Research Article

Anti-Angiogenic Activity of *Matricaria chamomilla* Flowers Methanol Extract - *In vivo* Study

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ABSTRACT

The methanol extract of *Matricaria chamomilla* flowers exhibited anti–angiogenesis Activity in ex-vivo and showed high free radical scavenging activity. The objective of the study is to investigate the anti-angiogenic activity of *Matricaria chamomilla flowers* methanol extract *in vivo*. Eggs were incubated for three days, small whole made on the fine pinpoint. The next day, the egg's sac was penetrated and a small frame was made in the shell. The window was resealed and the eggs were returned to the incubator until day 10 of chick embryo development, 20 µl of 500mg/ml of the methanol extract was transferred to the Chick Embryo Chorioallantoic Membrane (CAM), and eggs incubated for 72 hours (n = 6); The zone of inhibition was calculated as mean of inhibition area in millimeter (mm) ± standard means of errors (SEM). Functional groups of chemicals in the methanol extract were identified by using fourier transform infrared spectroscopy (FT-IR). Further investigations including determination of its chemical constitutes by gas chromatography/mass spectrometry (GC-MS). The methanol extract produced a significant inhibition zone of blood vessels. The qualitative analysis for active chemical compounds in methanol extract indicated presence of phenolic hydroxyl group as well as flavonoids and alkaloid groups. Gas chromatography/mass spectrometry (GC-MS) showed that the major constitutes of methanol extract were α-bisabolol, coumarin and apigenin. The methanol extract of *Matricaria chamomilla* Flowers has anti-angiogenic activity *in vivo* and this may be due to the presence of phenols and flavonoids compounds those are able to block the production of vascular endothelial growth factor.

Keywords: *Matricaria chamomilla* Flowers, *in vivo* study, anti-angiogenesis, CAM assay.

INTRODUCTION

Angiogenesis is about growth of new blood vessels from pre-existing one. It may be physiological that arises normally during wound healing or pathological that befalls in disease situations such as rheumatoid arthritis.[1] Hypoxia up-regulates the expression of diverse genes participant in numerous phases of angiogenesis.[2] Angiogenesis is facilitated by interplay and balance amid several anti-angiogenic factors. Though there are abundant factors persuading angiogenesis, vascular endothelial growth factor (VEGF) had been known as the dominate regulator in disease and health and disease.[3] Angiogenesis regulators are possible drugs for angiogenesis-related disorders treatment.[4] Alternatively, herbal medicines are progressively being employed to cure a variety of diseases. *Matricaria chamomile* flowers encompass volatile oil and flavonoids. It has anti-inflammatory and antioxidant properties. It was revealed antiangiogenic activity[5,6] as well as free radical scavenging activity.[5] The current study tries to investigate the possible antiangiogenic activity *in vivo* study of methanol extract from the flowers parts of *Matricaria chamomilla* cultivated from the Baghdad governorate area.

MATERIALS AND METHODS

Extraction

Five hundred grams of flower parts of *Matricaria chamomilla* were cultivated from the Baghdad governorate area. Authentication was done in department of Pharmacognosy/College of Pharmacy, Baghdad University prior to purchase.

Flower parts of *Matricaria chamomilla* specimen was labeled and annotated with date of collection and locality.

Plant material was then cut into smaller pieces and then first washed with tap water followed by washing with distilled water. It was than dried in an incubator at 37°C, until water droplets got completely evaporated.[7]

The dried powder of *Matricaria chamomilla* flower was extracted sequentially with four solvents starting with the non–polar one and ascending to the most polar one respectively (petroleum ether, chloroform, methanol and distilled water) with a ratio of 1:4 W/V (100gm of powder/400ml of solvent); the extraction with each solvent was repeated at least 3–4 times.

The powder of powder of *Matricaria chamomilla* flower was soaked with the solvent according to the ratio mentioned previously and was left for 24 hours in a shaking water bath at 40°C and then was filtered using Whatmann no.1 filter paper (20 cm) to obtain the clear extract. The filtrate kept for concentration with rotary evaporator (Buchi, Switzerland) each time before employing the solvent of higher polarity.

The residue was dried and extracted by the same procedure, mentioned above, with the other three

solvents (Chloroform, Methanol and then Water).[8,9] Closed amber vessels at +4 °C until tested.[8]

Chick Chorioallantoic Membrane Assay (CAM Assay)

Fertilized chicken eggs obtained from a local hatchery, Baghdad/Iraq were incubated for 72 h at 37°C with a relative humidity of 60 - 80%. The eggs were placed in horizontal position and rotated several times. After 72 h, 1-2 ml albumen were sucked off through a pinpoint hole pierced down by the side and sealed " in order to allow better view of the developed CAM, where the CAM will separate from the sack that is attached to the egg shell" then incubated again for another 24h. Then a round piece of shell (3-4 cm diameter) was removed from the top of the blunt end and the egg's sac punctured, then a round disc of filter paper which was impregnated previously with the test sample placed on the CAM and the eggs were sealed with a sterile adhesive tape and incubated for further 72 h.[10] The test sample was prepared as 50mg/ml and 20 µl (the final dose was 1mg/disc) placed on the disc of filter paper and left to dry prior to its transfer to the CAM. On day 7 the zone of inhibition photographed and calculated; 6 CAM were used for each control and test sample.[11]

Quantification and Immaging of CAM

The responses were graded + (3 - 6 mm); ++ (6 - 9mm); +++ (> 10mm). The quantification of zone of inhibition was done by using image analyzer. The results have been expressed as Mean ± Standard errors of means.[12]

Fourier Transform – Infrared Spectroscopy (FT – IR)

Potassium bromide (KBr) was transferred out of the oven into a mortar. About 1 to 2 % of the extract was added to the KBr, then mixed and grinded to a fine powder. The two stainless steel disks have been taken out of the desiccators; and placed a piece of the precut cardboard (in the tin can next to the oven) on top of one disk and filled the cutout hole with the finely grounded mixture. After that the second stainless steel disk had been put on top and transferred the sandwich onto the pistil in the hydraulic press. With a pumping movement, the hydraulic pump handle moved downward. The pistil started to move upward until it reached the top of the pump chamber. Then, the pump handle moved upwards and pumped until the pressure reaches 20,000 prf. Left for a few seconds and with the small lever on the left side, release the pressure (hold until the sample and pistil are all the way down). The disks removed and pulled apart. The removed film should be homogenous and transparent in appearance. Then Inserted into the Infra-Red (IR) sample holder and attached with scotch tape. After that the spectrum Run. The tests done for the active extract.[13]

Gas Chromatography – Mass Spectroscopy (GC – MS)

GC-MS analysis of the active extract was performed using a Shimadzu GCMS-QP2010 ultra system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with an Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25 µm ID × 0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 µl was employed (a split ratio of 10:1). The injector temperature was maintained at 240 °C, the ion-source temperature was 200 °C, the oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min to 200°C, then 5 °C/min to 280°C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 30 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas.[14]

RESULTS

In vivo chick chorioallantoic membrane (CAM) assay of methanol extract of *Matricaria chamomilla* Flowers

The results showed that the methanol extract of *Matricaria chamomilla* flowers produced inhibition of blood vessels growth in the CAM as recognized by appearance of avascular zone surrounding the disc that contained the extract.

The regression in blood vessels growth was measured, on day 7 of the experiment, according to the scoring system mentioned earlier. The methanol extract produced a zone of inhibition of blood vessels growth measuring more than 10mm and achieved significant scoring (+++) (Table 1 and Figure 1).

Table 1: The zone of inhibition of blood vessels growth and the corresponding scoring using the Chick Chorioallantoic Membrane (CAM) assay (n=6 for each group).

Egg	Zone of inhibition area (MM)	Scoring
1	11	+++
2	13	+++
3	7	++
4	11	+++
5	12	+++
6	8	++
Mean ± SEM	10.3±0.95	+++

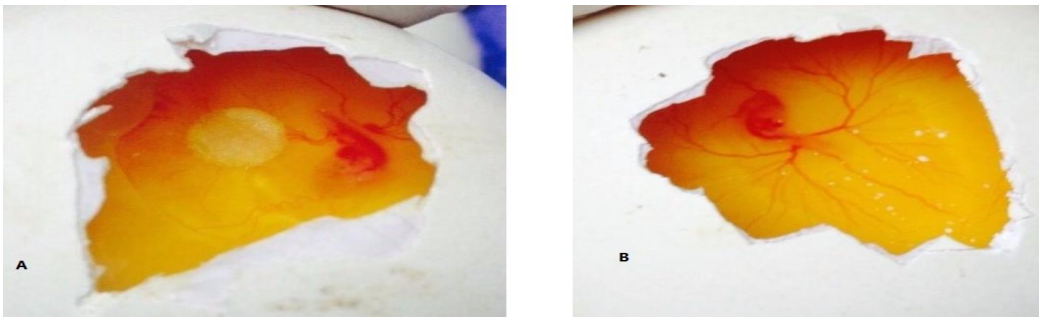

Figure 1: The *in vivo* (CAM) assay. (A) Represents the active group (treated with 1 mg of the methanol extract). (B) Represents the negative control (treated with DMSO 1%). The picture shows a clear avascular zone which indicates regression of blood vessels growth.

Table 2: The absorbance peaks of *Matricaria chamomilla* methanol extract functional groups.

No.	Wave number (cm^{-1})	Assignment Groups
1	3317	**O-H**, **N-H** Overlapping amide, alcohols, carboxylic acid
2	2885	**C-H** (-CH_3) stretching
3	2839	**C-H** (-CH_2) stretching
4	1697	**C=O** carboxylic acid
5	1604	**C=O** amide stretching
6	1473	**N-H** bending
7	1404	**C-O** (COO^-), **O-H** bending
8	1319	**C-H** bending alkanes
9	1242	**C-N** stretching, **C-O** stretching (COOH)
10	1056	**C-O** stretching alcohols, carboxylic acids
11	828	**O-H** bending carboxylic acids
12	617	**O-H** bending out of plan

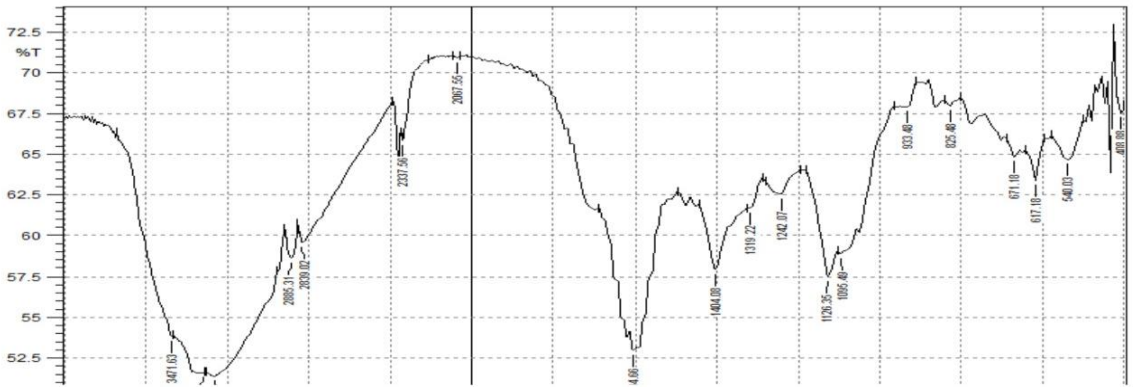

Figure 2: The absorbance peaks of *Matricaria chamomilla* methanol extract functional group.

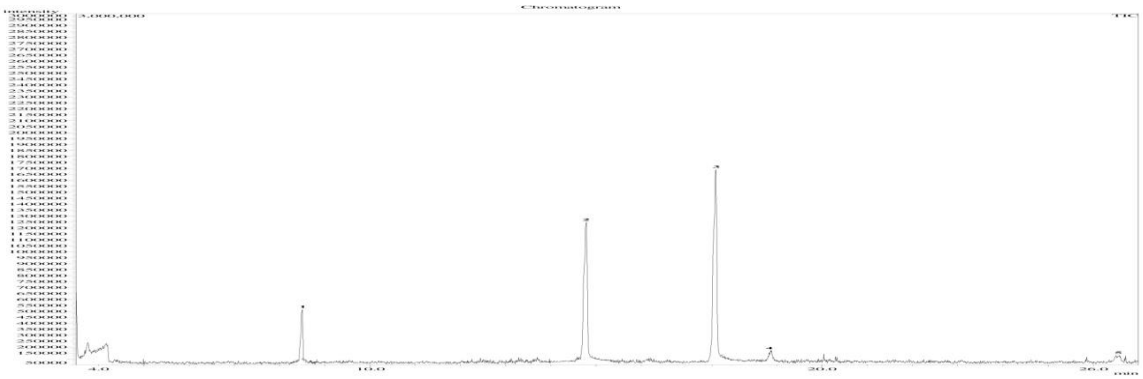

Figure 3: The chromatogram chart of GC-MS for methanol extract of *Matricaria chamomilla* flowers. (1), α-bisabolol (2), coumarin (3) apigenin.

Table 3: List of chemical fractions identified by GC/MS in the methanol extract of *Matricaria chamomilla* flowers.

Peak	R. Time	SI	Name
1	7.10	85	α-Bisabolol, α-Bisabolol oxide B, α-bisabolol oxide A
2	14.5	81	Coumarin, 7-methoxy, Ayapanin, Herniarin, 7-Methoxycoumarin
3	16.6	86	Apigenin, luteolin, *apigenin 7-glucoside*
4	18.2	78	Betaine Hydrochloride, 1-carboxy-N,N,N-trimethyl-, trimethylammonium chloride

Functional Group Identification (FT-IR) of *Matricaria chamomilla* Methanol Extract

The peak absorbance of the methanol extract of *Matricaria chamomilla* revealed existence of 12 functional groups with different absorbance as shown in Table 2 and (Figure 2). FT–IR appearance of broad peak at wave number 3317 cm^{-1} indicates presence of phenolic hydroxyl group (OH) which represents the stretching vibration because of presence of hydrogen bonding belonging to flavonoids.

The two medium bands at 2285 cm^{-1} and 2839 cm^{-1} represent stretching vibration of aromatic (C – H) bond. Also, appearance of a weak band at 1697 cm^{-1} belongs to stretching vibration of ketone (C = O) group. The appearance of sharp band at 1604 cm^{-1} belongs to stretching vibration of alkaloid (C = N) group.

Gas Chromatography Mass Spectrometry (GC-MS) Investigation of *Matricaria chamomilla* Methanol Extract

The main constituents of *Matricaria chamomilla* flowers methanol extract were identified by GC/MS are presented in Figure (3) according to their retention time (RT). Fifteen compounds were identified in the flower methanol extract as in Table (3) out of which, α-bisabolol was the principal compound followed by coumarin, apigenin respectively. Those three constituents were also showed the highest similarity index.

DISCUSSION

The present study showed that CAM treated with the methanol extract of *Matricaria chamomilla* flowers stopped new blood vessels formation and architecture of existing vasculature was distorted, the blood vessels number was declined significantly with appearance yellow light in treated CAMs.

This outlines a noticeable antiangiogenic effect. This result further supports the antiangiogenic activity observed by the *ex-vivo* study.[5] Fourier transform infrared spectroscopy (FT-IR) functional groups test for the chemicals in the methanol extract of *Matricaria chamomilla* showed that Phenols, Flavones, Tannins, Coumarins, Resins, Saponins and Alkaloids may exist in the extract.

Some studies ensured antiangiogenic effect of these compounds[15-17] that support the existence of the antiangiogenesis effect of the methanol extract of *Matricaria chamomilla* flowers.

Phenols are aromatic compounds hold hydroxyl group has been shown to have antiangiogenic effects.[18] Anti-angiogenic as well as antioxidant activities of varied classes of flavonoids have been assessed as potential novel anti-antiangiogenic agents. It has also been shown that cytotoxicity of flavonoids against selected cancer cell lines showed VEGF inhibition.[19]

Coumarins have been proved their antiangiogenic property and hydroxyl radical scavenging activity.[20] In parallel, analysis of the methanol extract of *Matricaria chamomilla* flowers revealed that they have varying components.

The highest peaks in the GC-MS chromatogram extract represented α-Bisabolol, Coumarin, and Apigenin. The presences of these components in the methanol extract of *Matricaria chamomilla* flowers has been documented in many reports.[21,22] It has been demonstrated that the flavonoid apigenin inhibited hypoxia-induced elevation of VEGF production at low oxygen conditions. Low oxygen (hypoxia) and transforming growth factor-β (TGF-β) are two major factors responsible for increased VEGF secretion. It has also been notice that VEGF expression is induced by TGF-β1 in human prostate cancer and treatment with apigenin markedly decreased VEGF production by inhibited TGF-β1-induced phosphorylation and nuclear translocation of Smad2 and Smad3.[23] Another experiments demonstrated that specific transient knockdown of Smad2 or Smad3 blunted apigenin's effect on VEGF expression.[24] Taken together, it may suggest that apigenin modulating TGF-β-activated pathways in particular the Smad2/3. Additionally, the antiangiogenic effects of apigenin are seen as inhibition of vascular endothelial growth factor–hypoxia inducible factor signaling pathways. These findings could provide an understanding for molecular mechanism underlying the antiangiogenic potential of apigenin.

CONCLUSION

Matricaria chamomilla flowers methanol extract mechanism of action may narrate to the presence of flavonoids and polyphenols that have been established to constrain angiogenesis. According to the results in the FT-IR and GS-MASS, the anti-angiogenesis activity may be credited apigenin compound inside the extract as this

compound has the ability to block the VEGF production, thereby inhibiting the angiogenesis process.

Abbreviations

CAM	Chorioallantoic membrane
FT-IR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography/mass spectrometry
KBr	Potassium promide
TGF-β	transforming growth factor-β
VEGF	Vascular endothelial growth factor

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