Research Article

Antiangiogenesis and Antioxidant Effect of *Anabasis articulata* Stems Extracts

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ABSTRACT

The objective is to find the potential anti–angiogenic antioxidant effect of *Anabasis articulate* stems extracts. *Anabasis articulate* stems powder was extracted serially with petroleum ether, chloroform, methanol and water utilizing the cold "maceration" method as extraction process. The direct antiangiogenic activity was evaluated using rat aorta ring assay; this assay was also used to conclude the dose–response effect of the active extract(s) by preparing serial concentrations. The antioxidant property was analysed using, 1, 1-diphenyl-2-picryl-hydrazyl free radical scavenging assay. All four extracts showed potent inhibition of the microvessels outgrowth in the rat aortic assay when compared to the negative control (received DMSO 1%) ($P<0.001$), but methanol extract displayed the highest percent of antiangiogenic activity. Methanol extracts of *Anabasis articulata* stems revealed a significant dose–dependent antiangiogenesis effect with IC 50 (18.27µg/ml). Furthermore, methanol extracts show a significant free radical scavenging activity ($P<0.05$) with IC 50 (94.7 µg/ml).Methanol extracts of *Anabasis articulate* stems showed the best and most significant antiangiogenesis activity as well as a significant free radical scavenging activity.

Keywords: Angiogenesis, E*x vivo* study, *Anabasis articulate* stems, antioxidant.

INTRODUCTION

Angiogenesis defined as a multifaceted process during which fresh blood vessels grow from a pre-existing vasculature. The term originates from two Greek words: angio, which means "blood vessel," and genesis, which means "beginning". It is an important component of the normal female reproductive cycle, embryogenesis, growth and successful repairing of the tissue. 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. Endothelial cells which are the principal building blocks of vasculature in process of angiogenesis, it must undergo four chief steps which include breaking through of the basal lamina that wraps surviving blood vessels, migration toward a source signal, proliferation and formation of tubes. Angiogenesis stimulation can be of therapeutic value in wound healing, ischemic heart disease and peripheral arterial disease; on the other hand inhibition of angiogenesis can be of therapeutic value in treatment of cancer, ophthalmic conditions, rheumatoid arthritis, and other diseases[1].The angiogenesis highly controlled system like most processes in homeostatic cellular systems. A great number of proangiogenic growth factors have been recognized, one of these factors is a protein called vascular endothelial growth factor (VEGF) [2]. *Anabasis articulata* (*AA*), 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. *Anabasis articulata* stems have many active constituents which may have more than one pharmacological effect[3].Phytochemical screening on *Anabasis articulate* show the existence of saponin**,** coumarins, flavonoids, phenolics, alkaloids, anthraquinones, irodoids, Cyanogenic glycosides , Cardiac glycosides, Carbohydrates or Glycosides, Unsaturated sterols or Triterpenoids, Tannins[4].

MATERIALS AND METHODS

Plant extraction

The stems of *Anabasis articulata* were collected from local herbal apothecary in Baghdad and authenticated by Botanic Department of Karbala University before purchased. The plant stems were air- dried indoor for one weak. The dried stems (2000 gram) were separated and then ground into powder. The powder was then separated into twenty portions and was extracted sequentially with four solvents beginning with the non – polar one and rising to the more polar one respectively petroleum ether (PE), chloroform(CH), methanol (ME) and water (W). 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 and left for 24 hours in a shaking water bath at 40°c 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 e final crude extract, which was stored in dry and tightly sealed bottle to be used later in the experiment[5].

Experimental animals

Albino rats were collected from Higher Institute for the diagnosis of infertility and assisted reproduction techniques of Al-Nahrain University. The experiments were approved by the Animal Ethical Committee of Al-Nahrain University, College of Medicine. All the animals were allowed to free access to food and tap water and kept at 24-28°C.Adult Male Sprague Dawley rats at age 12-14 weeks were used for the Rat aorta ring antiangiogenesis assay.

Rat aorta ring antiangiogenesis assay

The experiment was done in the tissue culture laboratory of Pharmacology Department in College of Medicine / Al-Nahrain University. The angiogenesis assay used in this technique is consistent with that developed by Brown and coworkers with minor modification[6]. Animals were humanely sacrificed via cervical dislocation under anesthesia with diethyl ether. A fresh 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. Medium M199 was used for the lower layer; fibrinogen and aprotinin at 3mg/mL and 5µg/ml respectively were added to this layer. Each 48-well plate a 300 µL of M199 medium was loaded and one aortic ring was then. To each well, add 10 µl 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% CO_2 for 30-60 min. The preparation of top layer medium was complete 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. To the top layer medium plant extracts were added at concentration of 100µg/mL, six replicates for each treatment was performed. Stock solution of the sample extract was formulated by dissolving the sample in dimethyl sulfoxide (DMSO), and diluted in M199 growth medium to make the final DMSO concentration 1%.The 48-well of the tissue rings were incubated at 37°C, 5% CO_2 in a humidified incubator. On the day 4, the top layer medium was replaced 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. On day 5, the results observed under inverted microscope and the blood vessel growth extent was quantified under 10X magnification with assistance of camera and software package. Data were collected from 10 X images using the Java-based NIH ImageJ image processing software version 1.43u [7]. The magnitude of blood vessel growth inhibition was determined according to the technique developed by Nicosia and coworkers (1997). The results are presented as mean percent inhibition to the negative control ± SE. The experiment was repeated three times using six replicate per sample. The percentage of blood vessels inhibition was determined according to the following formula:

Blood vessels inhibition = 1- (A_0/A) ×100 Where: A_0= distance of blood vessels growth for the test substance in µm. A= distance of blood vessels growth in the control in µm.

Rat aorta assay (antiangiogenesis) dose response studyof the methanol extract of *Anabasis articulata*

Serial dilutions from stock sample solution of the methanolic extract of *anabasis articulata* were prepared in the following concentrations: 6.25, 12.5, 25, 50, 75,100, and 200 (µg/ml) of the samples were in DMSO dissolved, and then diluted in the M199 growth medium to make the final DMSO concentration 1%. Wells with no samples treatment were received medium with 1% DMSO used as the negative control. The data was represented as mean ± SE. 100µg/ml aspirin was used as a positive control (8). The $IC_{50,}$ which is the concentration that inhibit 50% of blood vessels growth, was calculated by using the linear regression equation for the extract. Where Y= the percentage of inhibition, and X= concentration[9].

Free radical scavenging activity with 1, 1 -Diphenyl -2-Picrylhydrazyl (DPPH).

Free radical scavenging activity of the *Anabasis articulate* extracts were measured by utilizing (DPPH) scavenging activity assay. 200 µl of 0.1 mM DPPH dissolved in methanol was added to 100 µl of the active *Anabasis articulata* extracts in the following concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.813 µg) and for 30 min incubated. This procedure was performed using 96 well plates. Each concentration was tested in triplicate, and then the absorbance was measured at 517 nm using an ELISA reader. Ascorbic acid (Vitamin C) was utilized as a positive control and methanol alone used as blank. The negative control was prepared from 100µl of methanol and 200µl DPPH. The percentage of antioxidant activity was calculated according to the formula below [10,11]

$AA\% = 1- (A_S – A_B/A_C – A_B) \times 100$

A_S = absorbance of sample

A_B = absorbance of blank, A_C = absorbance of control

Statistical design and analysis

Rationalized Complete Block Design (RCBD) was utilized in the experiment design studies. The results were presented by means of means ± standard error of mean (SEM). One way analysis of variance (ANOVA) followed by Tukey test comparison *t*-test (2-tailed) was utilized to compare between groups. The differences between the means are studies as significant at the 0.05 confidence level. The concentration that inhibit 50% of the blood vessels growth, cells proliferation (IC_{50}) this value was analyzed by linear regression equation and logarithmic equation. The statistical analysis was done by using Windows SSPS 16.0 (SPSS Inc. Chicago, IL), the level of significance was set at $P<0.05$ as significant [12].

RESULTS

Anabasis *articulata* stems crude extract yields

The percentage yield of the extract was determined gravimetrically using the dry weight of extracts and weight of powdered sample material, the extraction process yielded 6.83 g (0.68%) petroleum ether extract, 18.19 g (1.8%) chloroform extract, 37.91g (3.79 %) methanol extract and 105.1 g (10.5 %) of water extract .

Antiangiogenesis (*Ex vivo* rat aorta ring assay)

Inhibition of blood vessels growth by petroleum ether, chloroform, methanol and water extracts of Anabasis articulata stems.

A concentration of 100 µg/ml of each of the four extracts was added on rat aorta embedded in complete growth medium of M199. There are a significant difference (P<0.0001) when comparing the four extracts (PE, CH, ME and WA) with the negative control in the expressions of inhibition of blood vessels growth. Among the four extracts, ME presented the highest percentage of antiangiogenic activity (94.3%) in comparison to the negative control while PE (70%), CH (61.3%) and WA (48.5%). On the other hand there is a significant difference in blood vessels growth inhibition when comparing PE, CH and WA to the positive control (P<0.0001), while there is no significant difference when comparing ME to positive control (P=0.479). In addition to above there is a significant difference when comparing ME with PE, CH, and WA in their anti – angiogenesis activity (P<0.0001). Lastly, all the extracts showed a significant inhibition of blood vessels growth but ME presented the best significant inhibition as shown in figure (1) and figure (2).The serial dilutions of the methanol extract of *Anabasis articulata* were added to the rat aorta rings. Seven concentrations were used (6.25, 12.5, 25, 50, 100, 200 µg/ml). These concentrations showed significant dose dependent inhibition activity (P<0.0001) in comparison with negative control, figure (3). The images of rat aorta rings displayed a dose related inhibition of tiny blood vessel outgrowths from the primary ex-plant, with lowest inhibition showed in image of (6.25 µg/ml) concentration, and highest shown in image of (200 µg/ml) concentration.

IC_{50} value was calculated from the logarithmicequation as illustrated in figure (4), (y = 20.523ln(x) - 9.625), Where: y = the percentage of inhibition and x = concentration. The data indicates significantdose related inhibitions, which with 50% inhibition of concentration equals to 18.27µg/ml.

DPPH scavenging activity Assay

The free radical scavenging activity of each of PE, CH, ME & WA extracts of *Anabasis articulata* was measured using the 2, 2-diphenyl-2-picrylhydrazyl (DPPH) assay. Seven serial concentrations were utilized to conclude the scavenging activity of each solvent as presented in table (3.4). The results displays that methanol extracts significantly diminished the DPPH free radical in a concentration dependent manner at (P<0.05). Also Statistical analysis revealed there is no significant difference when comparing ascorbic acid with methanol extract scavenging activity(P = 0.061), while there is significant difference when comparing ascorbic acid with PE, CH & WA extracts scavenging activity (P<0.05). IC_{50} of DPPH scavenging activities of the tested agents were calculated by the logarithmic regression equations. Where: Y= Percentage of DPPH scavenging activity and X=concentration. The IC_{50} of DPPH scavenging activity was calculated by the logarithmic equation of each tested agent by considering Y is to be 50%. The IC_{50} of DPPH for Ascorbic acid was (16×10^{-4} µg /ml), the IC_{50} of DPPH for PE extract was (200 µg /ml). The IC_{50} of DPPH for CH extract was (257 µg /ml), and the IC_{50} of DPPH for ME extract was (94.7 µg/ml), figure (5).

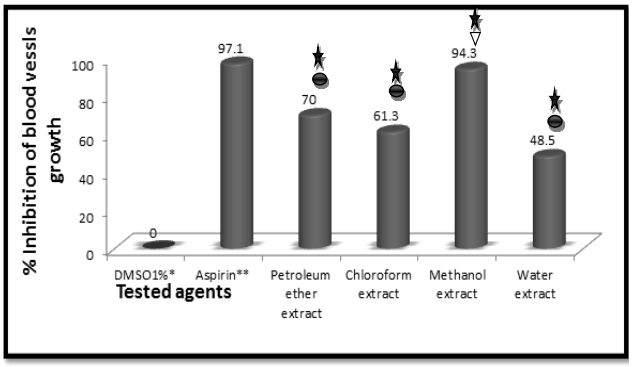

Figure (1): The effect of (100 µg/ml) of each of petroleum ether, chloroform, methanol and water extracts of *Anabasis articulata* on blood vessels growth.

* DMSO1% = Dimethyl sulfoxide serve as negative control.

** Aspirin = Serve as positive control (100µg/ml)

★ Significant difference when compare with negative control

● Significant difference when compare with positive control

▽ Significant difference when compare when compare with PE, CH &W

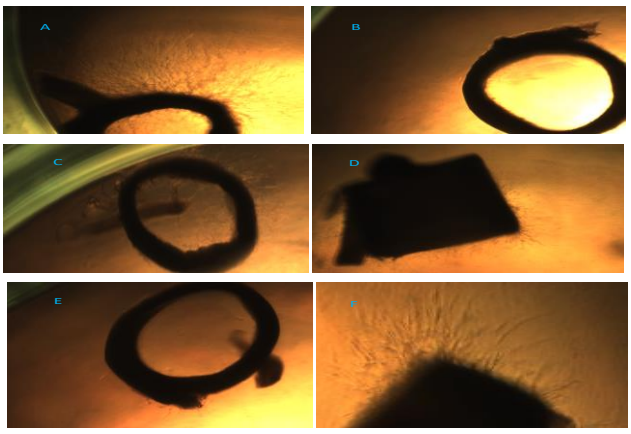

Figure 2: Images of aorta rings treated with aspirin, different solvents of *Anabasis articulata* extracts and controls. Were A, B, C, D, E and F represent the activity of the DMSO 1% (negative control), Aspirin (positive control), petroleum ether, chloroform, methanol, and water respectively.

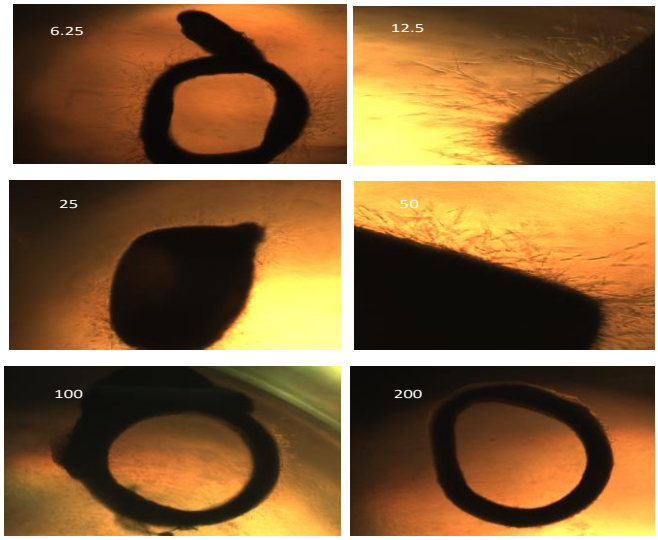

Figure 3: Dose response images of *Anabasis articulata* stems methanol extract, serial concentrations (6.25, 12.5, 25, 25, 50,100, and 200 µg/ml) have been tested against rat aorta.

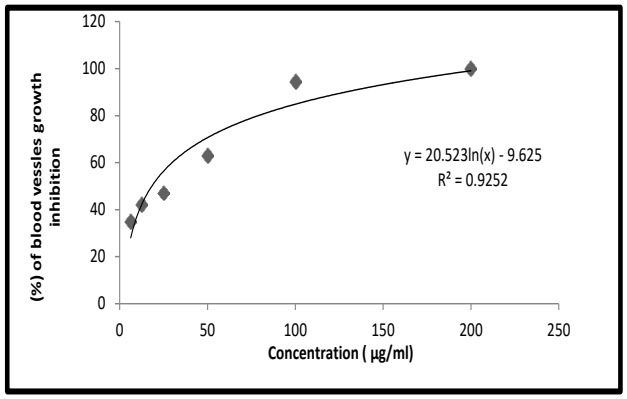

Figure 4: Dose response curve of *Anabasis articulata* stems methanol extract on rat aorta ring assay.

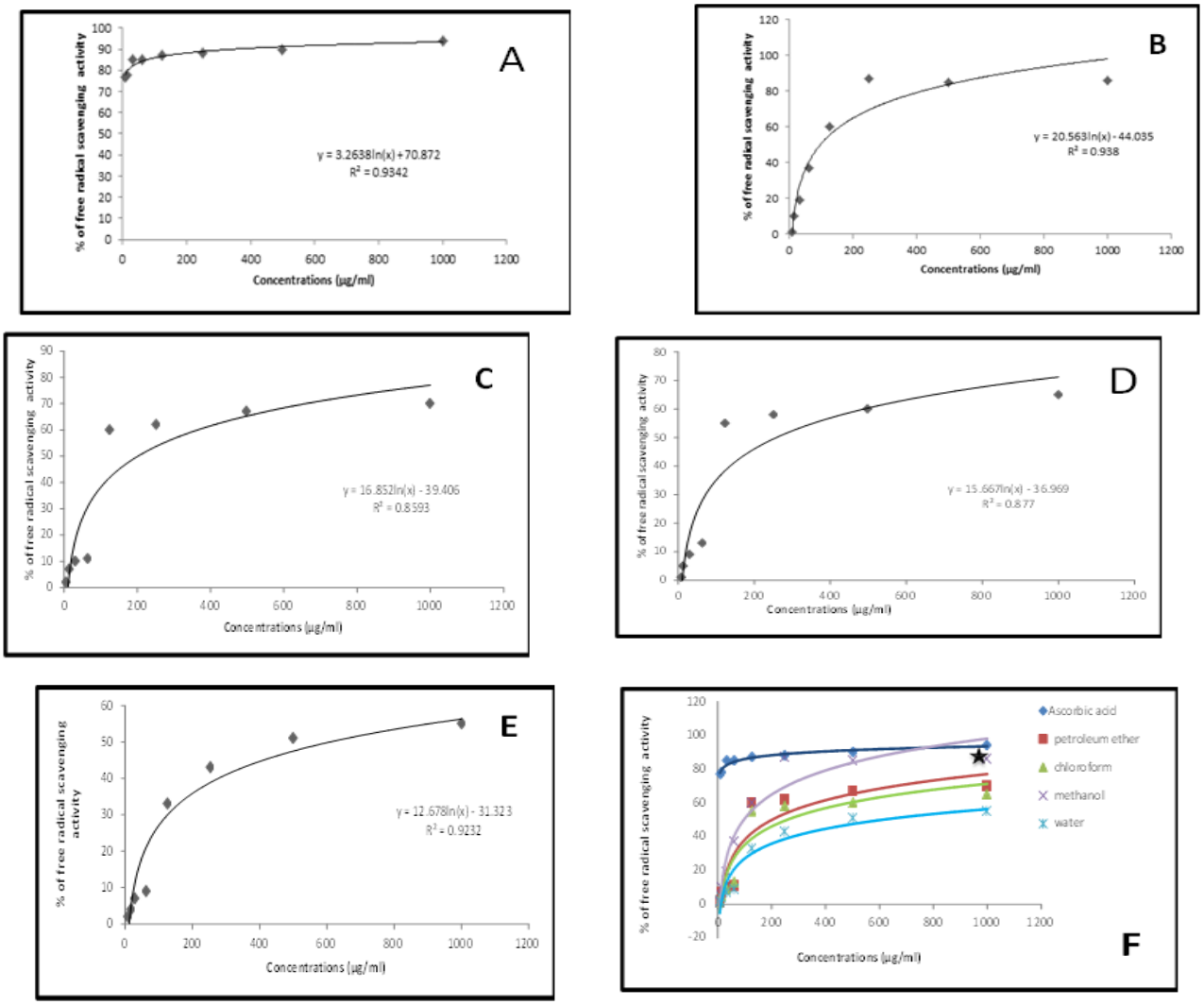

Figure 5: Dose response curve of serial dilutions of ascorbic acid (positive control) and *Anabasis articulata* methanol,petroleum ether,chloroform, *and* water extracts on DPPH free radical scavenging activity that represented respectively in A, B, C, D, E, F, and G.

- Non- significant when comparing with ascorbic acid (P >0.05)

DISCUSSION

Anabasis articulata extraction

Anabasis articulata is a one of the Saharan plants that is commonly called EshnanorAjrem.*Anabasis articulata* leaves, stems & roots used widely in folk medicine to treat diabetes, fever, skin diseases and kidney infections[13].Extraction process in this study was performed sequentially to confirm that the greater part of the active constituents have been extracted and isolated in accordance to their polarity to be complete for use in the experiments and to test their pharmacological activity either they have anti, pro or no angiogenic activities.Method of extraction used in this study was (maceration or cold method), this method was used so that avoid any loss or damage to the compounds inside the stems from exposure to high temperature [14]. Several factors affecting the yield of extraction; like shaking, maceration time, kinds and concentrations of the solvents that used, and temperature degree of the water used to warm the container that contain the powder of the stems of *AA*, all these factors clarify the differences in the percentage of yield of the extract. Additionally, literature reviews showed high concentrations of many chemical groups which may have a potent activity in angiogenesis process such as flavonoids, coumarins, saponin, glycine, alkaloids and others that exist in the stems of *Anabasis articulata*. These findings come in agreement with Hamdoon*et al* (2013) that proved the occurrence of the same constituents after phytochemical screening of *Anabasis articulata*.

Rat aorta anti-angiogenesis assay of *Anabasis articulata* stems extract

In excess of a quarter century after we first stated that explants of rat aorta have the ability to create vessels *ex vivo*, the aortic ring assay model has grown into one of the most commonly utilized tests of angiogenesis[15]. Several reasons have added to its popularity comprising reproducibility, cost effectiveness, simplicity of use and worthy correlation with *in vivo* studies. A significant revolution in the founding of this model was the innovation that endothelial sprouting from the aortic explants does not necessitate serum or external growth factors and is induced by endogenous components that activated by the injury of the dissection procedure. Quantification is attained by assessing the number and length of the micro vessel outgrowths beginning from the primary ex-plant. Because of the blood vessel growth reached their maximum on day five according to Blacher*et al* (2001) the activity of anti-angiogenesis of the crude extracts should counted after five days of cultured blood vessels, by quantifying the length of the blood vessel outgrowths. Aspirin was utilized in this assay, as a positive control as it is approved to have anti – angiogenesis influence that is interposed either through COX – dependent pathway, or through COX – independent pathway by hindering the NF-KB which is considered closely linked with inflammation and angiogenesis. In addition to that the vascular endothelial growth factor (VEGF)receptor fms-like tyrosine kinase 1 (FLT1) is a transmembrane protein that binds with high affinity to VEGF and placental growth factor (PlGF) and is required for angiogenesis, its expression was suppressed by aspirin[16].This study acknowledges that methanol extract (ME) of *Anabasis articulata* was found to have the peak percentage of anti-angiogenic activity in assessment to other extracts, while the other extracts, petroleum ether (PE), chloroform extract (CH) and water extract (W) of *Anabasis articulata*, showed inferior percentage of anti-angiogenic activity. However, the anti-angiogenic activity exhibited by PE, CH and WE remained significant, which was perhaps due to the presence of other active compounds having less concentrations or other compounds which may antagonized the active compounds. Because of its major anti-angiogenic influence, ME was nominated for further studies, to conclude their anti-angiogenic mechanisms and their chemical constituents those are essential in its pharmacological activity. Dose response curve was done for the ME against rat aorta anti-angiogenic assay; as stated by Wei and co-workers (2012). As the IC_{50} level decreases the safety decrease and vice versa. The outcomes of this study presented that the IC50 on blood vessels outgrowth was within the safe range 18.27µg/ml, these finding come in agreement Zeyad*et al* (2011) that revealed thedependent boarder concentration for herbs on angiogenesis process to be considered harmless was 20µg/ml of that extract. It was described by Pan *et al* (2011) that the coumarins compounds have numerous health benefits as well as effective anti – angiogenic properties. Also alkaloids in *anabasis articulata* may possess a strong angiogenic inhibitor effect with the ability to decrease the proliferation of vascular endothelial cells and to reduce expression of various pro-angiogenic factors. In addition to above phenolic contents can interfere with some angiogenesis-dependent pathologies[17]. Different types of polyphenols, alkalines&coumarins are existing in the *anabasis articulata* stems that could exhibit different polarities, thus the type of solvent and the temperature applied during the extraction highly affect the constituents in the extracts [18]. From the stated reasons above, it performs to be the inhibition of microvessels outgrowth made by ME in this screening assay may be attributed to the existence of different phenolic, coumarinic and alkalinic compounds with different polarities of extracts.

Free radical scavenging activity of *Anabasis articulata stems* extracts (DPPH Assay)

Free radical scavenging activity for the four extract was essential to be assessment to know the probable mechanism of action and the difference in the ability of blood vessels outgrowth inhibition within the four extracts[19]. The presence of alkaloids, phenols, glycine and coumarins in *Anabasis articulata* may explain the anti-angiogenesis mechanisms, as these groups may display their pharmacological effect through the antioxidant

possessions. These active compounds consider powerful in inhibiting angiogenesis progression[20]. Anti-oxidants are recognized for having strong anti-angiogenic activity, among those that have been recognized include, tocopherol, ascorbic acid, vitamin A, flavonoids, selenium, resveratrol, carotenoids, rosmarinic acid, 3-hydroxyflavone and 3', 4'-dihydroxyflavone and 2', 3'-dihydroxyflavone [21]. Free radicals are atoms or molecules with an unpaired electron and there are many evidences reveals that an excess of these free radicals give the idea to be linked with the development of many diseases like tumors, cardiovascular diseases, aging, inflammatory diseases and a variety of other disorders[22]. In this study we found that methanol extract of *Anabasis articulata* stems extract provided the most potent anti-oxidant activity when comparing to other extracts and it exhibited the highest percentage of anti-angiogenic activity, as revealed by the rat aortic ring assay. Its potency in stopping the new blood vessel growth could be added to its important antioxidant behavior, as revealed in the DPPH scavenging assay. This may end in a decrease in the free radicals existent, which are identified to trigger thehypoxia responsive element gene. Through the process of angiogenesis the concluding can acts as a trigger for VEGF, a basic cytokine in angiogenesis initiation [15].The existence of considerably high alkaloids, phenolic, glycine and coumarins contents in *Anabasis articulata* stems may perhaps play a vital role in contributing to its anti-angiogenic effect by inhibiting the activity of nitric oxide (NO) formation**.** Phenols are low molecular weight, secondary metabolites found in most land plants. Phenolic (Flavonoids) substance: 2-methoxy 4-vinylphenol protects plants against ultraviolet radiation, pathogens, and herbivores. Most of the protective effects of phenols in biological systems are recognized to their antioxidant activity, ability to transfer electrons, free radicals and chelating capabilities, activation of antioxidant enzymes, reduction of α-tocopherol radicals and inhibition of oxidase**.** This mechanism may be relatively accountable for the pharmacological effectiveness of several folkloric medicines. One of the potent angiogenic agents is TGFα; antioxidant agents can inhibit TGFα expression as one aspect of their ability to inhibit of angiogenesis [23]. In the search for worthy anti-angiogenic agents, powerful anti-oxidant property and good anti-inflammatory response are the two main selection standards necessary in the applicants extract. Antioxidants can considerably affect the angiogenesis progression and this can happen in a range of ways. In the tumor, antioxidants can affect the physiological redox balance that will mop up reactive oxygen species (ROS) that contribute to be prevalent in low oxygen tension locality [24].Hypoxia-inducible factor-1 (HIF-1) is one of the important mammalian transcription factors and displays augmented levels in both protein stability and intrinsic transcriptional activity in low oxygen tension. The action of HIF-1 is mainly determined via stability regulation of it is α subunit, which is stabilized under hypoxia but destroyed during normoxia. HIF-1 protein fixes to hypoxia-responsive elements (HRE) as the boosters of several genes including VEGF and recruits their mRNA expression[25]. The additional selection criterion is the capability of the candidate agents discusses anti-inflammatory response. The process of angiogenesis can also contribute to inflammatory response where the new blood vessels can transport inflammatory cells to the site of inflammation as well as nutrients and oxygen to the proliferating inflamed tissue. Angiogenesis shows a critical role in the pathogenesis of inflammation. The inflammation mechanistic pathways include proliferation, migration and recruitment of inflammatory cells[26].This phenomenon happens in diseases such skin psoriatic illness, rheumatoid arthritis and tumor where the proliferating tissue comprises a richness of inflammatory cells, angiogenic blood vessels and derivative of inflammatory mediators. Additionally, there are hypoxic areas where tissue proliferation has outperformed blood vessel growth, which stimulates further capillary expansion. This process can be extra enlarged by macrophages which can stimulate the release of enormous quantities of angiogenic factors. Directly or indirectly, inflammatory mediators themselves can also encourage angiogenesis. A widespread choice of inflammatory mediators have revealed to have angiogenic activities this includes tumor necrosis factor α (TNF α), prostaglandins E1 and E2 (PGE1, PGE2), interleukins 1, 6 and 8 (IL-1, IL-6, IL-8), and nitric oxide[27]. Usually, these agents can induce sides of inflammation, and their angiogenic action could be linked to the recruitment of inflammatory cells or elevated vascular permeability. New outcomes also propose that PGE2, IL-6 and IL-1 have been displayed to encourage VEGF expression by rising VEGF messenger ribonucleic acid (mRNA) levels. Therefore, through growth of inflammatory tissue, the hypoxic environments, joined with high concentrations of inflammatory mediators, can cause a rise of angiogenesis activity through VEGF formation[28].

CONCLUSION

Totally extracts of *Anabasis articulata* stems displayed important antiangiogenesis activity. Yet methanol extracts verified the best anti–angiogenesis activity in addition to significant dose–dependent antiangiogenic effect. Additionally methanol extracts revealed an important free radical scavenging activity by DPPH assay and in concentration dependent manner.

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