 3 and pH 7, and ethanol wild extract through the period of study.

Days	Wild methanol pH=7		Wild ethanol pH=7		Wild methanol pH=3		Control	
	PV	K_{232}	PV	K_{232}	PV	K_{232}	PV	K_{232}
0	16.97	0.219	15.4	0.219	15.42	0.219	16.8	0.219
5	31.8	0.237	31.74	0.229	33.6	0.228	34.3	0.233
8	34.05	0.239	33.02	0.244	35.3	0.242	35.1	0.247
12	41.3	0.247	43.7	0.254	48	0.248	42.7	0.264
14	48.6	0.257	50.6	0.256	50.67	0.259	49.76	0.266
18	55.6	0.257	56.4	0.265	57.03	0.264	61.25	0.279
20	65.83	0.269	67.11	0.274	68.85	0.276	70.59	0.282
25	82.5	0.284	83.43	0.276	85.94	0.281	88.58	0.299

From the results of PV and K factors for samples enriche with wild extracts, the methanolic extract has higher ability for inhibiting oxidation than ethanolic.

The results are in agreement with those obtained for total phenolic content and DPPH scavenging activity. Also, for the same type of olive leaves and solvent, the extract obtained at pH 7 exhibited the higher antioxidant activity than pH 3 which is compatible with total phenolic content and DPPH inhibition activity.

4. Conclusions

The present work, has shown that there are significant variations in total phenolic and oleuropein content and antioxidant activity of olive leaves extracts based on the type of leaves (wild or cultivated), the solvent used and pH of extraction. The highest oleuropein content in olive leaves extract was obtained from wild olive leaves using both methanolic and ethanolic extraction systems, which is considered as a valuable data about the potential for industrial mass production of oleoropein. The cultivated leaves extract at pH 7 has the highest TPC and antioxidant activity. These extracts exhibited oxidative stabilization effect of olive oil up to a greater extent than commonly used commercial synthetic antioxidant (BHT). Therefore, olive leaves can be considered as a potential antioxidant source of natural origin.

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6. References

Chu, Y. F., Sun, J., Wu, X., Liu, R. H. (2002). Antioxidant and anti proliferative activities of common vegetables. J. Agric. Food Chem. 50, 6910-6916.

Kaileh, M., Vanden Berghe, W., Boone, E., Essawi, T., haegeman, G. (2007). Screening of indigenous Palestinian medicinal plants for potential antinflamatory and cytotoxic activity. J. Ethnopharmacol., 113, 110-116.

Barbaro, B., Toietta, G., Maggio, R., Arciello, M., Tarocchi, M., Galli, A., Balsano, C. (2014). Effects of the Olive-Derived Polyphenol Oleuropein on Human Health. Int. J. Mol. Sci., 15, 18508-18524.

Brahmi, F., Mechri, B., Dhibi, M., Hammami, M. (2013). Variations in phenolic compounds and antiradical scavenging activity of Olea europaea leaves and fruits extracts collected in two different seasons. Industrial Crops and Products, 49, 256– 264.

Owen, R. W., Haubner, R., Wurtele, Hull, E., Spiegelhalder, B., Bartsch, H. (2004). Olives and olive oil in cancer prevention. Eur. J. Cancer Prev., 13, 319-326.

Tuck, K. L., Hayball, P. J. (2002). Major phenolic compounds in olive oil: metabolism and health effects. J. Nutr. Biochem., 13, 636-644.

Visiol, F., Poli, A., Galli, C. (2002). Antioxidant and other biological activities of phenols from olives and olive oil. Med. Res. Rev., 22, 65–75.

Carcia-Villalba, R., Larrosa, M., Possemiers, S. (2014). Bioavailability of phenolics from an oleoropein-rich olive (*Olea europaea*) leaf extract and its acute effect on plasma antioxidant status: comparison between pre- and postmenstrual women. Eur. J. Nutr., 53, 1015-1027.

Agalias, A., Melliou, E., Magiatis, P., Tsarbopoulos, A., Mitaku, S. (2005). Quantitation of polyphenols and secoiridoids in decoctions of *Olea europaea* leaves from ten Greek cultivated varieties. J. Liq. Chromatogr. Rel. Technol., 28, 1557-1571.

Lee, O. H., B. Y. (2010). Antioxidant and antimicrobial activities of individual and combined phenolics in Olea europaea leaf extract. Bioresource Technology, 101, 3751–3754.

Benavente-GarcõÂa , O., Castillo, J., Lorente, J., OrtunÄ, A., Del Rio, J.A. (2000). Antioxidant activity of phenolics extracted from Olea europaea L. leaves. Food Chemistry, 68, 457-462.

Talhaoui, N., Gómez-Caravaca, A. M., León, L., De la Rosa, R., Segura-Carretero, A., Fernández-Gutiérrez, A. (2014). Determination of phenolic compounds of 'Sikitita' olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents 'Arbequina' and 'Picual' olive leaves. Food Science and Technology, 58, 28-34.

Tripoli, E., Giammanco, M., Tabacchi, G., Di Majo, D., Giammanco, S., La Guardia, M. (2005). The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutrition Research Reviews, 18, 98–112.

Perona, J. S., Cabello-Moruno, R., Ruiz-Gutierrez, V. (2006). The role of virgin olive oil components in the modulation of endothelial function. J. Nutr. Biochem., 17, 429-445.

Sultana, B., Anwar, F., Asi, M. R., Chatha, S. A. S. (2008). Antioxidant potential of extracts from different agro wastes: Stabilization of corn oil. GRASAS Y ACEITES, 59, 205-217.

Lafka Theodora, I., Lazou Andriana, E., Sinanoglou Vassilia, J. (2013). Phenolic Extracts from Wild Olive Leaves and Their Pootential as Edible Oils Antioxidants. Foods, 2, 18-31.

Servili, M., Montedoro, G. (2002). Contribution of phenolic compounds in virgin olive oil quality. Eur. J. Lipid Sci. Technol., 104, 602-613.

Benavente-Garcia, O., Castillo, J., Lorente, J., Ortuno, A., Del Rio, J. A. (2000). Antioxidant activity of phenolics extracted from Olea Europaea Leaves. Food Chem., 68, 457–462.

Pereira, A. P., Ferreira, I. C. F. R., Marcelino, F., Valent¸o, P., Andrade, P. B., Seabra, R., Estevinho, L., Bento, A., Pereira, J. A. (2007). Phenolic compounds and antimicrobial activity of olive (Olea europaea L. Cv. Cobranc¸ osa) leaves. Molecules, 12, 1153–1162.

Micol, V., Caturla, N., Pérez-Fons, L., Mas, V., Pérez, L., Estepa, A. (2005). The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). Antiviral Res., 66, 129–136.

Visioli, F., Bellosta, S., Galli, C. (1998). Oleuropein, the bitter principles of olives, enhances nitric oxide production by mouse macrophages. Life Sci., 62, 541–546.

Jemai, H., Bouaziz, M., Fki, I., El Feki, A., Sayadi, S. (2008). Hypolipidimic and antioxidant activities of oleuropein and its hydrolysis derivative-rich extracts from Chemlali olive leaves. Chem. Biol. Interact., 176, 88–98.

Andrikopoulos, N. K., Antonopoulou, S., Kaliora, A. C. (2002). Oleuropein inhibits LDL oxidation induced by cooking oil frying by products and platelet aggregation induced by platelet-activating factor. Lebensm. Wiss. Technol., 35, 479–484.

Polzonetti, V., Egidi, D., Vita, A., Vincenzetti, S., Natalini, P. (2004). Involvement of oleuropein in (some) digestive metabolic pathways. Food Chem., 88, 11–15.

Malik, N. S. A., Bradford, J. M. (2006). Changes in oleuropein levels during differentiation and development of floral buds in “Arbequina” olives. Sci. Hort., 110, 274–278.

Al-Rimawi, F. (2014). Development and validation of a simple reversed phase HPLC-UV method for determination of oleuropein in olive leaves. Journal of food and drug analysis, 22, 285 -289.

Amiot, M. J., Fleuriet, A., Macheix, J. J. (1986). Importance and evolution of phenolic compounds in olive during growth and maturation. J. Agric. Food Chem., 34, 823–826.

Xynos, N., Papaefstathiou, G., Psychis, M., Argyropoulou, A., Aligiannis, N., Skaltsounis, A. L. (2012). Development of a green extraction procedure with super/subcritical fluids to produce extracts enriched in oleuropein from olive leaves. J. of Supercritical Fluids, 67, 89– 93.

Ahmad-Qasem, M. H., Cánovas, J., Barrajón-Catalán, E., Micol, V., Cárcel, J. A., García-Pérez, J. V. (2013). Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound. Innovative Food Science and Emerging Technologies, 17, 120–129.

Apostolakis, A., Grigorakis, S., Makris Dimitris, P. (2014). Optimisation and comparative kinetics study of polyphenol extraction from olive leaves (Olea europaea) using heated water/glycerol mixtures. Separation and Purification Technology, 128, 89–95.

Hussain Abdullah, I., Chatha Shahzad, A. S., Noor, S., Arshad Muhammad, U. , Khan Zulifqar, A., Rathore Hassaan A., . Sattar Munavvar, Z. A. (2011). Effect of Extraction Techniques and Solvent Systems on the Extraction of Antioxidant Components from Peanut (Arachis hypogaea L.) Hulls. Food Anal. Methods, 5, 890-896.

Ja`pon-Lujan, R., Luque-Rodriguez, J. M., de Castro, M. D. L. (2006). Dynamic ultrasound-assisted extraction of oleuropein and related biophenols from olive leaves. J. Chromatogr., A1108, 1, 76- 82.

Bouaziz, M., Fki, I., Jemai, H., Ayadi, M., Sayadi, S. (2008) . Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves. Food Chemistry, 108, 253–262.

Gamel, T. H., Kiritsakis, A. (1999). Effect of methanol extracts of rosemary and olive vegetable water on the stability of olive oil and sunflower oil. Grasas y. Aceites, 50, 345- 350.

Savournin, C., Baghdikian, B., Elias, R., Dargouth-Kesraoui, F., Boukef, K., Balansard, G. (2001). Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in Olea europaea leaves. J. Agric. Food Chem., 49, 618–621.

Afaneh, I., Yateem, H., Al-Rimawi, F. (2015). Effect of Olive Leaves Drying on the Content of Oleoropein. Journal of Analytical Chemistry, 6, 246-252.

Mau, J. F, Ryan, P. R., Delhaize, E. (2001). Aluminum tolerance in plants and the complexing role of organic acids. Trends Plant Sci., 6, 273-278.

Capasso, R., Evidente, A., Scognamiglio, F. (1992). A simple thin layer chromatographic method to detect the main polyphenols occurring in olive oil vegetation water. Phytochemical analysis , 3, 270-275.

Ismaili, A., Heydari, R., Rezaeepour, R. (2016). Mointoring the Oleoropein content of olive leaves and fruits using ultrasound- and salt-assisted liquid-liquid extraction optimized by response surface methodology and high-performance liquid chromatography. J. Sep. Sci., 39, 405-411.