STUDY OF CHEMICAL COMPOSITION AND PHYTOCHEMICAL COMPOUNDS OF LOCAL OLIVE (*OLEA EUROPAEA* L.) LEAVES

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ABSTRACT

The current study aimed to estimate the chemical composition and detect bioactive compounds, such as polyphenols and flavonoids, in the aqueous and alcoholic extracts of olive leaves. The study involved collecting olive leaves from areas in Al Za'franiya, Baghdad, Iraq. According to the chemical composition, olive leaves have a good protein content (9.89%), which may play a major role in alleviating global malnutrition. The results revealed that the leaves contain high total Ash (22.20%), carbohydrates (55.66%), energy (273.52 kcal/100g), and low total fat content (1.28%). Ethanol extract 70% contained the highest concentration of oleuropein (298.0 mg/L), which is responsible for antioxidant and pharmacological potential, as well as enhancing the immune system. The study concluded that olive leaves represent a rich and diverse natural source of active compounds, which opens horizons for their use in the food and health industries.

***Keywords: Oleuropein, Antioxidant, HPLC, Chemical composition, By-product**
***Part of M.Sc. thesis for the [1st]author**

دراسة التركيب الكيميائي والمركبات الفعالة لأوراق الزيتون المحلي

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المستخلص

هدفت الدراسة الحالية الى تقدير التركيب الكيميائي والكشف عن المركبات الفعالة مثل البوليفينولية والفلافونويدات لمستخلص المائي والكحولي لأوراق الزيتون. شملت الدراسة جمع أوراق الزيتون من مناطقة الزعفرانية في بغداد/العراق. ووفقاً للتركيب الكيميائي فإن أوراق الزيتون تحتوي على نسبة جيدة من البروتين (9.89%) والذي قد يلعب دوراً رئيسياً في تقليل فرص الاصابة بسوء التغذية على مستوى العالم. كما أظهرت النتائج أن الأوراق تحتوي على نسبة عالية من الرماد الكلي (22.20%) والكربوهيدرات (55.66%) والطاقة (273.52 كيلو كالوري/100 غم) ونسبة منخفضة من الدهون الكلية (1.28%). كما احتوى مستخلص الإيثانول 70% على أعلى تركيز من الأوليوروبين (298.0 ملغم/لتر) وهو المسؤول لكبح الجذور الحرة (مضاد أكسدة طبيعي) واستخدامه في الصناعات الدوائية فضلاً عن تعزيز الجهاز المناعي. وخلصت الدراسة إلى أن أوراق الزيتون تمثل مصدراً طبيعياً غنياً ومتنوعاً للمركبات الفعالة النشطة مما يفتح آفاقاً لاستخدامها في الصناعات الغذائية والصحية.

***الكلمات المفتاحية: أوليوروبين، مضادات أكسدة، كروماتوغرافيا السائل عالية الدقة، تركيب كيميائي، مخلفات ثانوية**

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INTRODUCTION

Olive trees are among the oldest fruit trees in the world and the original homeland of this type of tree is the Mediterranean region, including Iraq (41). The Mediterranean basin contains 95 % of the world's olive trees as a major agricultural crop. The United States and Iraq ranked 14 and 23, respectively in olive-producing countries worldwide (13). Mainly in Greece, Italy, Spain, Australia, Portugal, France, Cyprus, the United States, and Tunisia (20, 22). The nutritional value of olive leaves has obtained attention due to the rich composition of bioactive compounds such as phenolic compounds and flavonoids, which have been significantly studied for their potential health benefits (10, 19). According to several studies, olive leaf extract improves coronary artery blood flow, and leaves have long been used as a folk remedy to treat fever and other illnesses (32, 34). According to Machado et al. (22), olive leaves are a valuable raw material that may be utilized as a natural antioxidant and stabilizer for vegetable oils. Throughout the nineteenth tropical century, the British used olive leaves to cure ailments like malaria that were common in the colonies, and the ancient Egyptians used them to embalm the bodies of the pharaohs (7). Olive tree leaves have been used very commonly in traditional herbal medicine to treat and prevent various diseases, especially in the Mediterranean region (8). Olive leaves have important biomedical properties such as antiviral, antimicrobial, anti-Alzheimer, antioxidant, treat fever, and anti-inflammatory activities (28, 29). Olive leaf extracts are particularly important due to their therapeutic effects, as these extracts contain of bioactive phenols (30). Most studies have shown that oleuropein is the main phenolic component of olive leaves. Oleuropein has a significant optimistic effect on the technical and nutritional properties of the plant, including its anti-inflammatory and antioxidant effects, which may be related to its ability to prevent several diseases, such as atherosclerosis also works as an antibacterial and antihypertensive (17). The chemical composition of olive leaves can be influenced by several factors: age of the tree, time of harvesting, and environmental fluctuations (26). About 38% of the weight of olive leaves consists of organic content, ranging from 76.4 to 92.7 g/100 g dry matter (2). Several scientific studies show that olive leaf phenolic and polyphenols are responsible for numerous biological properties. Reactive oxygen species are effectively inhibited by phenolic compounds, which are categorized as secondary metabolites and present in olive leaves (24). Olive leaf extracts are particularly important due to their therapeutic effects, as these extracts contain of bioactive phenols (30). Most studies have shown that oleuropein, the main phenolic component of olive leaves, Oleuropein has a significant optimistic effect on the technical ad nutritional properties of the plant, including its anti-inflammatory and antioxidant effects, which may be related to its ability to prevent several diseases such as atherosclerosis also worked as an antibacterial and antihypertensive (17). This study aimed to analyze phytochemicals and chemical compositions as well as evaluate the antioxidant concentration of olive leaves.

MATERIALS AND METHODS

Plant material : Fresh leaves of olive (*Olea europaea* L.; Nabali variety) were directly collected from tree plantation farms from the Horticulture Department /Ministry of Agriculture in Al-Za'franiya, Baghdad, Iraq. This variety of olive leaves was scientifically defined by the Crop Science and Horticulture Department, College of Agricultural Engineering Science. University of Baghdad, Iraq. Olive leaves were collected in early September 2024, washed with tap water, and air-dried in a dark place, then ground into a fine powder, and stored in a closed plastic container until use for extract.

Olive leave extracts (OLE): The extraction of the olive leaves powder was carried out by maceration method using three organic solvents of different polarities (70% ethanol, 80% methanol, and 100% Aqueous). Fifty grams of olive leaves powder of each sample were macerated separately in 500 mL of extraction solvent at room temperature for 24 h then stirred by a magnetic stirrer for 4 h. Then the macerate was filtered through the Whatman paper (No. 1). Finally, a rotary evaporator under a vacuum at 40 °C was used to concentrate and remove the solvent. The

resulting extracts were stored at 4 °C in a dark container until use (5, 39).=

Chemical composition and total energy

AOAC (1) methods were used for the chemical analysis of olive leaves powder: Moisture content was determined according to method item (No.934.01) by drying an appropriate amount of the sample in an air circulation oven at 105 ºC until constant weight. Method in item (No.920.39) was applied for the determination of crude fat content using Soxhlet apparatus. Crude fiber content was measured with method No.978.10. Crude protein was determined on the leaves of olive from Kjeldahl nitrogen using a 6.25 conversion factor (method No.990.03). Ash content was measured via method item (No.923.03) by heating samples in a muffle furnace at 550 ºC for 6 h to constant weight as described in the AOAC manual. Energy value was calculated based on the energy nutrient results achieved using the conversion of the Atwater general factor system, as described by Hammoud and Saddam (15), considering 9 kcal/g (37 kJ/g) for fat, 4 kcal/g (17 kJ/g) for carbohydrate, and 4 kcal/g (17 kJ/g) for protein.

Qualitative phytochemical analysis of olive leaves: Qualitative analysis techniques were focused on identifying the presence or absence of numerous chemical groups in the various alcoholic and aqueous extracts of leaves. The presence of tannins in olive leaves extract was proven by the formation of a red precipitate when 1 ml of the extract was mixed with a few drops of 1% lead acetate (40). A graduated cylinder containing one ml of extract and four ml of distilled water was shaken for twenty minutes. The formation of foam is a sign that saponines are present. A few drops of Meyer reagent per milliliter were added to the extract. A yellow or cream-white precipitate occurs as a result of the alkaloids (31). A test tube was filled with 1 milliliter of olive leave extract and a few drops of sodium hydroxide solution. The ability of flavonoids to produce a vivid yellow hue that goes away when a few drops of diluted hydrochloric acid are added indicates their presence. A 10% FeCl3 solution, 1.0 ml of olive leave extract, and 1.0 ml of distilled water were added. A bluish-black hue indicates the presence of phenol (44).

HPLC for quantification of oleuropein

Oleuropein in the olive leaves extract was identified using an HPLC system (SYKAM Germany) equipped with a quadrupole pump and a C18 column and a mobile phase of acetonitrile (ACN)/methanol; oleuropein was detected by injecting 0.1 ml of the previously prepared extract. Water with acetic acid (0.1%) (Mobile phase A) and acetonitrile (mobile phase B). The solvents were monitored according to the following conditions with some modifications: from 0 to 22 min, 90% A to 50% A; from 22 to 32 min, 50% A to 0% A; from 32 to 40 min, 100% B; and from 40 to 50 min, 0% A to 90% A. The flow rate used was set at 0.50 mL/min. The reading was at a wavelength of 285 (42).

Phenolic compounds determination

Determination of phenolic compounds using Folin-Ciocalcium analysis at 1n concentration and gallic acid standard curve at 200 (µg/ml) concentration (stock solution), the method described by Bennour et al., 2020 (5) was used to determine the total phenolic content of olive leaf extract.

Determination of flavonoids

The method described by Bennour *et al.* (2020) (5) was followed. Based on the standard curve of rutin at a concentration of 2 µg/g. and at a wavelength of 510nm.

Antioxidant activity of olive leaves

DPPH radical scavenging assay was assessed by the stable 2, 2-diphenyl-1-(DPPH) radical with some modifications. A 400 µg/ml DPPH solution was prepared by dissolving 0.04g of DPPH in 100 ml of methanol. Weigh out 0.5g of vitamin C and mix was added 100 ml of distilled water and methanol to make a 5000-ppm solution to produce the samples and the vitamin C standard solution. The dilution law was used to create solutions for the samples and vitamin C with different concentrations (30, 60, 120, 250, and 500 ppm). After shaking the mixture well, it was left at room temperature for 30 minutes, and a UV-visible spectrometer produced by Shimadzu Company was used to detect absorption at a wavelength of 517 nm (27, 37). DPPH consumption was converted to ascorbic acid equivalent (mg VCE/g dried leaves) using a calibration curve

(R2 =0.9997) with ascorbic acid standard solutions.

The inhibition ratio was obtained using the following formula: Inhibition ratio = A0 - A1 / A0 × 100

Where A0 represents the absorbance of the blank sample and A1 represents the absorbance when the test sample is present (44).

Statistical analysis : The results were expressed as mean ± standard deviation by a one-way analysis of variance (ANOVA) test followed by Tukey's test using (IBMSPSS Statistics 21.0)

RESULTS AND DISCUSSION

Chemical composition of olive leaves powder: The proximate chemical composition of olive leaves is shown in Table 1. The moisture content of olive leaf powder was recorded at (4.08±0.1%). Low humidity in dried olive leaves indicates preservation quality, as low humidity levels reduce the growth of microorganisms. A study by Haboubi *et al.* (14) showed that olive leaves with low humidity show higher stability of bioactive substances such as phenols. The total fat percentage in the model was (1.28 ± 0.11%), the total fat content is low, which is expected since olive leaves are not a storehouse of fat compared to seeds or oil. According to Bouaziz *et al.* (9), olive leaves are low in fat but rich in essential fatty acids such as oleic acid, which makes them nutritionally valuable. The percentage of total protein was (9.86 ± 0.23%), a relatively high percentage of protein, indicating that olive leaf powder may be an alternative plant source of protein. A study by Ibrahim *et al.* (16) also implied that proteins in olive leaves may play a main role in alleviating global malnutrition. Total ash was (6.93±0.11%), the ash content represents the essential minerals in the leaves, such as potassium, calcium, and magnesium, whose benefit in improving body functions is confirmed by a study Benavente-García *et al.* (4). Crude total fiber was (22.20±1.21%), this high percentage reflects the health value of olive leaves as a rich source of fiber, which contributes to improving digestive health and reducing cholesterol levels, as documented in a study Olmez *et al.* (23). Carbohydrates were (55.66 ± 1.0%), the high percentage of carbohydrates indicates that olive leaves are considered an energy source, making them a potential nutritional component, which is in line with the results of Machado *et al.* (22). Finally, Energy was (273.52±0.52 Kcal), the caloric value reflects carbohydrates, fats, and proteins combined, which adds to the importance of olive leaf powder as a low-calorie, high-health food source (16).

Table 1. Chemical composition of dried olive leaves powder (g/100g)

Component	Mean ± Standard Deviation*
Moisture	4.08 ± 0.1
Total Fat	1.28 ± 0.11
Total Protein	9.86 ± 0.23
Total Ash	22.20 ± 1.21
Total Fiber	6.93 ± 0.11
Total Carbohydrates	55.66 ± 1.0
Energy (Kcal)	273.52 ± 0.52

The ± values represent the arithmetic mean of the replicates

Qualitative chemical detection of active compounds in olive leaf extracts: Table 2 shows the results of the tests to detect the nature and type of active compounds present in olive leaves for the three extracts (70% ethanol, 80% methanol, 100% aqueous) respectively. Phenolic compounds were detected using 1% ferric chloride reagent, where all extracts gave positive results, but the strongest response was shown in ethanol and methanol extracts, indicating that organic solvents have a higher extraction capacity for these compounds than water. In flavonoids, the concentration of ethanol Extract was the highest (33). while that of methanol and water was lower. This can be explained by the fact that flavonoids are better soluble in modest amounts of alcohol. As for alkaloids, the aqueous extract contained the largest amount, while the percentages of ethanol and methanol were lower. This indicates that there is a higher solubility of alkaloids in water. The methanol extract showed the highest level of detection of glycosides compared to the other extracts, indicating that glycosides have a moderately soluble nature in alcohol. Since Terpenes are relatively nonpolar and therefore dissolve better in organic solvents, we found that they were present in good proportions in ethanol extracts but lacked in water. While the table shows that coumarin were present only in methanol and water extracts, but were not

detected in ethanol (18). Tannins were present in high levels in all extracts. Saponins were detected by Vortex shaking, and were present in all extracts (38). but at higher concentrations in ethanolic and methanolic extracts and lower in water, as saponines dissolve well in alcoholic solvents but are also present in water. The table shows the pH values for each extract, where the ethanol extract was 5.04, the methanol extract was 4.61, and the water extract was 5.50. These values show that the extracts are relatively acidic, which may affect the stability of some chemical compounds.

Table 2. Qualitative analysis (colorimetric) of active compounds in olive leaves extracts

Active ingredient	Test	Ethanol Extract 70%	Methanol Extract 80%	aqueous extract 100%
Phenols	Ferric chloride 1%	+++	+++	++
Flavonoids	Ethyl alcohol 95% + Potassium hydroxide 50%	+++	++	+
Alkaloids	Mayer's reagent	++	+	+++
Glycosides	Benedict's reagent	+	++	-
Terpenes	Chloroform + Acetic acid Anhydrous+ Sulfuric acid	++	++	-
Coumarins	Filter paper moistened with 10% sodium hydroxide solution	-	+	+
Tannins	A- Lead acetate 1% B- Ferric chloride 1%	+++	++	++
Saponines	Shake with vortex device	++	++	+
pH	PH meter	5.04	4.61	5.50

(-) indicates negative detection, (+) indicates positive detection, and (++ and +++) indicates positive detection with high concentrations of active compounds

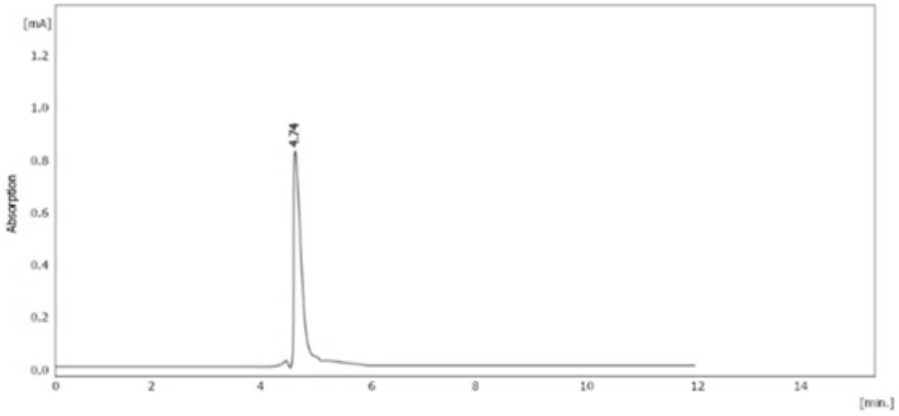
Total phenolic, flavonoid, and oleuropein in olive leaf extract: Results of quantitative analysis are shown in Table (3) and Figure (1). The most efficient method for extracting these chemicals is ethanol (152.9 mg Gallic /100 mg), followed by methanol (130.1 mg/100 mg) and aqueous extract (124.7 mg/100 mg), respectively. Ethanol extract is more beneficial in food and supplement industries because it provides rich doses of antioxidant compounds (7). Flavonoids are an important compound of dietary nutrients that enhance the immune system and protect against inflammation and chronic diseases. Ethanol solvent is the most effective solvent for extracting flavonoids (98.8 mg rutin /100 mg), followed by methanol (91.8 mg/100 mg) and aqueous extract (80.9 mg/100 mg), as evidenced by table 3. Olive leaves extract by ethanol solvent may have higher nutritional value for enhancing the health of individuals through inclusion in functional foods and food industries (6). Oleuropein is a very common compound in olive leaves which has antioxidant and anti-inflammatory properties. In addition, this compound has a potential role in improving cardiovascular health (3, 35). Table 3 shows that the highest percentage of oleuropein was in 70% ethanol extract (298.0 mg/100 mg), followed by 80% methanol (261.0 mg/100 mg), with the lowest reported was in aqueous (2.970 mg/100 mg). This confirms previous studies that indicated that oleuropein is more soluble in organic solvents than water, and therefore, ethanol extract is best suited for extracting active compounds from leaves (36). The antioxidant properties of phenolic compounds have a role in decreasing the chances of chronic diseases including diabetes and heart disease making them one of the vital ingredients that have health benefits (26).

Table 3. Quantitative chemical detection of active compounds in olive leaves extracts

Active ingredient	Ethanol extract 70%	Methanol extract 80%	Aqueous 100%
Total phenolic (mg Gallic/100mg) (spectro)	152.9	130.1	124.7
Total Flavonoid (mg Rutin/100mg)(spectro)	98.8	91.8	80.9
Oleuropein (mg/ L) (HPLC)	298.0	261.0	2.970

HPLC chromatogram shows that there are major peaks in certain retention times and these peaks correspond to the compounds found in olive leaves extract. Oleuropein is used as a standard compound to determine the substances and measure the concentration of the leaves extracts. Three major peaks (Figure 1) for the ethanol extract were observed indicating that the main peak at 4.78 min corresponds to oleuropein accounting for 50% of the total area. The extract shows the highest total area than the methanol and aqueous

extracts, indicating that ethanol could be a better solvent for olive leaf phenolic extraction (6). This is consistent with the literature showing that ethanol is commonly used for the extraction of phenolic compounds due to its high ability to extract these compounds with strong activities. (22). As for the methanol extract, the main peak appears at 4.71 minutes, which is slightly less efficient than the ethanol extract. The last one corresponds to the aqueous extract, which presents three peaks, as shown in Figure 1 (D), at retention times of 3.00, 4.60, and 6.05 min, where a good distribution of the percentage of each compound regarding area and height can be observed. The peak corresponding to the main one is that from 4.60 min, oleuropein, representing 50% of the total area, shows the high concentration of this compound within the aqueous extract. This corresponds with literature that shows oleuropein is among the major constituents of olive leaves and possesses numerous health-promoting activities, including antioxidant and anti-inflammatory effects. (12).



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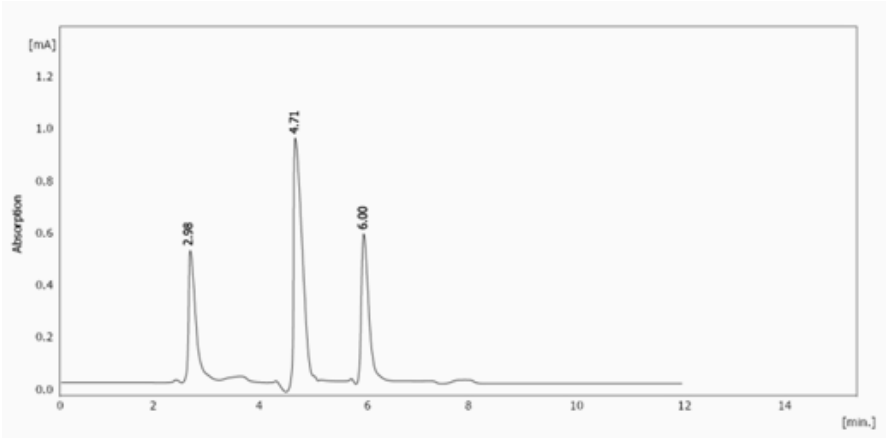

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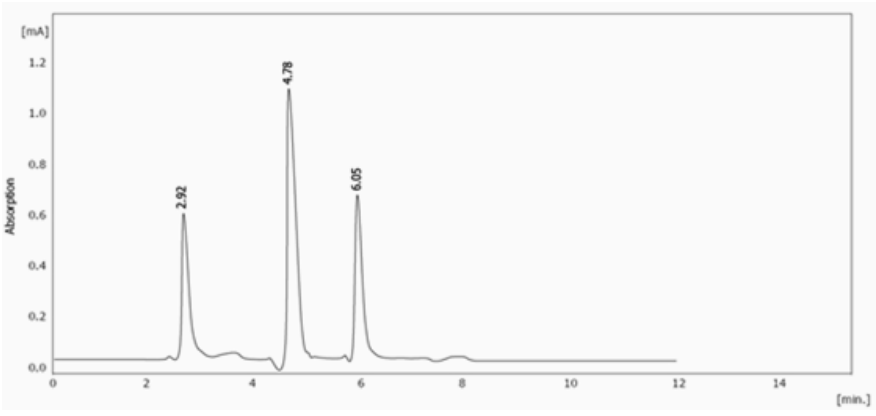

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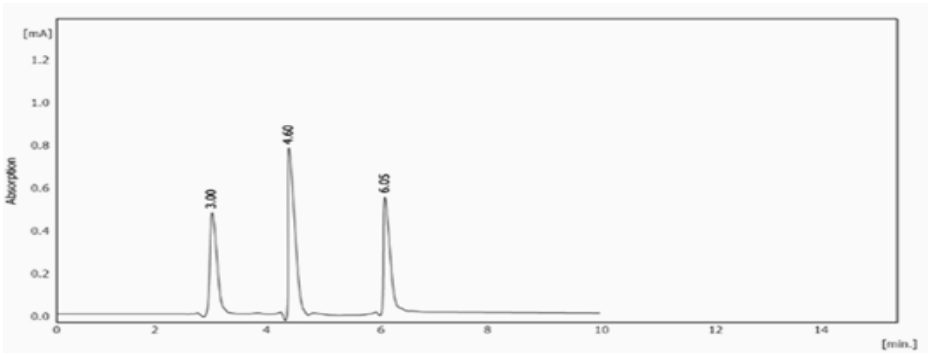

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Figure 1. High-Performance Liquid Chromatography (HPLC) Chromatogram of standard and olive leaves extracts (OLE); A) Oleuropein, B) 70% ethanol extract, C) 80% methanol extract, and D) 100% Aqueous extract

Antioxidant activities of olive leaf extracts

DPPH assay commonly used to estimate antioxidant activity was applied to determine the activity. This method is based on the ability of antioxidants to scavenge free radicals. Table 4 demonstrates the different concentrations of three extracts compared with vitamin C as standard at concentrations of 500 ppm, 250 ppm, 120 ppm, 60 ppm, and 30 ppm of 70% ethanol extract, 80% methanol extract, and 100% aqueous extract antioxidant effectiveness. The 70% ethanol extract is more effective than vitamin C, especially at high concentrations (90.66% at 500 ppm). However, efficiency decreased significantly at lower concentrations. This suggests that ethanol-soluble active components (such as phenolics and flavonoids) contribute significantly to antioxidant activity. 80% methanol extract, efficacy recorded values close to ethanol extract at most concentrations (89.00% at 500 ppm and 22.30% at 30 ppm). This is attributed to the efficiency of methanol in extracting polyphenol compounds, which are mainly responsible for antioxidant activity (21). Aqueous extract showed relatively lower potency compared to ethanol and methanol but still maintained significant potency (87.55% at 500 ppm and 22.25% at 30 ppm). Water-soluble components, such as carbohydrates and proteins, likely play a less active role compared to phenolic compounds (25).

Table 4. Efficacy of extracts as natural antioxidants compared with vitamin C as a standard

Samples	30 ppm	60 ppm	120 ppm	250 ppm	500 ppm
Vitamin C (Standard)	12.25	39.58	51.25	69.00	75.65
Ethanol extract 70%	22.41	46.22	69.88	80.22	90.66
Methanol extract 80%	22.30	46.00	66.08	77.44	89.00
Aqueous extract 100%	22.25	45.89	64.58	74.56	87.55

Conclusions

The results of this study indicate that local olive leaves (*Olea europaea* L.) represent a rich source of bioactive compounds, especially polyphenols and flavonoids, known for their antioxidant properties. These substances help fight free radicals and reduce the chance of developing chronic diseases. The active chemicals isolated from olive leaves have a lot of potential for use in the food and pharmaceutical industries, making them a natural alternative to manufactured chemical compounds. This makes them a promising ingredient for the food and health industries. Chemical analyzes showed that the leaves contain a good percentage of protein (9.86%), which enhances their nutritional value. They are also characterized by a high content of carbohydrates (55.66%) and energy (273.52 kcal/100g), which indicates the possibility of using them as a complete food source. The study showed that the 70% ethanol extract was the most efficient in extracting oleuropein (298.0 mg/L), the main compound responsible for the antioxidant activity and pharmacological properties of olive leaves, which enhances its potential for use in the prevention of chronic diseases such as atherosclerosis and heart disease. Alcoholic extracts also showed higher efficiency in extracting phenolic compounds and flavonoids compared to the aqueous extract, indicating that organic solvents are more capable of dissolving biologically active compounds. The

results of the DPPH test showed that the ethanolic extract had the highest antioxidant capacity compared to other extracts, recording a free radical scavenging rate of 90.66% at a concentration of 500 ppm, even outperforming vitamin C. This indicates the potential of olive leaf extract as a natural antioxidant in the food and pharmaceutical industries. Furthermore, the results indicate that it could be a useful by-product for sustainable agriculture instead of being disposed of.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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