

Research Article



The Anti – Angiogenic Activity of *Olea europaea* Seeds Extracts: *ex vivo* and *in vivo* study

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ABSTRACT

The objective is to identify the possible anti – angiogenic activity of *Olea europaea* Seeds extracts. The powder of the *Olea europaea* seeds was extracted sequentially with chloroform, methanol and water using the cold method "maceration" as extraction process. The *ex vivo* rat aorta ring assay was used to screen the extracts for possible anti – angiogenesis activity, this assay was also used to determine the dose – response effect of the active extract(s) by preparing serial concentrations. Free radical scavenging activity of the active extract(s) was determined using DPPH (1, 1 – diphenyl – 2 – picrylhydrazyl) assay. The obtained data revealed that the three extracts exhibited significant inhibition of blood vessels growth when they were compared to the negative control (received DMSO 1%) ($P < 0.05$), but chloroform and methanol extracts showed the highest percent of inhibition of blood vessels growth. According to the screening results, both chloroform and methanol extracts were selected for further investigation. Each of chloroform and methanol extracts of *Olea europaea* seeds exhibited a significant dose – dependent anti – angiogenesis effect with IC_{50} (22.379 μ g/ml and 24.85 μ g/ml) respectively. Furthermore chloroform and methanol extracts exhibited a significant free radical scavenging activity ($P < 0.05$) with IC_{50} (37.61 μ g/ml and 52.69 μ g/ml) respectively. The results revealed that each of chloroform and methanol extracts of *Olea europaea* seeds exhibited the best and most significant anti – angiogenesis activity as well as a significant free radical scavenging activity.

Keywords: Anti-angiogenesis, herbs, extraction.

INTRODUCTION

Angiogenesis is the process that involves the formation of new blood vessels from pre - existing ones. The primary step of it is thought to be initiated by activation of endothelial cells of pre-existing vessels in response to angiogenic stimuli. This process is typically initiated within hypoxic tissues where additional new blood vessels are required to maintain oxygenation and nutritional supply.¹ When the tissue is hypoxic, cellular oxygen sensing mechanisms are activated, which induce gene expression of various pro – angiogenic proteins. The primary activated factors are HIFs (hypoxia inducible factors) which in turn they up – regulate multiple pro – angiogenic genes directly or indirectly. Among the up – regulated genes, VEGF-A (vascular endothelial growth factor – A) is the major one and also responsible for the proliferation and migration of cells during this process.² There are two types of angiogenesis and they can both occur in utero and in adults; the sprouting angiogenesis (non-splitting), which is the most commonly studied mechanism, involves degradation of capillary basement membrane, followed by migration of endothelial cells, starting with a tip cell that moves along a gradient of pro-angiogenic factors. Endothelial cells proliferate and migrate, following the tip cell. These endothelial cells will form a lumen and then recruit pericytes or smooth muscle cells to surround the vessel, and the basement membrane will be formed.³ The second type is the intus susceptible angiogenesis

(splitting), which involves the extension of the vessel wall into the lumen causing a single vessel to split in two. This type of angiogenesis is thought to be fast and efficient compared with sprouting angiogenesis because, initially, it only requires the reorganization of existing endothelial cells and does not require the immediate proliferation or migration of endothelial cells.⁴ The process of angiogenesis is very essential physiologically as in ovulation, embryogenesis and wound healing, and also in pathological conditions like, rheumatoid arthritis, psoriasis, age related macular degeneration, Alzheimer's disease, cancer and others.⁵ *Olea europaea*, which is also known as the olive tree, belongs to the family Oleaceae. While olive oil is well known for its flavor and health benefits, the leaf has been used medicinally in a variety of times and places. Olive leaf and olive leaf extracts (OLE) are now marketed as anti-aging, antioxidant, immunostimulator, cardio protective, blood sugar regulating, anti-inflammatory and antibiotic agents.⁶ The objective of this study is to investigate the possible anti – angiogenic activity of *Olea europaea* seeds extracts.

MATERIALS AND METHODS

Extraction process

Two hundred grams seeds of *Olea europaea* were rinsed with tap water and cleaned from the remaining flesh then left to air dry. The dried seeds were ground into very fine powder. The powder extracted sequentially with (chloroform, methanol and water), using Maceration



method. The mixture filtered using whatmann no.1 filter paper to obtain the extract. The extract was concentrated using a rotary evaporator with vacuum (Buchi, Switzerland), crude extract, stored in dry and tightly sealed container to be use later in the experiment.

Ex vivo Rat Aorta Ring Anti – angiogenic Assay

The rat aortic ring assay experiment was conducted after the experimental procedures were revised and approved by Ethics Committee of Al-Nahrain University/College of Medicine. The assay was performed according to the standard protocol developed by Brown and his colleagues⁷, with minor modifications. Twelve to fourteen weeks old Albino male rats were obtained from the animal house of Institute for diagnosis of infertility and assisted reproduction techniques/Al-Nahrain University. The animals were humanely sacrificed via cervical dislocation under anesthesia with diethyl ether. Thoracic aorta was excised, rinsed with serum free media, cleaned from the fibro adipose tissue and was cross sectioned into thin rings of 1 mm thickness. M199 medium was used for the lower layer after adding fibrinogen and aprotinin at 3mg/mL and 5µg/ml respectively. A 300 µl of M199 medium was loaded in each 48-well plate and one aortic ring was seeded in each well. To each well, 10µl of thrombin; prepared at 50 NIH U/mL in 0.15 M NaCl and then was incubated and allowed to solidify at 37°C in 5% CO₂ for 30-60 min. The top layer medium was prepared by adding the following to M199 medium: 20% of heat inactivated fetal bovine serum (HIFBS), 1% L-glutamine, 0.1% aminocaproic acid, 1% amphotericin B and 0.6% gentamicin. Plant extracts were added to the top layer medium at concentration of 100µg/mL and each treatment was performed in six replicates. A stock solution of the sample extract was prepared by dissolving the sample in dimethyl sulfoxide (DMSO), and diluted in M199 growth medium to make the final DMSO concentration 1%. The tissue rings were incubated at 37°C, 5% CO₂ in a humidified incubator. On day 4, the top layer medium was changed with fresh medium prepared as previously mentioned. The DMSO (1% v/v) and acetyl salicylic acid "Aspirin" (100µg/mL) were used as negative and positive controls respectively. The results examined on day 5 under inverted microscope and the extent of blood vessel growth was quantified under 40X magnification with aid of camera and software package. The magnitude of blood vessel growth inhibition was determined according to the technique developed by Nicosia and coworkers (1997)⁸. The results are presented as mean percent inhibition to the negative control ± SD. The experiment was repeated three times using six replicate per sample. The percentage of blood vessels inhibition was determined according to the following formula:

$$\text{Blood vessels inhibition} = 1 - (A_0/A) \times 100$$

Where:

A₀= distance of blood vessels growth for the test substance in mm.

A= distance of blood vessels growth in the control in mm

Dose Response Study of the Active Crude Extract with Rat Aorta Ring Anti – angiogenic Assay

Serial dilutions of the active extract were prepared in the following concentrations: 200, 100, 50, 25, 12.5, 6.25 and 3.12µg/ml, of the samples were dissolved in DMSO, and diluted in the M199 growth medium to make the final DMSO concentration 1%. Wells without test samples were received medium with 1% DMSO used as the negative control. The data was represented as mean ± SD. The concentration that inhibits 50% of the growing blood vessels "IC₅₀" was calculated by using the linear regression equation or the logarithmic equation for the extract. Where Y= the percentage of inhibition, and X= concentration⁹.

Free radical scavenging activity with DPPH assay

The free radical scavenging activity of the active extract was measured by using the DPPH method. 200 µl of 0.1 mM DPPH dissolved in methanol was added to 100µl of the active extract in the following concentrations (500, 250, 125, 62.5, 31.25 and 15.625 µg) and incubated for 30 min. this procedure was executed using 96 well plate and each concentration was tested in triplicate, then the absorbance was measured at 517 nm using an ELISA reader. Ascorbic acid (Vitamin C) was used as a positive control and methanol alone as blank. The negative control was made of 100µl of methanol and 200µl DPPH. The percentage of antioxidant activity (AA) was calculated according to the formula below¹⁰

$$AA\% = 1 - (A_s - A_b / A_c - A_b) \times 100$$

A_s = absorbance of sample
A_b = absorbance of blank

A_c = absorbance of control

Statistical Analysis

The experiment design used for this study was Rationalized Complete Block Design (RCBD). Results were presented as means ± SD (Standard Deviation). The differences between groups were compared by the one way ANOVA followed by Tukey Post-hoc test (t – test) and considered significant at P < 0.05, 0.01 and 0.001. The concentration that inhibited 50% of blood vessels and caused reduction of free radicals (IC₅₀) was calculated using logarithmic equations. The statistical analysis was carried out by using SPSS edition 17.0.

RESULTS

Extraction Process

Three solvents were used to extract 200gm of *Olea europaea* seeds powder, which are chloroform, methanol and water. Of the three extracts, Chloroform extract gave the best yield percentage (15%) as shown in table (1)



Table 1: Weight and yield percentage obtained from *Olea europaea* seed crude extracts.

Type of extract	Weight (g/500g)	Yield (%)
Chloroform	30	15
Methanol	19	9.5
Water	5	2.5

200gm *Olea europaea* seed powder used in the extraction process

Ex vivo rat aorta ring anti – angiogenesis assay for CE, ME and WE of *Olea europaea* seeds:-

Aortic rings embedded in complete growth medium have received a concentration of 100 µg/ml of each of the three extracts (chloroform, methanol, and water). The blood vessels growth inhibition was presented as mean ± SD (Table 3-2).

The screening showed that all the extracts significantly inhibited blood vessels growth at day five of the experiment, there was a significant difference in blood vessels growth inhibition among each of the three extracts of *Olea europaea* seeds and the negative control (DMSO) ($P < 0.05$). Among these three extracts, the chloroform extract showed the highest anti-angiogenic activity (in term of blood vessels growth inhibition) in comparison with the other extracts. Also, there was a significant difference between the chloroform extract and the positive control (acetylsalicylic acid), and there were significant differences among each of chloroform, methanol and water extracts ($P < 0.05$) as shown in table (2), figure (1) and image (1).

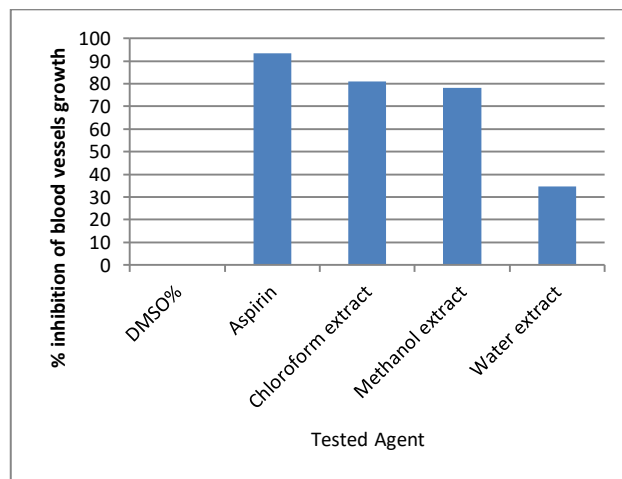
Table 2: The inhibition percentage of blood vessels growth produced by the tested extracts, negative and positive controls

Compound	% of inhibition ± SD
Negative control "DMSO 1%"	0
Positive control "aspirin"	93.5 ± 0.227
Chloroform extract	80.96 ± 0.385
Methanol extract	78.08 ± 0.372
Water extract	34.64 ± 0.295

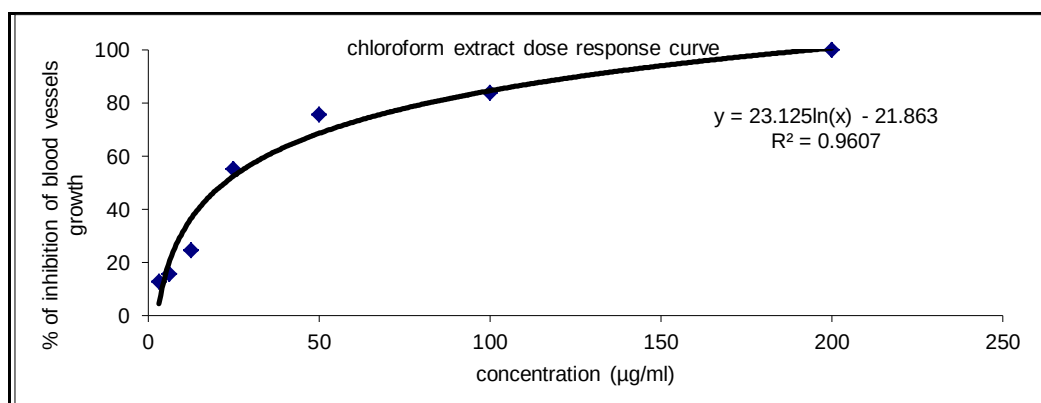
The results are presented as mean percent inhibition to the negative control ± SD. The experiment was repeated three times using six replicate per sample (n=18).

Dose response effect of chloroform extract of *Olea europaea* seeds on rat aortic ring model

Five serial dilutions of each of chloroform extract was prepared and added to the embedded rat aortic rings to determine the dose response curve. Chloroform extract showed significant dose dependent inhibition of blood vessels growth when compared to the negative control (DMSO 1%) ($P < 0.05$) at day five of the experiment as shown in table (3), figure (2) and image (2).

**Figure (3.1):** Anti – angiogenesis activity of 100µg/ml of each of chloroform, methanol and water extracts along with the positive and negative controls in *ex vivo* aortic ring model**Table (3):** Serial concentrations and their respective inhibition percentage for chloroform extract of *Olea europaea* seeds

Concentration (µg/ml)	% of inhibition ± SD
200	100 ± 0
100	83.28 ± 0.346
50	75.76 ± 0.368
25	55.28 ± 0.261
12.5	24.72 ± 0.168
3.125	12.88 ± 0.178

**Figure 2:** Dose response curve of chloroform extract of *Olea europaea* seeds in rat aortic rings model

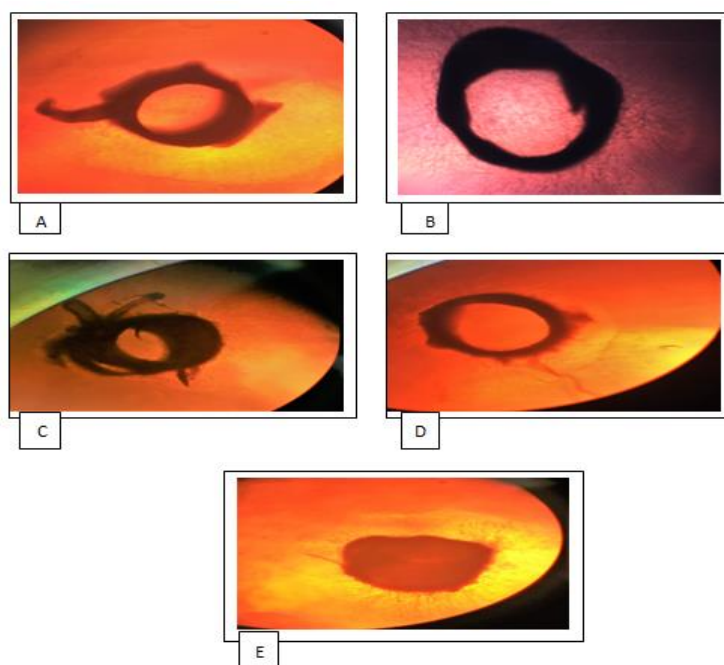


Image (3.1): Images showing the effects of *Olea europaea* seeds extracts on blood vessels growth in rat aorta rings, where A, B, C, D, and E represent the activity of Acetylsalicylic acid (positive control), Dimethyl sulfoxide (negative control), chloroform, methanol and water extracts, respectively.

The results are presented as mean percent inhibition to the negative control $\pm$ SD. The experiment was repeated three times using six replicate per concentration (n=18).

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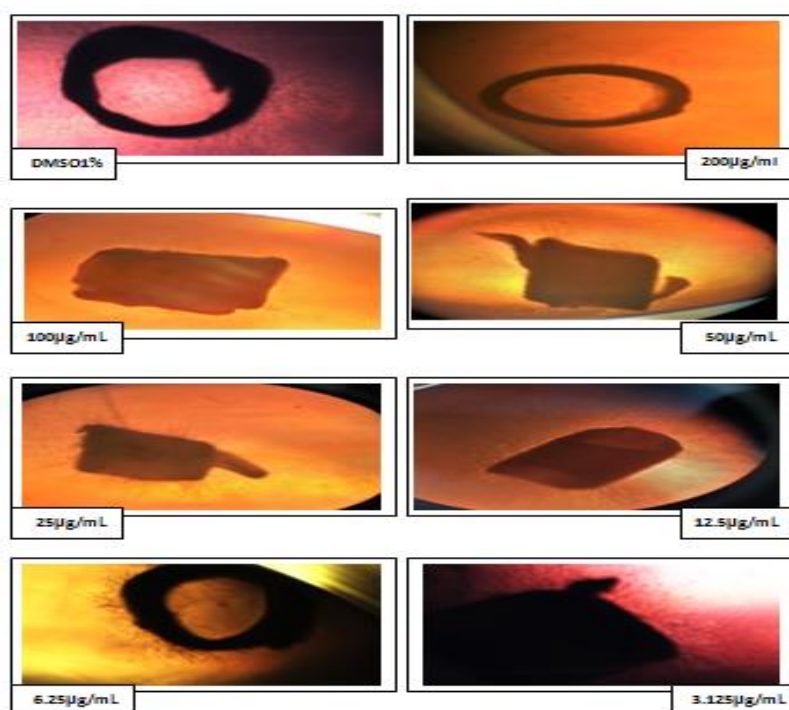


Image 2: The dose response effect of the serial concentrations of chloroform extract of *Olea europaea* seeds in rat aortic rings model DMSO1% was used as negative control

Free radical scavenging activity of methanol and chloroform extracts of *Olea europaea* seeds:

The free radical scavenging activity of each of chloroform and methanol extracts was measured by the use of the

DPPH assay. Seven serial concentrations were used to determine the scavenging activity as shown in Table (3.5). The results discovered that each of chloroform and methanol extracts significantly reduced the DPPH free radical in a concentration dependent manner at ($P < 0.05$).

The IC₅₀ was determined for each of chloroform and methanol extracts, and ascorbic acid (positive control) from the logarithmic equation shown in figures (3.4), (3.5) and (3.6) and it was found to be: IC₅₀ for ascorbic acid

(positive control) = 1.55µg/ml; IC₅₀ for chloroform extract = 37.61µg/ml; IC₅₀ for methanol extract = 52.69µg/ml

Where Y= the percentage of reduction in DPPH free radical, and was set at 50%; X= the concentration

Table 5: The percentage of DPPH free radical scavenging activity for methanol and chloroform extracts of *Olea europaea*

Concentration (µg/ml)	DPPH free radical scavenging activity (%)		
	Ascorbic acid	Chloroform Extract	Methanol Extract
500	94.39 ± 0.042	85.06 ± 0.040	83.08 ± 0.040
250	89.52 ± 0.036	83.52 ± 0.036	81.41 ± 0.0005
125	83.48 ± 0.056	78.81 ± 0.059	74.75 ± 0.069
62.5	78.97 ± 0.045	62.97 ± 0.181	61.12 ± 0.016
31.25	71.85 ± 0.018	41.85 ± 0.091	37.32 ± 0.063
15.125	68.19 ± 0.061	32.86 ± 0.285	20.27 ± 0.017

Ascorbic acid used as positive control, each concentration has been triplicated (n=3)

DISCUSSION

Extraction process

The extraction process used in this study was cold maceration method. This approach is suitable for extraction of thermo-labile compounds since high temperature may cause the destruction of these compounds¹¹. In the present study, chloroform extract produced the highest yield of crude extract followed by methanol extract and finally water extract which produced the lowest yield of crude extract (Table 3.1). It appears that there are several factors seem to influence the variation in the yield and the composition of phytochemicals in each extract. These include the type of the extraction method, the length of the extraction process, the temperature of the water bath, agitation or shaking, type of solvent used and its pH, concentration, and polarity; particle size of the powdered plant part and solution to sample ratio¹².

Effect on *ex vivo* rat aorta ring anti – angiogenesis assay

The rat aortic ring model bridges the gap between *in vitro* and *in vivo* models¹³. In the current study, the primary objective was to identify whether the three extracts have any antiangiogenic activity and which extract has the uppermost activity, it was important to screen the extracts against rat aorta *ex vivo* assay to recognize the most biological active extract for further *in vitro* and *in vivo* tests. The rat aorta ring assay was employed to study the antiangiogenic properties of the three extracts on the blood vessel formation. The results demonstrate that all extracted samples using the cold method (maceration) with the successive solvent extraction, have antiangiogenic property at the concentration of 100µg/ml. It was found that at this concentration of the extracts inhibited the endothelial formation of blood vessels of the rat aorta significantly compared to controls.

In the present study, aspirin was used as a positive control in the rat aorta ring experiment because it is approved to have anti – angiogenesis effect. That is

mediated either through COX – dependent pathway or COX – independent pathway by blocking the NF-KB which is considered directly associated with inflammation and angiogenesis¹⁴. The results of the present study revealed that each of chloroform extract and methanol extract had the most active anti – angiogenic activity and they had comparable effect against the inhibition of blood vessel growth. Both extracts were selected for additional investigations when compared to water extract. This may be due to the existence of a higher concentration of biologically active compounds or other compounds with biological activity in chloroform extract and methanol extract. The quantification of angiogenesis on this system implies the determination of the number and length of branching microvessels¹⁵. Antioxidants have been reported to inhibit angiogenesis in several *in vitro* and *in vivo* assays. These include

1. natural substances (polyphenols, flavonoids, isoflavones, lycopene, catechins, pigment epithelium-derived factor, glutathione, and resveratrol).
2. nutritional substances (β-carotene, selenium, and vitamins C, D, and E).
3. synthetic and semi-synthetic compounds (N-acetylcysteine, sodium pyruvate, L-NIO, L-NAME, pyrrolidine dithiocarbamate and organoselenium compounds)¹⁶.

Among the chemical components of olive oils, most of the minor components are responsible for the antioxidant potential. Phenolic components of olive oil (such as hydroxytyrosol and oleuropein) are widely studied for their antioxidant activity¹⁷. Other minor components are regarding health applications. These include esters of fatty acids, aliphatic alcohols, terpenes, hydrocarbons, sterols, and vitamin C (ascorbic acid)¹⁸. From the mentioned reasons, it appears to be the inhibition of micro vessels outgrowth produced by CE and ME in this screening assay may be ascribed to the presence of this

different compounds. These findings were supported by the results obtained from FT – IR, and GC – MS analysis.

Free radical scavenging activity by DPPH assay for chloroform and methanol extract of *Phoenix dactylifera* seeds

In this study, the DPPH radical was used to determine the radical scavenging activity of the active extracts. The DPPH radical is a stable nitrogen-derived organic free radical, which can be reduced to a non-radical form (DPPH-H) by accepting an electron or hydrogen in the presence of a hydrogen-donating antioxidant. Because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has usually been used for screening antiradical activities¹⁹.

Free radical scavenging activity test for both chloroform and methanol extracts of *Olea europaea* seeds was critical to understanding better the possible mechanism of action behind their ability to suppress blood vessels growth. In the present study, chloroform extract and methanol extract showed significant reduction of free radicals by the DPPH assay and in a concentration – dependent manner. This could be due to the presence of phenolic compounds in both extracts. Olive oil can be rich in phenolic compounds. Phenolic acids were the first group of phenolic compounds identified in virgin olive oil. Tyrosol, vanillic acid, luteolin, and apigenin were identified and quantified by LC-MS²⁰. Other important components are tocopherols and tocotrienols, in a range of 12_ 400 mg/kg. Hydrophilic phenols have been identified as being responsible for most of the antioxidant properties of the virgin olive oil. In addition to its antioxidant potential in biological systems, olive oil is more resistant to thermal oxidation during frying than other edible oils²¹. During thermal oxidation, tocopherols and phenols are the major compounds contributing to the stability. The high concentration of oleic acid in the oil also contributes to the stability. The following mechanisms can mediate the antioxidant properties of phenolic compounds: scavenging radicals as ROS/RNS; suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals responsible for free radical production; or by up-regulating antioxidant defenses²². Phenolic compounds possess ideal structure chemistry for free radical scavenging activities because they have phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; and extended the conjugated aromatic system to delocalize an unpaired electron²³. Still, chloroform extract showed less high antioxidant activity than that produced by methanol extract as the IC₅₀ of chloroform extract was lower than that of methanol extract (37.61µg/ml for ME and 52.69µg/ml for CE). The higher antioxidant activity produced by CE of *Olea europaea* seeds was not only due to the presence of polyphenols, but also ascorbic acid was found in CE more than in ME; this was supported by the results of the GC-MS analysis.

The presence of ascorbic acid in the extract enhanced its free radical scavenging activity since it is considered one of the powerful antioxidant agents as it was shown from the results of the present study when pure ascorbic acid was used as positive control in DPPH assay (Table 5)

CONCLUSION

All extracts of *Olea europaea* seeds exhibited significant anti – angiogenesis activity, however chloroform and methanol extracts demonstrated the best anti – angiogenesis activity as well as significant dose – dependent anti – angiogenic effect. Furthermore, each of chloroform and methanol extracts exhibited a significant free radical scavenging activity by DPPH assay and in concentration – dependent manner.

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