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Comparison of extraction yields, antioxidant, antimicrobial activity and concentration of main components of *Olea europaea* leaf samples at different seasons and of different areas in Palestine

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Abstract : *Olea europaea* is widely cultivated tree for oil production in the Mediterranean area. The benefits of olive leaves refer to their vital polyphenols components. In this study, the optimum way for the extraction of olive leaves using Soxhlet extractor and various concentrations of ethanol as solvent was examined. In order to find the effect of environmental conditions on the extraction yield and chemical composition of olive leaves, phenolic components of olive leaf extract (Oleuropein and Rutin) were analyzed area-wise and season-wise by a new and reliable RP-HPLC method. Extracts were screened for two pharmacological effects, namely antioxidant and antimicrobial activities. Thus, olive leaves were collected from three different areas in Palestine [south (Hebron), center (Beit Jala) and north (Tulkarm)] at two maturation stages [June 2014 (season 1) and October 2014 (season 2)]. Results showed that 75% EtOH was the best extracting solvent and season 2 gave higher yields of extraction. Beit Jala samples showed higher extracts than the two other areas with higher concentrations of Oleuropein, while Rutin was not detectable. Antioxidant activity was higher for untreated samples and samples of Hebron and Beit Jala had similar values. All samples showed good antimicrobial activity against Gram positive bacteria, while no inhibition was detected against Gram negative bacteria.
Keywords: Olive leaf, Soxhlet extraction, Oleuropein, Rutin, antimicrobial activity.

Introduction

Olive tree, *Olea europaea*, is a species of fruit trees belongs to the family *Oleaceae*. It is widely cultivated in the Mediterranean Basin where 98% of the world crop is produced[1]. Olive tree is of a vital importance in Palestine; it is considered as a major commercial crop that occupies around 48% of agricultural land of West Bank and Gaza Strip[2]. Several Palestinian industries depend on olive tree for food, wood, cosmetics and medicine industries. Finally it has a significant symbolic meaning that represents Palestinians'

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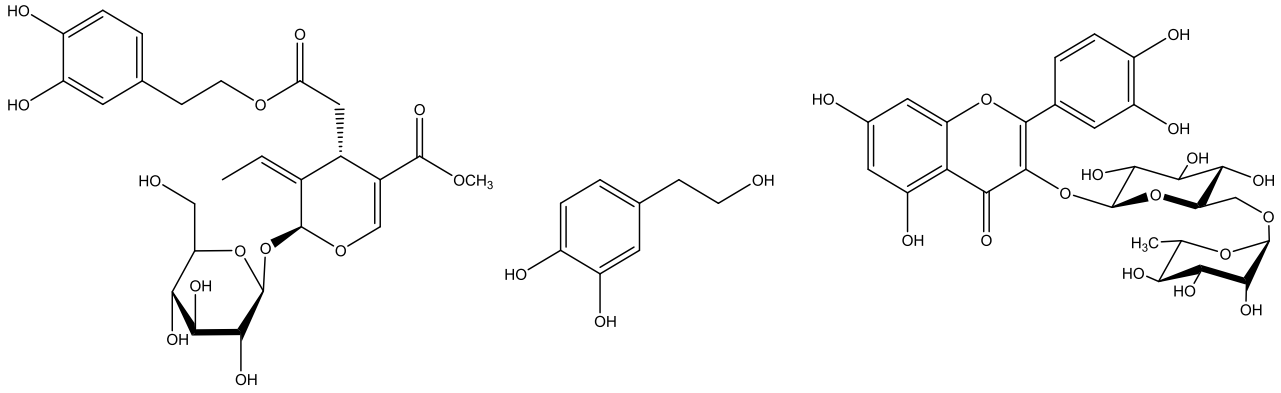
attachment to their land due to the fact that such tree dates back to the ancient times of Palestine, and was used to give light.

Olive fruit and oil have been used since ancient times in the Mediterranean diet and traditional medicine due to their nutritional and pharmacological effects. Health effects of olive leaves were first noticed in 1854 when the extract was used to treat fever, and now many of their health effects are reported. Olive leaf extract (OLE) has been found to have beneficial health effects like antioxidant, anticancer[1], anti-inflammatory, and antibacterial effects[3]. These effects are attributed to its flavonoid and phenolic components. The predominant polyphenol in OLE is Oleuropein, followed by Hydroxytyrosol while Luteolin-7-glucoside, apigenin-7-glucoside, and Verbascoside are present in detectable amounts[4]. Figure 1 shows three of the main components of OLE. Oleuropein is the ester of *β*-glycosylated elenolic acid with Hydroxytyrosol, and considered as a natural antioxidant reagent due to its activity as radical scavenger[5,6]. It has other pharmacological properties including antimicrobial activity[7], hypotensive and antiarrhythmic activities[8]. Hydroxytyrosol is a precursor of Oleuropein, and has a noticeable antioxidant and antibacterial activity[5,6,9]. Rutin, one of the important flavonoids of OLE, is a flavonol glycoside that combines quercetin and rutinose (Fig.1). It is present in OLE in low quantities compared to other polyphenols like Oleuropein, but its presence enhances OLE beneficial health effects[10]. Rutin has been reported to have several pharmacological effects including anti-inflammatory[11], antioxidant[10], anti-hypercholesterolemic[12] and anticancer[13] effects. It also inhibits platelet aggregation and prevents blood coagulations[14]. The chemical composition of olive leaves varies qualitatively and quantitatively among trees depending on environmental and seasonal conditions such as climate, soil and moisture content[15].



a. Oleuropein b. Hydroxytyrosol c. Rutin

Fig. 1. Chemical structure of some polyphenols in OLE.

Materials and methods

Reagents and chemicals

Oleuropein analytical standard, Rutin 98%, HPLC-grade acetonitrile, ethanol (EtOH) and phosphoric acid were purchased from Sigma-Aldrich. Water which was used in buffer preparation was first filtered by vacuum filtration over 0.45µm filter paper. Oleuropein 40% was purchased from local company that was standardized by Oleuropein analytical standard. Olive leaves were collected from three different areas in Palestine: south (Hebron), middle (Beit Jala) and north (Tulkarm) at two maturation stages [June 2014 (season 1) and October 2014 (season 2)]. Before injection to HPLC samples of OLE were filtered using 0.22 µm syringe filters. Bacterial strain of *E. coli* and *S. aureus* were obtained from the Department of Biology at Bethlehem University.

Instruments and Chromatographic Conditions

Chromatographic separation was achieved with LC system (Waters 1525 Binary HPLC pump) coupled with (Waters 2487 Dual Absorbance) detector, using a reversed-phase (EC150/4.6 Nucleodur C18 Gravity, 5 µm) column. Gradient elution using acetonitrile and phosphate buffer (pH 3.00 by H_3PO_4) was used as mobile phase. Dual wavelength UV detector was set at 256nm until the 11^{th} min of the run and at 245nm from the 11^{th} min until the end of the run. The injection volume was 20µL and the flow rate was set to 1mL/min. Biochrom Libra S22 UV-VIS spectrophotometer was used for the determination of the antioxidant capacity and for the preparation of the McFarland reagent.

Pretreatment of Olive leaf

Six different samples of olive leaves were collected from different areas of the West Bank and at different seasons and were treated accordingly: Leaves were washed with tap water and dried at room temperature for a week in a well-ventilated area. 25g of the dried leaves were soaked with 450mL EtOH for 10 min at 60^{o}C, then dried again before extraction. The volume of the solvent used for pretreatment was reduced to 150mL by Rotary Evaporator. The part which was pretreated with alcohol was called the treated sample, while the part of the dried leaves which was directly extracted was called the untreated sample. Dry leaves were ground by a coffee grinder and sieved through a mesh to obtain a fine powder to be extracted by Soxhlet extraction.

Soxhlet Extraction

Around 11g of the ground and sieved leaves were extracted by Soxhlet extractor for three hours using 150mL of absolute EtOH. For the extraction of treated leaves, the EtOH used in pretreatment was reused for the extraction. The obtained liquid extract was evaporated by Rotary Evaporator to get dry solid extract. At this stage, the percentage of the total extract was calculated for each sample. In order to determine the most efficient alcohol concentration for olive leaf extraction, different concentrations of EtOH were used (75%, 50% and 25% EtOH).

Determination of Oleuropein and Rutin Content in OLE by HPLC

For determination of the concentration of both Oleuropein and Rutin in each sample, High-Performance Liquid Chromatography (HPLC) was used. A new and reliable method was worked out with the following conditions:

- Injection volume = 20 µL
- Flow rate = 1 mL/ min
- Gradient mobile phase:

Time (min)	% Acetonitrile	% Water (pH= 3.0 by H_3PO_4)
0	10	90
20	40	60
21	10	90

- Dual wavelength λ:

Time (min)	λ (nm)
0-11	256
11-21	245

Preparation of standards: 10mg of Rutin (98%) was transferred to a 100mL volumetric flask and dissolved with 20mL EtOH, then made up to volume by water. 10mL of this solution was transferred into a 100mL volumetric flask containing 100mg Oleuropein (40%). Volume was made up to 100mL with 20% EtOH (standard final concentration 40mg Oleuropein and 0.98mg Rutin/100mL).

Preparation of samples: About 250 mg accurately-weighed dry extract was transferred to a 100mL volumetric flask. 20mL EtOH was added and sonicated and volume was made up to 100mL by water (sample final concentration 250mg/100mL).

Antioxidant Activity

Ferric Ion Reducing Antioxidant Power (FRAP) method described by Benzie and Strain[16] was used for the determination of the antioxidant activity of OLE using an appropriate spectrophotometer.

Preparation of FRAP reagent: 2.5mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution (0.031g of TPTZ was dissolved in 10mL of 40mM HCl at 50°C) and 2.5mL of 20mM $FeCl_3.H_2O$ were mixed with 25mL of 0.3M acetate buffer (pH 3.6). The resulting solution was incubated at 37°C for at least 10min.

Preparation of standard solutions: a stock solution of 2mM $FeSO_4.H_2O$ was prepared, then serial dilutions were made from it to obtain standard solutions with concentrations of 0.6, 1.2 and 1.6 mM.

Preparation of samples: an accurately-weighed amount of each dry extract was dissolved in 20% EtOH and diluted to obtain a final concentration of 90mg/100mL.

Standard calibration curve: 1mL of water and 80µL of each of the four different concentrations standard solutions were pipetted into a standard plastic cuvette. 600µL of the incubated FRAP reagent was added, then the change in absorbance at 593nm (a result of reduction of the Fe^{+3} – TPTZ to the blue Fe^{+2} – TPTZ complex) was recorded after exactly 4 minutes, using water as a blank.

Testing antioxidant activity: sample solutions with known concentrations were tested as described above but 80µL of the solutions was added instead of the standards. Different concentrations of Hebron pre-treated sample (30, 50, 80, 100 and 120µL) were studied to construct the linearity of the concentration dependence on absorption.

Antimicrobial activity

Three different methods (Disk Diffusion Method[17], Agar Dilution Method, and the "well" method) were worked out to test the antimicrobial activity of OLE against *E. coli* and *S. aureus*. 0.5 McFarland solution was used as a standard to prepare bacterial dilutions.

Preparation of positive control: 50mg of Amoxicillin was transferred to 100mL volumetric flask and dissolved and made up to volume with 0.1M phosphate buffer (pH=6.0) to give a final concentration of 50mg/100mL.

Preparation of samples: an accurately-weighed amount of each dry extract was dissolved in 0.1M phosphate buffer (pH=6.0) and diluted to obtain a final concentration of 50mg/100mL. Another set of sample solutions were prepared in 20% EtOH to give concentrations 50, 200, 1000, 2000 mg/100mL.

Preparation of standard: an accurately-weighed amount of Oleuropein (40%) was dissolved with 20% EtOH and diluted to obtain a final concentration of 320mg/100mL.

Disk Diffusion Method: 10mm disks of each of bacterial strains were cultured on a Petri dish of Mueller-Hinton agar. Using 10mm disks, the negative control (empty disk), and 20µL of both positive control (Amoxicillin) and sample disks were applied on the agar surface. Bacterial strains were introduced to the Petri dishes and they were incubated at 37°C overnight. Finally, zones of inhibition were examined.

Positive control preparation: a solution of a concentration of 8mg Amoxicillin/100mL was prepared to be used with *E. coli* while a 50mg /100mL solution was prepared to be used with *S. aureus*. The diluent was 0.1M phosphate buffer (pH=6.0).

Preparation of samples: an accurately-weighed amount of each dry extract was dissolved with 20% EtOH and diluted to obtain a final concentration of 2000mg/100mL.

Agar Dilution Method: 1mL of each negative control (sterile D.W), positive control and the sample were mixed with 19mL of cooled melted agar in separate Petri dishes. The dishes were dried at 37°C in an incubator for

30min. Bacterial strains were cultured on the surface and incubated at 37°C for at least 36 hours and then bacterial growth was examined.

The "well" method was done by digging three holes or wells of 10mm of diameter each into the agar plate for positive (Amoxicillin) and negative controls (DI water) as well as for sample. Bacteria was introduced on the plate and holes were filled with 95µL of samples and controls. Plates were incubated at 37°C for at least 36 hours then bacterial growth was examined.

Results and Discussion

Soxhlet Extraction

It was found that the total extract values for EtOH-treated samples were higher than the values for untreated samples in both seasons for the three areas. Moreover, Beit Jala samples showed the highest total extract value in both seasons, while those of Tulkarm showed the lowest values. Results are shown in Table 1.

Table 1: Total extracts for EtOH-treated and untreated samples of the three areas.

% Total Extract	Season 1			Season 2		
	Hebron Leaves	Beit Jala Leaves	Tulkarm Leaves	Hebron Leaves	Beit Jala Leaves	Tulkarm Leaves
EtOH-Treated Sample	36.82	60.74	31.48	43.05	49.60	35.77
Untreated Sample	29.5	43.18	20.54	34.69	40.26	30.97

As Soxhlet Extraction was done using different EtOH concentrations for the three areas untreated-samples in season 2, it was found out that extraction with 75% EtOH gave the highest total extract values for all samples as shown in Table 2.

Table 2: Total extracts for untreated samples of season 2 using different EtOH concentrations.

	% Total Extract		
% EtOH	**Hebron Leaves**	**Beit Jala Leaves**	**Tulkarm Leaves**
25%	16.73	25.17	13.35
50%	19.63	19.99	18.55
75%	41.7	42.86	42.56

Determination of Oleuropein and Rutin Content in OLE by HPLC

By applying the previously mentioned HPLC method, peaks for Rutin and Oleuropein were well defined and retention times were around 10 and 13 mins, respectively. Fig. 2 shows chromatograms for both the standard (a) and extracted sample (b). Oleuropein and Rutin content were calculated by comparing the area under peaks of these two polyphenols with standards. Results are shown in Tables 3 and 4.

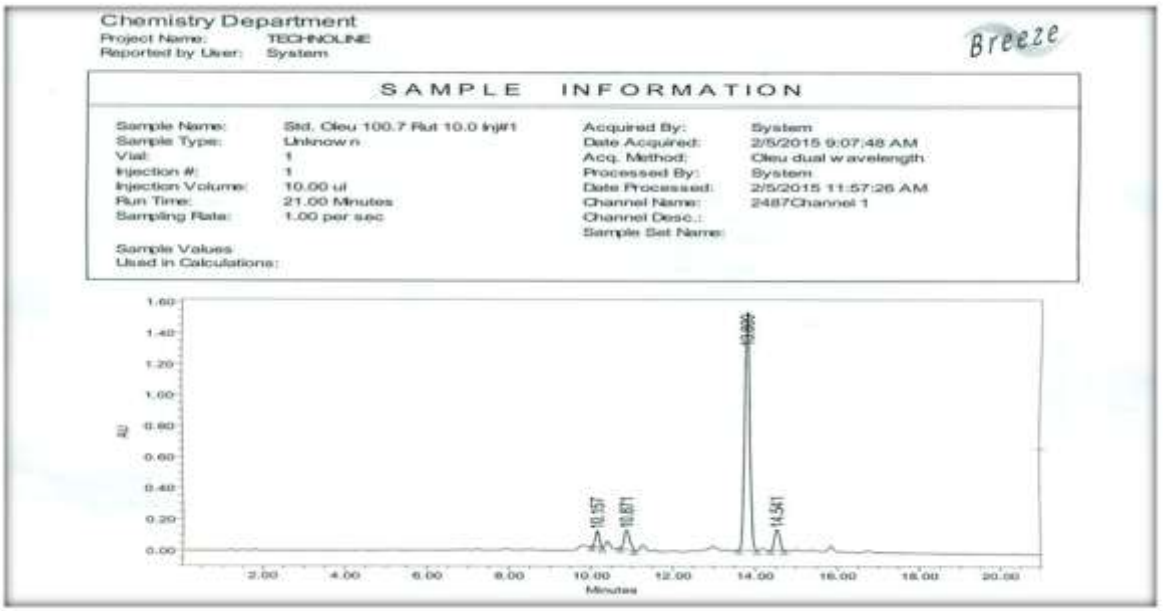
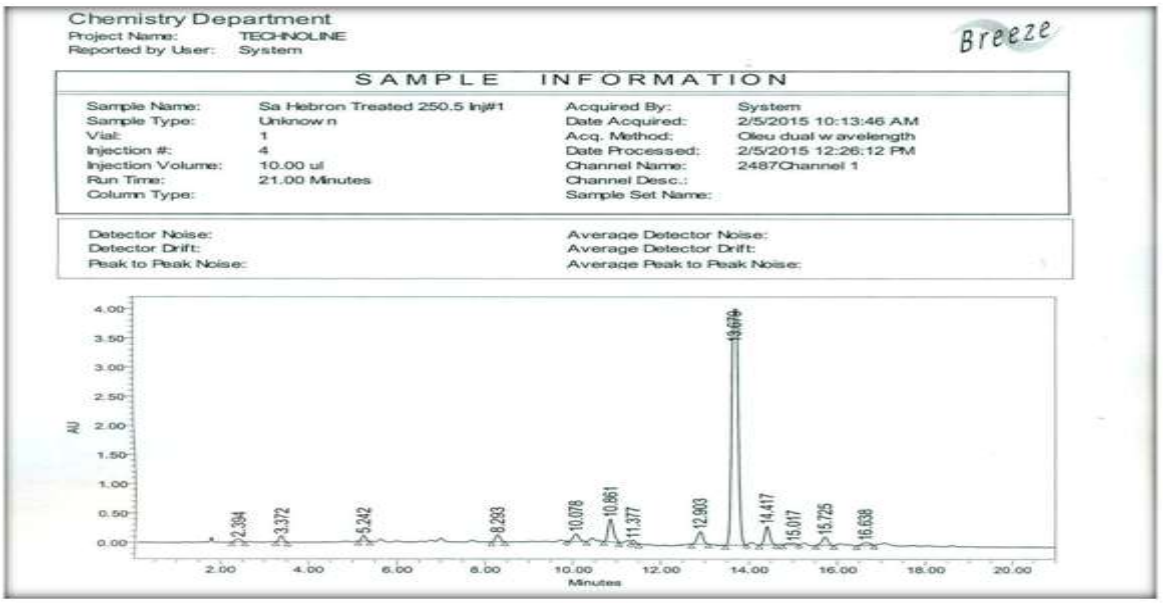


Fig. 2. a) HPLC chromatogram for standard Oleuropein and Rutin.



b) HPLC chromatogram for a sample of OLE.

In Season 1, Oleuropein content in EtOH-treated samples was lower than in untreated samples for Hebron and Tulkarm leaves, but for Beit Jala sample, it was higher in the EtOH-treated sample. However, in Season 2 all EtOH-treated samples had higher Oleuropein content than untreated samples. Moreover, Oleuropein content in Season 2 for the three areas was higher compared to those of Season 1. The high concentration of Oleuropein in season 2 is attributed to the fact that October is the olive picking season. Consequently, polyphenolic compounds would be at peak concentrations. Beit Jala samples were the highest in Oleuropein concentration for both seasons, while those of Tulkarm were found to be the lowest. (Table 3).

Table 3: Oleuropein content for EtOH-treated and untreated samples of the three areas.

Concentration of Oleuropein (mg/ g dry leaves)	Season 1			Season 2		
	Hebron	Beit Jala	Tulkarm	Hebron	Beit Jala	Tulkarm
EtOH-Treated Sample	75.99	102.27	38.9	137.14	159.16	79.09
Untreated Sample	84.14	76.67	63.2	130.52	143.9	76.58

Generally, Rutin is found in low concentrations in OLE. As shown in Table 4, Rutin was undetectable in Beit Jala leaves in both seasons, in Tulkarm leaves in season 2 and in the untreated sample of Hebron in season 1. However, Hebron leaf extract gave relatively high Rutin content in season 2 and lower content in the EtOH-treated sample in season 1. (Table 4).

Table 4: Rutin content for EtOH-treated and untreated samples of the three areas. ND= Not detectable.

Concentration of Rutin (mg/ g dry leaves)	Season 1			Season 2		
	Hebron	Beit Jala	Tulkarm	Hebron	Beit Jala	Tulkarm
EtOH-treated Sample	0.66	ND	0.391	0.951	ND	ND
Untreated Sample	ND	ND	0.29	1.432	ND	ND

Antioxidant Activity

Since season 2 had higher extract yields and higher component concentration than season 1, antioxidant activity was measured for season 2 only. A calibration curve was constructed (Figure 3) based on data obtained from absorbance values for different concentrations of $FeSO_4.H_2O$ standards (Table 5). This graph shows excellent linearity and regression to be used as a reference.

Table 5: Absorbance of $FeSO_4.H_2O$ Standards.

Standard Concentration (mM)	Absorbance
0.0	0.000
0.6	0.590
1.2	1.202
1.6	1.617
2.0	2.041

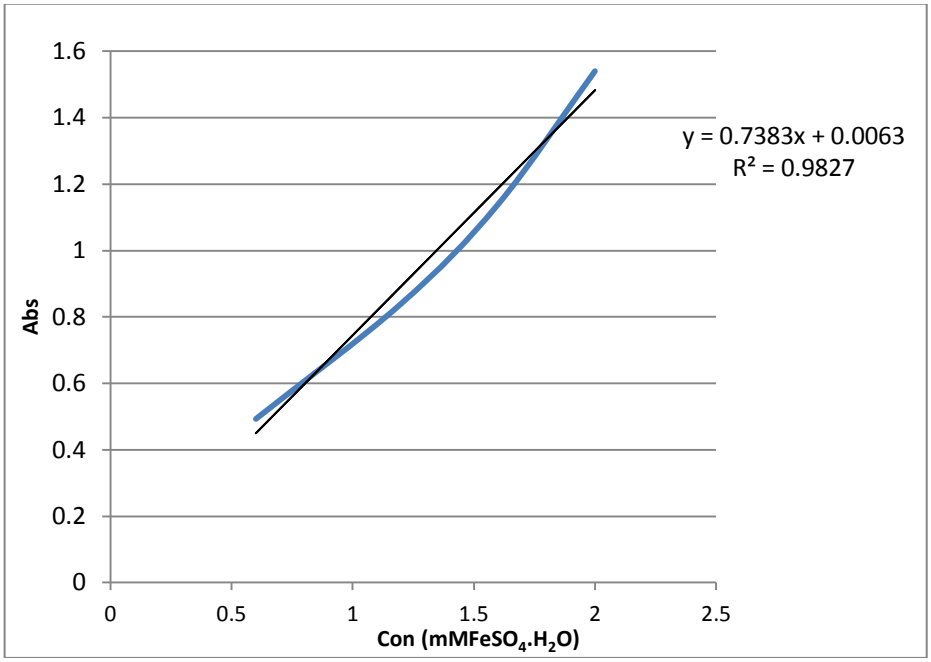


Fig. 3. Calibration curve (Graph of Absorbance Vs Concentration of $FeSO_4.H_2O$ standards).

From Figure 3, antioxidant capacities for three areas leaf extracts were calculated and expressed as mM Fe^{+2}/1g dry leaves. Results are demonstrated in Table 6.

It was observed that antioxidant capacities for Hebron and Beit Jala leaf extracts were high and within the same range. Meanwhile, Tulkarm sample capacity was lower.

Table 6: The Antioxidant capacity of the three areas leaf extracts expressed in equivalent (mMFe^{+2} /1g dry leaves).

Season 2						
	Hebron Leaves		Beit Jala Leaves		Tulkarm Leaves	
	EtOH-Treated	Untreated	EtOH-Treated	Untreated	EtOH-Treated	Untreated
Absorbance	0.908	0.943	0.889	0.965	0.695	0.728
Conc. (mM Fe^{+2})	1.222	1.269	1.197	1.30	0.935	0.980
Eq (mM Fe^{+2}/1g dry leaves)	5508.59	5760.22	5379.62	5913.99	4253.53	4448.40

The linearity of the antioxidant activity was tested by measuring the absorbance of different concentrations of Hebron EtOH- treated samples. Results showed linearity from at least 50% to more than 150% of the target measuring concentration. Results are summarized in table 7 and shown in Fig. 4.

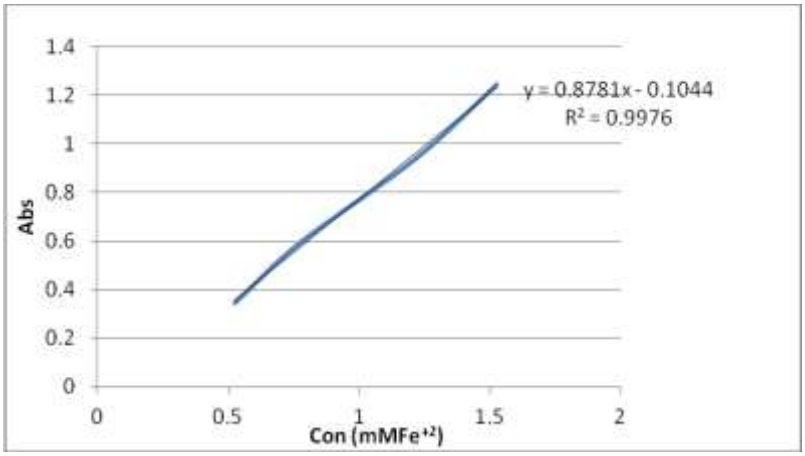


Fig. 4. Linearity of absorbance used in antioxidant activity for Hebron EtOH-treated samples.

Table 7: The Antioxidant capacity for different concentrations of Hebron EtOH-treated leaves.

Hebron EtOH-treated sample (Season 2)				
Vol. (µL)	**Conc. ($mMFe^{+2}$)**	**Amount (mg)**	**Abs.**	**Eq ($mM\ Fe^{+2}$/1g dry leaves)**
30	0.524	0.02736	0.391	6301
50	0.774	0.0456	0.596	5584
80	1.222	0.07296	0.946	5510
100	1.527	0.0912	1.247	5508

Antimicrobial Activity

Disk diffusion and agar dilution methods were not successful since extracts did not develop any zone of inhibition, although Amoxicillin (positive control) and water (negative control) worked efficiently as expected. On the other hand, "well method" was the only effective procedure, where inhibition appeared for Gram-positive species, while no inhibition was observed for Gram-negative species. Finally, to calculate the MIC value, serial dilution of samples was done. It was found that 40µg of samples showed good inhibition for bacteria.

Conclusion

This research was based on the idea that olive tree consists of an important component i.e. leaf. Olive leaves are considered cheap raw materials that can be used as a source for high-added value compounds such as Oleuropein and Rutin.

The aims of the research were determining the amount of Oleuropein and Rutin Season-wise and area-wise also testing the extract for antioxidant and antimicrobial activity. So three samples of Beit Jala, Hebron and Tulkarm leaves were collected in two seasons June and October 2014, extracted through Soxhlet extraction for 3 hours and total extract was calculated. Oleuropein and Rutin Contents were measured by using HPLC with a validated method. Antioxidant Activity of the extract was tested with FRAP method while antimicrobial activity was performed by the "well" method against Gram positive (*S. aurous*) and Gram negative (*E-coli*). Beit Jala leaves were the best maybe due to the fact that it is a mountain place and soil has more nutrients than the other examined samples.

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