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The Anti-angiogenic Activity of *Matricaria Chamomilla* Flowers Extraction

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Abstract: ***Background**: The Matricariachamomilla is widely used in Asian countries as the traditional medicine to treat various diseases. **Objective**: The possible anti-angiogenic activity of Matricaria chamomilla flowers extractswas aimed to be investigated. **Subjects and methods**:The powder of the Matricariachamomilla flowers was extracted sequentially with petroleum ether, 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 by preparing serial concentrations. Free radical scavenging activities of the extracts were determined using 1, 1–diphenyl–2–picrylhydrazyl) assay (DPPH). **Results**: The obtained data revealed that the four extracts exhibited significant inhibition of blood vessels growth when they were compared to the negative control (received DMSO 1%) (P<0.05) amidst them methanol extract was shown to have significant inhibition of blood vessels growth with comparable effect to positive control (acetylsalicylic acid). Methanol extract of Matricariachamomillaflowers exhibited a significant dose–dependent anti–angiogenesis effect with IC_{50}(29.85µg/ml). Furthermore, methanol extracts exhibited a significant free radical scavenging activity (P<0.05) with IC_{50} (81 µg) compared to other extracts. **Conclusion**: the results revealed that methanol extracts of Matricariachamomilla flowers exhibited the most significant anti–angiogenesis activityand this activity may be due to the high free radical scavenging capacity*

Keywords: Anti-angiogenesis, *Matricariachamomilla*, free radical scavenging activity

1. Introduction

The creation of new blood vessels from pre-existing ones called Angiogenesis. The main step of it is believed to be originated by activation of endothelial cells of pre-existing vessels in reaction to angiogenic incitements. This process is usually started in hypoxic tissues as extra fresh blood vessels are vital for oxygenation and nutritional supply [1]. Cellular oxygen sensing mechanism will be activated as tissue is hypoxic. This may induce gene expression of numerous pro–angiogenic factors. The principally activated one are HIFs (hypoxia inducible factors) which then pro–angiogenic genes directly or indirectly may up–regulate. Amidstthem vascular endothelial growth factor–A (VEGF-A) is the main one and also accountable for the proliferation and migration of cells during this process [2]. Severalkinds of factors that inhibited angiogenic factors have been revealedsuch asangiostatin, which decreases VEGF-mediated activation of mitogen-activated protein kinases (MAPK) in endothelial cells leading to inhibition of proliferation and induction of apoptosis [3]. Angiogenesis is animportantaction in certain physiological circumstancesfor example growth, wound healing and action of female reproductive organs. However angiogenesis has been shown pathological process in much condition such as arthritis, cancers, asthma and retinopathies [4].The use of *Matricariachamomilla*for medicinal uses backs to ancient Greece and Rome [5]. *Matricariachamomilla*is native to the old World and is a member of the daisy family (*Asteraceae*or *Compositae*). It is generally represented by two known varieties, *Matricariachamomilla*(German chamomile) and *Chamaemelumnobile* (Roman chamomile). *Matricariachamomilla* is considered the more potent of the two, has received more scientific evaluation, and is more widely cultivated than Roman chamomile. Its primary uses are as a sedative, anxiolytic and anti-inflammatory. The main constituents of *Matricariachamomilla* flowers are chamazulene, apigenin, and bisabolol[6].

2. Materials and Methods Extraction

Five hundred grams of *Matricariachamomilla* flowers were cultivated from the Baghdad governorate area/ Iraqi. The dried flowers were then grounded in to a very fine powder. The dried powdered were extracted by employing successive extraction method using different organic solvent in increasing polarity order (petroleum ether, chloroform, methanol, and water) using Maceration method. The mixture filtered using Whatman no.1 filter paper to obtain the extract. The extract was concentrated using a rotary evaporator with vacuum (Buchi, Switzerland). The driedextract was stored in a refrigerator until used later in the experiment [1]

3. Rat Aorta Ring Anti-Angiogenesis 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. This assay was performed according to the standardprotocol of Brown with a simple modification[7]. Theanimals were sacrificed by cervical dislocation underanesthesia with chloroform. Thoracic aorta were rapidlyexcised, rinsed with serum free media, cleaned as muchas possible from peri-adventitial fibro adipose tissue andresidual blood clots. These were then cross sectioned intothin rings of 1 mm thickness. The assay was carried out ina 48 well tissue culture plate. The lower layer, consistingof 500 µl Serum free M199 growth mediumsupplemented with fibrinogen (3mg/ml) and aprotinin(5µg/ml) was added and one aortic ring was seeded ineach well. After that, 15 µl of thrombin prepared at 50NIH U/ml in 0.15 M NaCl was

added to each well and themixture was allowed to solidify at 37°C in 5% CO2 for 60–90 min. A stock solution of sample in dimethyl sulfoxide(DMSO) (10 mg/ml) was prepared and added to the toplayer to obtain a final concentration of 100µg/ml with afinal DMSO concentration of 1% (v/v). After that, the toplayer; consisting of 500 µl of M199 supplemented with20% of heat inactivated fetal bovine serum (HIFBS), 0.1%6-aminocaproic acid, 1% L-Glutamine, 0.6% gentamicin,1% amphotericin B and 100 µg/ml of the sample wasadded to each well. Dimethyl sulfoxide (1% v/v) andacetyl salicylic acid (ASA) (100 µg/ml) were used asnegative and positive controls respectively. The tissuerings were incubated in a humidified incubator at 37°C,5% CO2 for five days. On day four of the experiment, thetop layer medium was replaced with fresh mediumprepared as mentioned above. The experiment wasrepeated three times. In each experiment, six replicateswere performed. The extent of blood vessels growth wasquantified under 40X magnification using an invertedmicroscope (Olympus, Japan) on day five of theprocedure with the aid of a camera (Lieca CCD, Japan)and (LiecaQWin) software packages. The magnitude ofblood vessels growth inhibition was determinedaccording to the technique developed by Nicosia andcoworkers which include measuring the length of theminute blood vessels outgrowths from the primary explants [8].The percentage of blood vessels growthinhibition was determined using the following formula:

Blood vessel growth inhibition% = [(L0 - L) / L0] × 100%
Where
L: Distance of blood vessels growth in µm
L0: Distance of blood vessels growth in the control in µm

Rat aorta ring assay (antiangiogenesis) dose response study of the methanol extract of *Matricariachamomilla* flower

Serial dilutions of the most active extract in rat aorta ring assay wereprepared in the following concentrations: 200, 100, 50, 25, and 12.5µg/ml, fromthe original stock samples that were dissolved in DMSO and diluted in the M199growth medium to make the final DMSO concentration 1%. Wells without testsamples were received medium with 1% DMSO used as the negative control. Thedata was represented as mean ± SEM. The concentration that inhibits 50% of thegrowing blood vessels (IC_{50}) was calculated by using the logarithmic regression equation for the extract. Where Y= the percentage ofinhibition, and X= concentration [9].

Radical scavenging activity

The 1,1- diphenylpicrylhydrazyl (DPPH) assay is widely used in plantbiochemistry to evaluate the properties of plant constituents for scavenging freeradicals. The method is based on the spectrophotometric measurement of theDPPH concentration change resulting from the reaction with an antioxidant. It is adark–colored crystalline powder with stable free radical molecules that gives adeep violet color when in solution and becomes colorless or pale yellow in colorupon reacting with an antioxidant agent [10].The free radical scavenging activities of the extracts were measured by usingthe DPPH method. 200 µl of 0.1 mM DPPH dissolved in methanol was added to 100 µl of the extracts in the following concentrations (500, 250, 125, 62.5, 31.25, 15.625 and 7.813 µg) and incubated for 30 min. This procedure was executed using96 wellsplate and each concentration was tested in triplicate, then the absorbancewas measured at 517 nm using an ELISA reader. Ascorbic acid was used as apositive control and methanol alone as blank. The negative control was made of100µl of methanol and 200µl DPPH. The percentage of antioxidant activity (AA)was calculated according to the formula below [11].

AA% = 1- (AS – AB/AC – AB) x 100
AS = absorbance of sample AB = absorbance of blank
AC = absorbance of control

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

4. Results

***Matricariachamomilla* extract yields**
The extraction yields were calculated for each sample extracted with a solvent system as percentage of the weight of resulting extract to the weight of *Matricariachamomilla* flowers that was originally used (500g). The highest yield obtained by the extraction procedure was for the water extract (42.5g; 8.5%) while the lowest yield was for the methanol extract (22g; 4.4%). On the other hand, the chloroform and petroleum ether extracts gave 39g 7.8%) and 27.5g (5.5%), respectively; Table (3.1)

Table 1: Extract yields obtained from *Matricariachamomilla* flowers

No.	Extracts*	Weight (g)	(%)
1	Petroleum ether	27.5	5.5
2	Chloroform Extract	39	7.8
3	Methanol Extract	22	4.4
4	Water Extract	42.5	8.5
Total		131	26.2

*These extracts were obtained from 500g of *Matricariachamomilla* flowers that represent 100%

***Ex vivo* rat aorta rings antiangiogenesis assay of petroleum ether, chloroform, ethanol and water extracts**
The inhibition in growth of blood vessels was presented as mean percentage ± SEM as shown in Table (2). The extracts of petroleum ether, chloroform, methanol and water were significantly inhibited blood vessels growth compared to negative control; DMSO (a vehicle used to dissolve the samples) ($P<0.05$). Amongst these four extracts, the methanol extract displayed the highest antiangiogenic activity in comparison to the other extracts ($P<0.05$).In comparison to the positive control (acetyl salicylic acid; aspirin), there was a significant difference in terms of blood

vessels growth inhibition compared to negative control and petroleum ether, chloroform as well as water extracts ($P<0.05$). However, there was no significant difference between positive control and methanol extract of *Matricariachamomilla* flowers ($P>0.05$). The comparisons amid the four extracts in terms of blood vessels growth inhibition with both the negative and positive controls revealed that the methanol extract is the most biologically active as shown in figure (1). Images of the extracts and controls (positive and negative) are shown in figure (2).

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

Tested agents	*Mean Percentage(%) ± SEM*
Negative control (DMSO)*	0.0
Positive control (Aspirin)	$91\pm0.25^{\#++}$
Petroleum ether	$37\pm0.5^{++}$
Chloroform extract	$70\pm0.5^{++}$
Methanol extract	$89\pm0.5^{\#++}$
Water extract	$78\pm0.5^{++}$

***** DMSO: Dimethyl sulfoxide, $^{\#}$significantly different from other groups and $^{++}$significantly different from negative control.

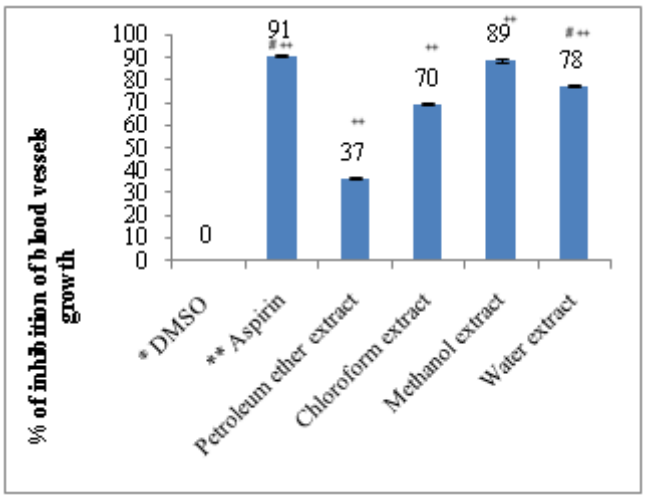

Figure 1: The effect of 100µg/ml of petroleum ether, chloroform, methanol and water extracts of *Matricariachamomilla* flowers on blood vessels growth in ex vivo aortic rings model. * DMSO (Dimethyl sulfoxide) served as negative control, ** Aspirin served as positive control, $^{\#}$ significantly different from other groups and $^{++}$ significantly different from negative control.

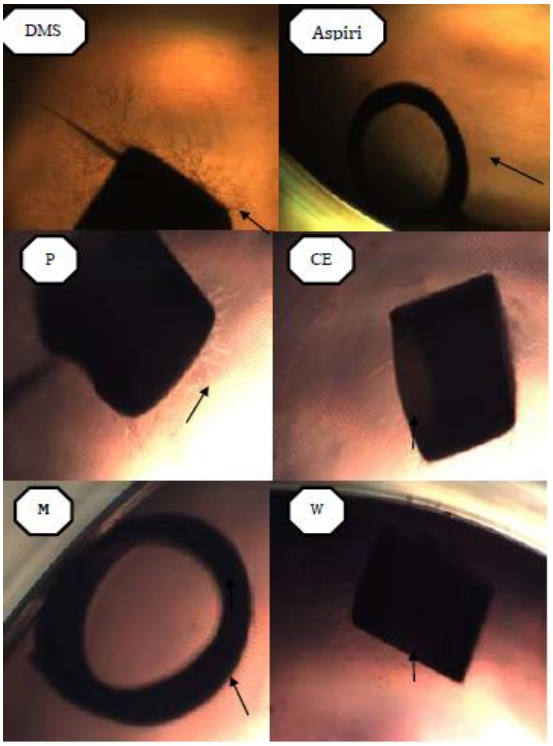

Figure2: Images of aortic rings treated with the four extracts of *Matricariachamomilla* flowers and controls. The pointed black arrows indicate the growth of micro blood vessels.DMSO 1% as a negative control, aspirin as a positive control, PE: petroleum ether extract, CE: chloroform extract, ME: methanol extract and WE: water extract.

Dose-Response curve of the effect of the methanol extract of *Matricariachamomilla* flowers on rat aorta

Different concentrations (6.25, 12.5, 25, 50, 100 and 200µg/ml) of the methanol extract of *Matricariachamomilla* flowers were added to the rat aortic rings. These extract showed significant concentration-dependent inhibitory effect ($P<0.05$) in comparison with the negative control (DMSO 1%), yet the (6.25 µg/ml) concentration showed no significant effect ($P>0.05$). The percentage of inhibition for each concentration is shown in Table (3).In addition, a dose-response curve was constructed as in Figure (3) that showed significant concentration-dependent inhibition of blood vessels growth with the concentration that produced 50% inhibition (IC_{50}) is approximately 29.85µg/ml.On the other hand, the images of rat aortic rings Figure (4) showed dose-related inhibition of tiny blood vessel outgrowths from the primary ex-plant, with the lowest inhibition showed in image A and the highest shown in image F. Images G and H represent the negative and positive controls, respectively.

Table 3: Percentage of blood vessel growth inhibition by different concentrations of the methanol extract of *Matricariachamomilla* flowers in rat aortic rings assay. The experiment was repeated three times using six replicates per concentration (n=18).

Concentration (µg/ml)	Percentage of inhibition±SEM
6.25	7.07±0.14
12.5	31.7±0.16
25	42.5±0.20
50	58.5±0.20
100	87.3±0.21
200	100

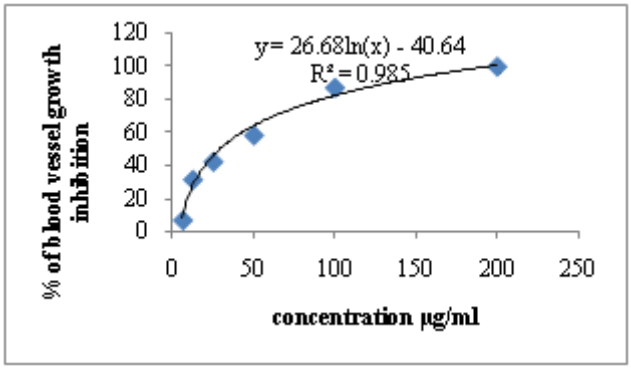

Figure 3: Dose-response curve of the inhibitory effect of different concentrations of the menthol extract of *Matricariachamomilla* flowers on blood vessels growth using rat aortic rings assay, y is the percentage of inhibition and x is the concentration.

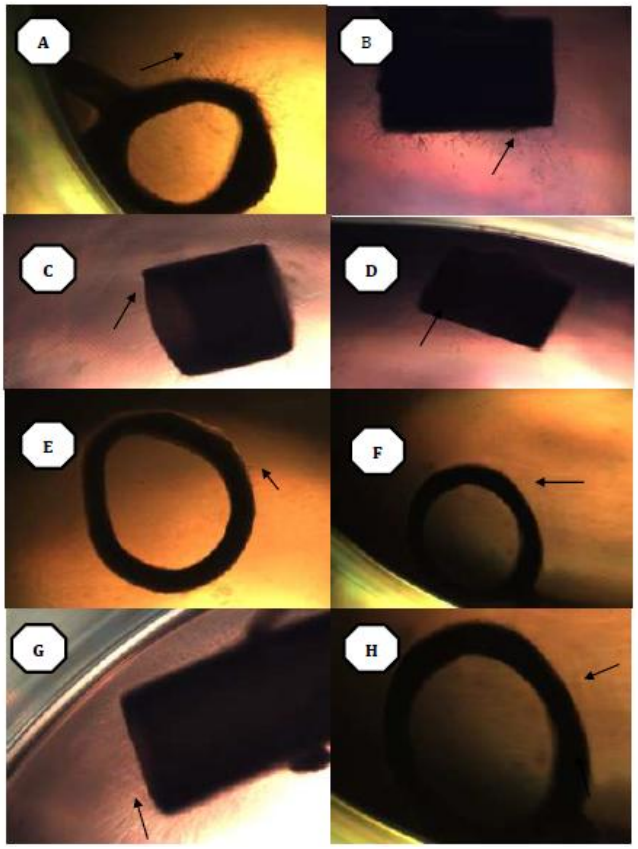

Figure 4: The dose-response effect of different concentrations of the methanol extract of *Matricariachamomilla*flowers in rat aortic rings assay. A, B, C, D, E, F, G and H stand for the serial concentrations (6.25, 12.5, 25, 50, 100, 200µg/ml) of the methanol extract and the controls (negative and positive), respectively.The arrows indicate the growth of micro blood vessels. The images were taken at day five of the experiment

The free radical scavenging activity assay

The free radical scavenging activity of the chloroform, petroleum ether, methanol and water extracts of *Matricariachamomilla* flowers was tested by measuring the 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of a range of concentrations (7.81, 15.125, 31.25, 62.5, 125, 250500 and 1000 µg/ml) with the use of ascorbic acid as a positive control. The results showed that the percentage of DPPH scavenging activity of ascorbic acid and the four extracts of *Matricariachamomilla* flowers is concentration-dependent (Table 3.4). In addition, a dose-response curve was constructed for each of the five tested agents figures (5) from which the IC_{50} for each tested agent was found to be 3.6µg/ml, 6118µg/ml, 1305µg/ml, 81µg/ml and 245µg/ml, respectively.Moreover, the results showed that of the methanol extract has more powerful free radical scavenging activity than the other extracts of *Matricariachamomilla*flowers, ($P<0.05$), (Figure 3.10). However, the free radical scavenging activity of the positive control (ascorbic acid) was shown to be more powerful than that of the methanol extract.

Table 4: Comparing the percentage of DPPH scavenging activity of ascorbic acid and the four extracts of *Matricariachamomilla* flowers. Data are expressed as (Mean ± SEM). Each concentration has been triplicated (n=3)

Tested agents / Conc µg/ml	ascorbic acid (+ve control)	Petroleum ether extract	Chloroform extract	Methanol extract	Water extract
7.825	48.0± 0.001	5.4± 0.001	6.0± 0.002	8.4± 0.003	6.08± 0.003
15.625	58.0± 0.007	5.7± 0.003	6.4± 0.009	21.0± 0.014	8. 1± 0.005
31.25	65.3.4± 0.005	11.9± 0.004	12.5± 0.003	43± 0.011	12.5± 0.005
62.5	75.0± 0.005	21.6± 0.004	21.9± 0.002	55± 0.016	23.6± 0.011
125	77.0± 0.002	29.1± 0.002	30.7± 0.002	64.2± 0.006	39.5± 0.034
250	77.0± 0.002	31.4± 0.005	38.2± 0.002	70.9± 0.004	58.7± 0.02
500	78.3± 0.005	31.7± 0.005	42.9± 0.015	72.6± 0.115	65.2± 0.005
1000	79.7± 0.005	33.7± 0.005	43.9± 0.015	73.64± 0.115	66± 0.115

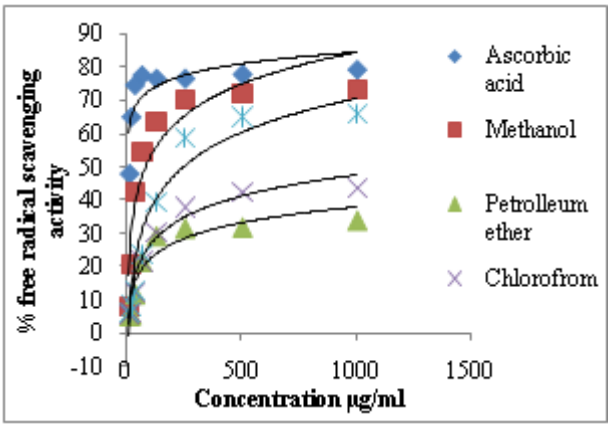

Figure 5: Dose-response curves of the scavenging activities of ascorbic acid (positive control) and the four extracts of *Matricariachamomilla* flowers

5. Discussion

It has been documented that different parts of plant show
It has beenshown that the dried flowers of *Matricaria chamomilla* contain alkaloids andflavonoids and phenol,

which contribute to its medicinal properties [6].The extraction process used in this study was cold maceration method.The flowers of *Matricaria chamomilla* were extracted successively withincreasing polarity order of solvents, from petroleum ether (non-polar) towater (highly polar); to ensure that a wide range of bioactive compounds havebeen extracted and separated according to their polarity [2].In the present study, water extract produced the highest yield of crudeextract followed by the extracts of chloroform, petroleum ether and finally methanol extract which produced the lowest yield of extract. Many factorsaffecting the extraction yield; such as the shaking, time of macerations, types,concentrations and pH of the solvents used, heat degree of the water used towarm the container that contain the powder of the extract , particle size of the powdered plant part and solvent to sample ratio [1]. Methanol extract had the most effective anti–angiogenic activity amidst the other extracts of *Matricaria chamomilla* flowers and it had comparable effect against the inhibition of blood vessel growth as Aspirin. The results of the present study showed that methanol extract significantly inhibited blood vessels growth in a dose dependent manner. The results of this study showed that the IC_{50} on blood vessels outgrowth was 29.85µg/ml. It has been documented that as the IC_{50} level decreases, the safety decrease and vice versa (Wei *et al*., 2012). It has been mentioned that the dependent boarder concentration for herbs on angiogenesis process to be considered safe was ranging from 20-40µg/ml of that extract [12]. In here the IC_{50} of methanol extract of *Matricariachamomilla*flowers considered within the safe range.In this study, the DPPH radical was used to determine the radicalscavenging activity of the four extracts yield. Free radical scavenging activity tests for petroleum ether, chloroform, methanol and water extracts of *Matricaria chamomilla* flowers were veryimportant in order to better understand the possible mechanism of actionbehind their ability to suppress blood vessels growth. In the present study,results showed significant free radical scavenging activity of methanol extractin compared with petroleum ether, chloroform and water extracts in aconcentration–dependent manner. The IC_{50} of DPPH for methanol extract of*Matricaria chamomilla* flowers was 81µg/ml. This finding was in consonancewith other finding made by Seddik and his colleagues 2013 as they foundalmost the same outcome (69µg/ml) [13].In consistence, it had beenshown that the extract solution from *Matricaria chamomilla* flower exhibiteda significant dose-dependent inhibition of DPPH activity. The flavonoidsapigenin-7-*O*-(6″-acetyl)-glucoside, luteolin, apigenin that were considered inthe antioxidant activities of the extract solution from *Matricaria chamomilla*flowers [14].Moreover, Free radical scavenging activity of *Matricaria chamomilla*flowers methanol extracts could be due to their higher content of phenoliccomponents which chlorogenic acid, Caffeic acid, p-coumaric acid and ferulicacid. Such hydroxylphenolic compounds can donate hydrogen atoms to DPPHand scavenge it [15]. fradical scavenging capacity increased with increasing extract concentration [15].This may be explaining the non-significant effect compared to ascorbic acid,the positive control used in the current study Results of this studyshowed positive correlation when comparing the *Matricaria chamomilla*flowers extracts in both antiangiogenic activity shown by the rat aorta assay,and antioxidant power shown in the DPPH scavenging assay. These findingsmay explain the anti–angiogenesis activity of methanol extract as well as itsantioxidant properties. It has been documented that agents with goodantioxidant activity are well known to possess anti–angiogenic activity [1, 2].

6. Conclusion

Matricaria chamomilla flowers extracts showed potential inhibition activity against angiogenesis with highest effect exerted by the methanol extract. This herb may have promising activity against angiogenesis related diseases. Possible correlation of antioxidant activity of *Matricaria chamomilla*flowers extracts and anti-angiogenesis activities is suggested.

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