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Antiangiogenic Activity of Iraqi *Anabasis articulata* Stems *In vivo* Study

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Abstract: ***Background*: *Anabasis articulata*, also called Eshnan, Ajremor Berry bearing glasswort, is widely distributed in Syrian, Algerian, Egyptian and Iraqi desert. *Anabasis articulata* is broadly used in folk medicine to treat diabetes, fever, eczema and kidney infections. *Objective*: to examine the antiangiogenic action of *Anabasis articulata*in vivo.*Subjects and methods*: The powder of the *Anabasis articulata* stems was sequentially extracted with four solvent types of different polarity.Eggs were gestated for three days, small whole prepared on the fine pinpoint. The following day, the egg's sac was penetrated and a small frame was made in the shell. Eggs were returned to the incubator until day 10 of chick embryo growth, methanol extract was moved to the Chick Embryo Chorioallantoic Membrane (CAM), and eggs incubated for 72 hours (n = 6); the zone of inhibition was estimated as mean of inhibition area. Functional groups of the chemicals component private to the extract has been recognized by Fourier transform infrared spectroscopy FT-IR and Gas chromatography mass spectrometry was utilized to recognize the most likely relevant agent. *Results*: The results displayed that the zones of inhibition area range between 7 and 10 mm. FT-IR displayed that some of the recognized functional group may relate to flavones, coumarins, alkalines, saponins and tannins. Scopoletin, 2-Methoxy-4-vinylphenol, glycine and 1, 2-dimethyl-Piperidine, have been recognized in methanol extract.Conclusion: the results revealed that the mechanism of anti-angiogenic activity for the methanol extract of the *Anabasis articulata* stems may be related to these substances that have the capacity to block the VEGF- receptor, thereby inhibiting the angiogenesis.***

Keywords: *Anabasis articulata*, *in vivo* study, anti-angiogenesis, CAM assay, Scopoletin, 2-Methoxy-4-vinylphenol, glycine and Piperidine.

1. Introduction

Angiogenesis defined as a multifaceted process during which fresh blood vessels grow from a pre-existing vasculature. It is an important component of the normal female reproductive cycle, embryogenesis, growth and successful repairing of the tissue(1).Angiogenesis, in other hand, is dangerous to the organism, permitting growth and metastasis of tumor cancers, contributing to the blindness in diabetic retinopathy and relevant cause in rheumatoid arthritis.The angiogenesis highly controlled system like most processes in homeostatic cellular systems. A great number of proangiogenic growthfactors have been recognized, one of these factors is a protein called vascular endothelial growth factor (VEGF).*Anabasis articulata*in Iraq used traditionally under (Eshnan) name, used commonly in folk medicine to treat diabetes, fever, headache and skin diseases such as eczema. Chick Chorioallantoic Membrane assay (CAM) have been broadly used to examine angiogenesis, tumor cell invasion and metastasis. The CAM model has various benefits, such as (a) the greatly vascularized nature of the CAM importantly stimulates the effectiveness of tumor cell grafting; (b) cost effectiveness and simplicity, (c) high reproducibility; and lastly (d) the half-life of several investigational molecules such as small peptides tends to be much elongated in comparison to animal models, permitting experimental study of possible anti-metastatic compounds that are only presented in small quantities because the CAM assay is a closed system. *In vivo* angiogenesis assays have permitted essential progress in studying the efficacy and in explaining the mechanism of action of several agents, weather angiogenic stimulators or inhibitors(2).

2. Materials and Methods

Plant extraction

The stems of *Anabasis articulata* were collected from local herbal apothecary in Baghdad and the stems were authenticated by Dr. Ibrahim Salih Abbas, Assistance Professor (Ph.D. Medicinal plants, Botanic Department, Karbala University, Iraq) before purchased. The plant stems wereair- dried indoor for one weak. The dried stems were separated and then ground into powder (3).

Preparation of the extracts

The powder extracted sequentially with four solvents beginning with the non – polar one and rising to the more polar one respectively petroleum ether, chloroform, methanol and water.The extraction ratio was 4:1 W/V ratio of each solvent. Maceration process (cold method) included soaking plant stems (powdered) in a stoppered container with a solventand permitted to stand at room temperature in water bath for 3 days with frequent agitation. The work was done in the phytotherapy laboratory of Department of Pharmacology in College of Medicine / Al-Nahrain University. The powder of *Anabasis articulata* stems was soaked with the solvent consistent with the ratio stated previously and was left for 24 hours in a shaking water bath at 40℃ and then was filtered using whatmann no.1 filter paper to get the clear extract. The extract was concentrated by a rotary evaporator with vacuum (Buchi, Switzerland)to achieve the final crude extract, which was stored in dry and tightly sealed bottle to be used later in the experiment (4).

Chick chorioallantoic membrane assay (CAM assay)

Fertilized chicken eggs were taken from Ebaa Research center, Baghdad, Iraq. The fertilized chicken eggs are

positionedhorizontally and rotated several timesin an incubator, as soon as embryogenesis commenced and are saved under relative humidity of 60 - 80%.at 37°C. Small whole made on the fine pinpointon day three, and the egg left to be incubated at 37 °C for one more day. The egg's sac punctured on day four and a small window (3-4 cm) was made in the shell afterremoving 2-3 ml of albumen to detach the CAM from the shell, then window was resealed with adhesive tape and eggs were returned to the incubator until day 10 of chick embryo development. On day 10, sample prepared as (50mg/ml), 20 µl placed on round disc of filter paper left to desiccate and then transported to the CAM and eggs were resealed and returned to the incubator for 72 hours(until day 14) (n = 6 chicken embryos per sample); the zone of inhibition photographed and calculated(5).

Quantification and imaging of CAMs

Six CAMs were utilized in each experimental and control groups. The responses may be ordered as: (+) 3-6 mm; (++) 7-9mm; (+++) > 10 mm(6).Data were collected using the Java-based NIH ImageJ image processing software version 1.43u(7).

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR assay is vital for functional group documentation in plant extracts. Methanol extract of *Anabasis articulata*was milled into fine powder by utilizing agate mortar and the FT-IR spectrometer in the area of 4000-400cm^{-1} by using standard KBr pellet technique. Potassium bromide (KBr) was transferred out of the oven into a mortar; about 1 to 2 % of the extract was added on the KBr, then mixed and grinded to a very fine powder. Both stainless steel disks are utilized; by a placed portion of the precut cardboard on top of one disk and fill the cutout hole with the finely grounded blend. After that the second stainless steel disk is putted on the top and then transferred as sandwich onto the pistil in the hydraulic press. With the hydraulic pump handle passaged downward, the pistil started to passage upward until it arrive the top of the pump chamber. Then, pump until the pressure reaches 20,000 PRF. Then disks are isolated and pulled apart and introduced into the Infra-Red (IR) sample holder and attached with scotch tape. After that the spectrum runs (8),the test done for all extracts.

Gas chromatography–mass spectrometry (GC-MS)

GC-MS analysis was accomplished on a Shimadzu GC-MS QP-2010. Inert Cap 1MS capillary column was utilized (30 m x 0.25 mm x 0.25 µm film thickness) with helium as carrier gas at a flow rate of (1.6 ml/min). The source was worked in positive ionization mode (electron impact energy: 70eV) and the revealing was performed in full-scan mode. The inlet and the transfer line temperatures were both preserved at 250°C while the ion source was reserved at 200°C. Samples were injected in splitless mode (10:1) and detached using a temperature gradient program as follows: 100°C for 1 min, to 200°C at 12°C/min and then kept at 200°C for 5 mins; then to 300°C at 5°C/min and kept at 300 C for further 5 mins. GC-MS spectra were assessed by Postrun software and searched in the National Institute of Standards and Technology (NIST) MS Search V2.0 browsers .The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were determined(9).

3. Results

The *in vivo* Chick Chorioallantoic Membrane Assay (CAM Assay)

Images showed the blood vessels growth inhibition at day 7, were images in (Figure 1) representing the control images, and (Figure 2)represent the treated blood vessels of CAM with the*Anabasis articulata* methanol extract. The results showed that the zones of inhibition ranged from 7 mm to more than 10 mm, so the score is two plus as shown in table (1).

Table 1: Zone of blood vessels growth inhibition in chick chorioallantoic membrane assay (n=6) for each group.

Eggs no.	Zone of inhibition Area (mm) / Scoring
1	11 / +++
2	11 / +++
3	8 / ++
4	12 / +++
5	13 / +++
6	9 / ++
(Mean ±SE)	10.66±0.86

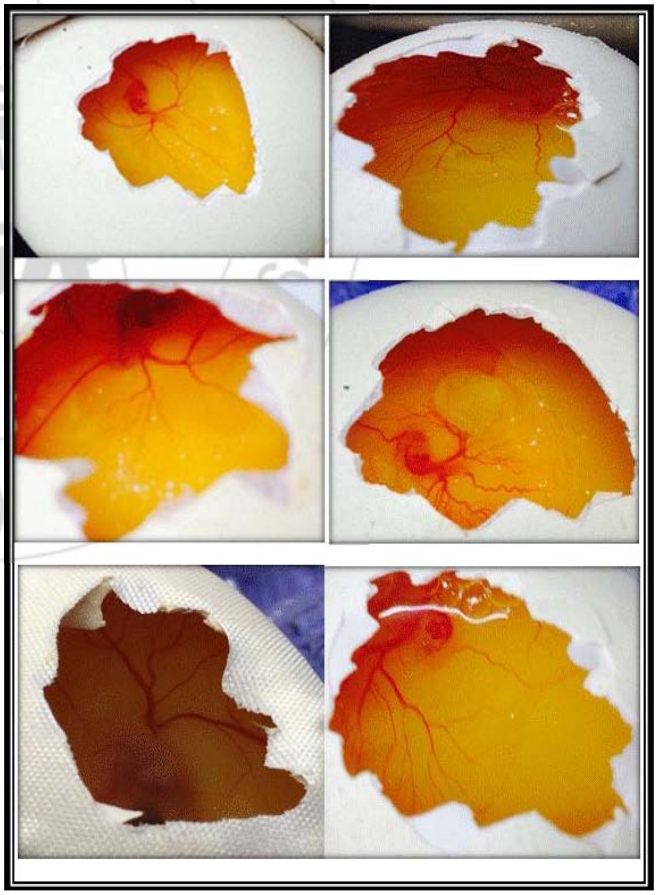

Figure 1: Chick chorioallantoic membrane assay images of control eggs

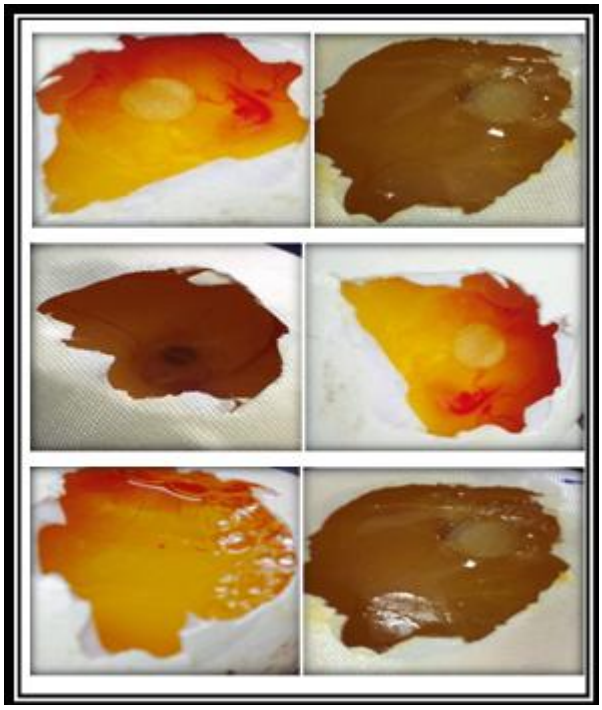

Figure 2: Chick chorioallantoic membrane assay images of treated eggs with *Anabasis articulata*methanol extract

Functional group identification (FT-IR) of *Anabasis articulata*methanol extract

The peak absorbance of the methanol of *Anabasis articulata*(revealedexistence of twelve functional groups with different absorbance respectively) as illustrated in Table(2), Figure (3)).

Table 2: The peaks of absorbance of *Anabasis articulata*stems.

Wavenumber (cm^{-1})	Assignment groups ((Iqbal M., Saeed A and Zafar S. I, 2009), (Silverstein R. M, Webster F. X. and Kiemle D. J., n.d.))
7933	O-H, N-H Overlapping amide, alcohols, carboxylic acid
2939	C-H ($-CH_3$) stretching
2831	C-H ($-CH_2$) stretching
1728	C=O carboxylic acid
1620	C=O amide stretching
1450	N-H bending
1396	C-O (COO^-), O-H bending
1334	C-H bending alkanes
1242	C-N stretching, C-O stretching (COOH)
1072	C-O stretching alcohols, carboxylic acids
810	O-H bending carboxylic acids
601	O-H bending out of plan

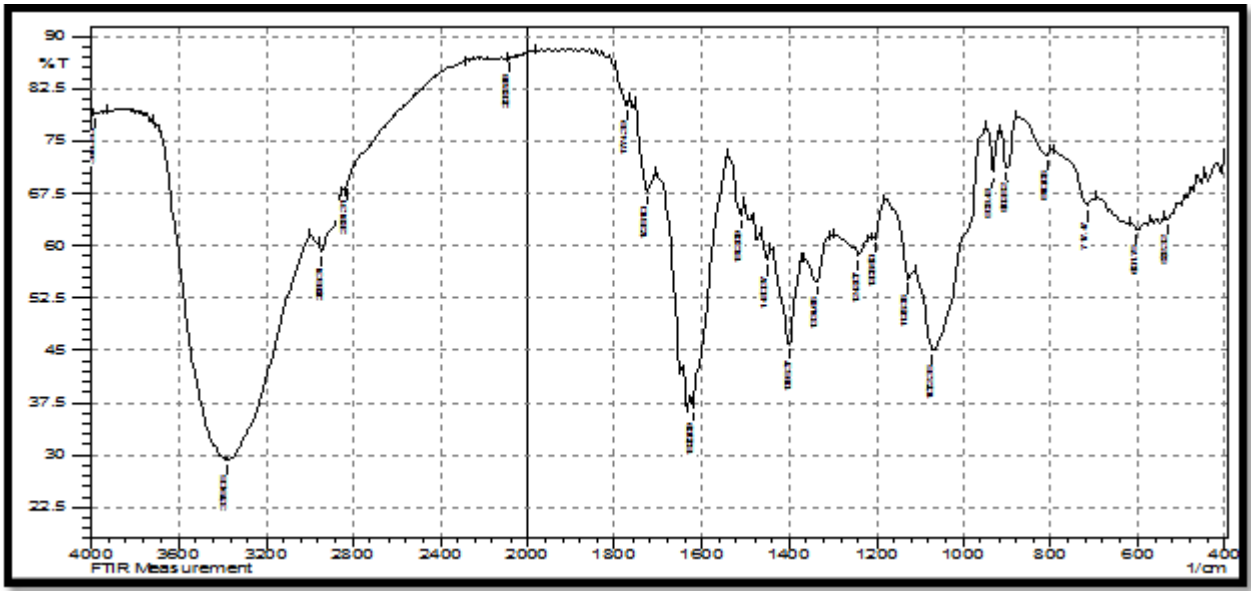

Figure 3: The peaks absorbance of *Anabasis articulata*methanol extract functional group

Analysis of Active Chemical Compounds

The qualitative analysis for active chemical compounds(10)is presented in Table (3).

Table 3: Active chemical compounds of the adsorbents (methanol extract of *Anabasis articulata*)

Active organic compound	Methanol extract of *anabasis articulata*
Tannins	+
Saponins	+
Alkaloids	+
Resins	+
Coumarins	-
Flavones	+
Terpenes	-
Steroids	-

Gas Chromatography Mass Spectrometry (GC-MS) investigation of *Anabasis articulata* methanol extract

The data of the results obtained through GC-MS analysis of the methanol extract of *Anabasis articulata* showing different peaks of the different constitutes of the methanol extract with three predominant peaks and single minor peak, the four peaks are presented as shown in table (4) and figure (4).

Table 4: Phytocomponentsidentified in the methanolic extract of *Anabasis articulata*by GC-MS

R. Time	Area%	Molecular formula	MW	Height area%	Name
11.5	40.76	$C_{10}H_8O_4$	192	31.95	Scopoletin
17.74	16.18	$C_9H_{10}O_2$	150	23.23	2-Methoxy-4-vinylphenol
13.65	24.70	$C_4H_9NO_2$	103	23.23	N,N-Di-methyl glycine
12.75	18.36	$C_7H_{15}N$	113	21.95	Piperidine, 1,2-dimethyl-

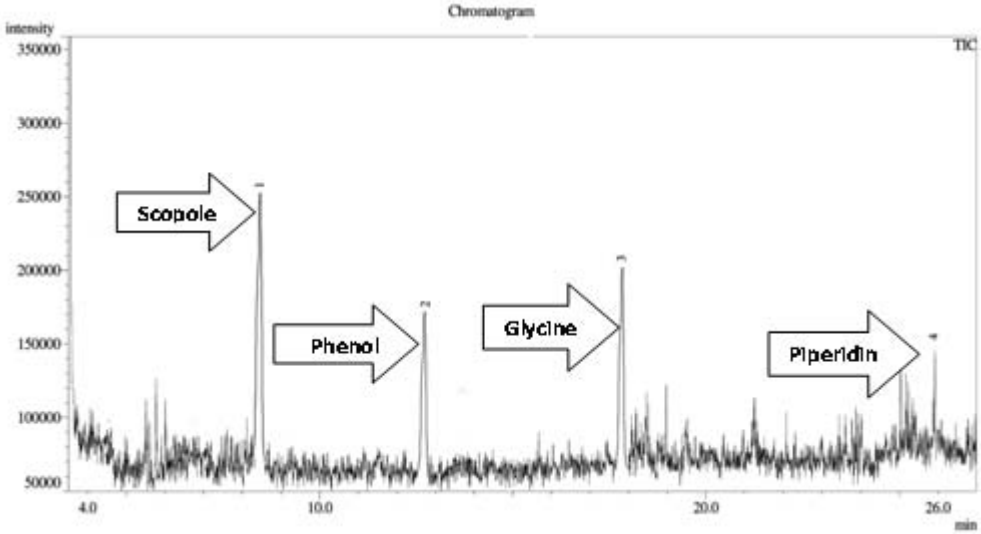

Figure 4: GC-MS chromatogram of the methanol extract of *Anabasis articulata*.

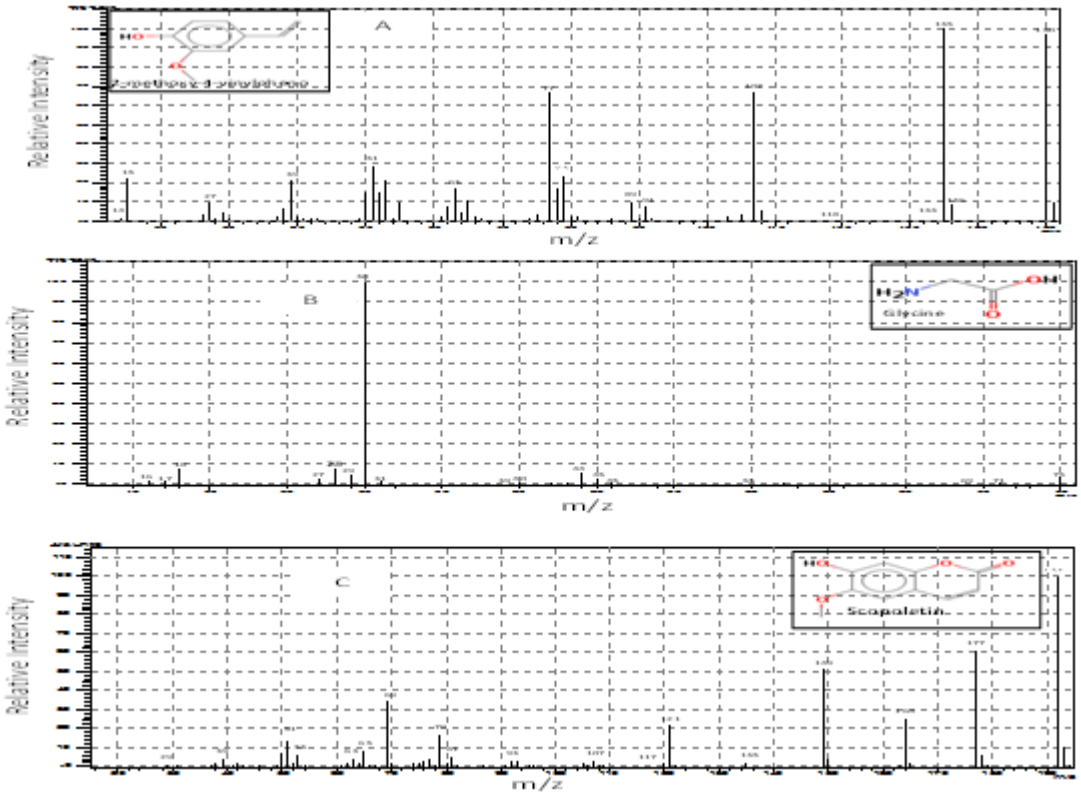

Figure 5: Mass spectrum and structure of phytocomponents identified by GC-MS in the methanolic extracts of ***Anabasis articulata***.

4. Discussion

Chick Embryo Chorioallantoic Membrane (CAM) assay *In vivo* study

The results of CAM treated with methanol extract of *Anabasis articulata* stems displayed noticeable anti-angiogenesis. Radiation of huge number of vessels was stopped from underneath the disc which carried the methanol extract. Furthermore, the vessels were thin, disordered, with a light yellow look. In this study the result displayed that methanol extract which had a potent anti-angiogenic activity, such findings backing their results achieved from the *ex vivo* assay. It appears that the anti – angiogenic activity of methanol extract was due to the existence of variety of phytochemicals as was shown by FT-IR and GC-MS analysis. The functional groups of the chemicals in the *Anabasis articulata* stems extracts tested by FT-IR; revealed the presence of alkaloids, coumarins, flavones & saponins. Shazia and coworkers'(2014) stated that alkaloids have potent anti angiogenic activity. Alkaloids prevent angiogenesis and metastasis through inhibition of NF-κB that controls the inflammatory gene expression and recently it has been suspected to be involved also in the control of tumor development. In addition to that Eun and Koh (2004)showed that alkaloids inhibits the VEGF-induced endothelial cell migration, sprouting and survival *in vitro*, and blocks blood vessel formation *in vivo* in different experimental models.The nuclear factor NF-κB pathway has long been regarded a prototypical proinflammatory signaling pathway; mostly depend on the role of NF-κB in the expression of proinflammatory genes comprising cytokines, chemokines, and adhesion molecules.NF-κB has important role in the stimulation of VEGF in human primary macrophages, which are the chief cytokine producers in chronic inflammatory diseases like rheumatoid arthritis(11).Pan *et al* (2011) find that ascopoletin (a coumarins compounds) down-regulated the VEGF expression through NF-κB.Rocha *et al* (2012) studies the antiangiogenic actions of flavones in the ECV304 human endothelial cell line, Rocha *et al* revealed flavones reduced the mRNA levels of two important angiogenic factors VEGF and bFGF that stimulate permeability, proliferation, and tube formation of endothelial cells and these findings are consistent with the finding of this study. All these above findings might clarify the anti – angiogenesis activity of *Anabasis articulata* stems methanol extracts in both *ex vivo* and *in vivo* assays.

Phytochemical Analyses of *Anabasis articulata* stems methanol extract:

Functional group analysis by FT-IR for methanol extract of *Anabasis articulata* shown the presence of flavones, alkaloids, tannins, saponins and resins, although GC-MS supports these results; it presented some cross likeness in some of the phytochemical constituents.

The major three constituents detected by GC-MS of methanol extract were scopoletin, glycine& 2- methoxy 4-vinylphenolwith minorone was1, 2- dimethyl piperidine. These results agree with Ghembaza and coworkers in (2016); moreover,Hamdoon and coworkers (2013) studies also displayed that *Anabasis articulata*stems has quantity of phenols, coumarins, alkalines and amino acids that come in agreement with our finding, the dissimilarities was simply in the concentrations of these active compounds. The information of this study may interpret the vital anti-angiogenic activity perhaps via its inhibition of the vascular

endothelial growth factor (VEGF), fibroblast growth factor (FGF), cyclooxygenase receptor (COX-2) and epidermal growth factor receptor (EGFR). Scopoletin (coumarin compound) might obstruct the proliferation, migration of endothelial cells induced by VEGF at mRNA and protein levels. These results propose that scopoletin is substantially able to attenuate VEGF induced angiogenesis, and it might act by preventing the production of VEGF. Scopoletin might down-regulated the VEGF expression through NF-κB rather than PI-3K/Akt signaling pathway(12). Furthermore, Scopoletin could diminish IL-6, VEGF and FGF-2 expressions in rat synovial tissues. The antiangiogenic activity of scopoletin in our study strengthened by Rong and coworkers provedthat scopoletin selectively down-regulated serum-induced ERK1/2 phosphorylation, without affecting endothelial cell p38 MAPK or JNK phosphorylation. These outcomes validate that scopoletin has anti-angiogenic properties that are apparent chiefly through constraining migration and tube formation of endothelial cells via down-regulating ERK1/2 stimulation(13).

The glycine detected in the present extract has the ability to inhibit angiogenesis *in vivo* as shown in our study and these findings strengthened by Bruns *et al.* (2014) that found the inhibitory effect of glycine to the VEGF receptors. Several evidences recommend a possibility that glycine is valuable as an immuno-modulating amino acid. Glycine most likely stops the lipopolysaccharide (LPS)-induced elevation of intracellular $Ca^{(2+)}$ concentration in cells of Kupffer, thus diminishing LPS receptor signaling and cytokine making. Vascular endothelial growth factor (VEGF) shows a critical role in rheumatoid arthritis evolution by supporting new blood vessel formation. Triggering of VEGF receptor has been shown to result in stimulation of phospholipase C-gamma and rises in the concentration of intracellular $Ca^{(2+)}$. The VEGF-induced cell proliferation is reliant on intracellular $Ca^{(2+)}$ concentration. The VEGF augmented intracellular $Ca^{(2+)}$ concentration quickly, but glycine diminished increases in intracellular $Ca^{(2+)}$ concentration due to VEGF. More, the preventer's effects of glycine were prohibited by small concentrations of strychnine (1 micromol/L) or cultivation with chloride-free buffer. Lastly, glycine considerably weakened serum-stimulated proliferation and migration of endothelial cells. These data specify that the inhibitory effect of glycine on growth and migration of endothelial cells is due to activation of a glycine-gated chloride channel. This hyperpolarizes the cell membrane and blocks influx of $Ca^{(2+)}$, in this manner minimizing growth factor-mediated signaling(14). A naturally occurring phenolic compound (2- methoxy 4-vinylphenol) that is used as flavoring agent, exert potent anti-inflammatory effects by inhibiting LPS-induced NO, PGE_2, iNOS, and COX-2 in RAW264.7 cells. These effects are mediated by suppression of NF-κB and MAPK activation and histone acetylation(15). 2-Methoxy4-vinylphenolexert*ex vivo* and *in vivo* antiangiogenesis effect in this study, these findings strengthened by Venugopalan and Kathirvel that find the 2-Methoxy-4-vinylphenol of ***Plectranthusamboinicus*** leaves presented a strong free radical scavenging activity and have antiangiogenesis consequence on chorioallantoic membrane and tumor induced angiogenesis.

5. Conclusions

Methanol extract of Iraqi *anabasis articulata* stems considerably inhibited the blood vessels growth in CAM. The mechanism may relate to the presence of scopoletin, glycine and phenols which have been identified by GC-mass and FT-IR.

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