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RESEARCH ARTICLE

Investigation of the Possible Anti-angiogenic Activity of Iraqi Scabiosa palaestina L. Using Ex Vivo Rat Aorta Ring Assay

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ABSTRACT

Background and aim: Angiogenesis is defined as the creation of new blood vessels that have been generated from pre- existing vessels by the stimulation of endothelial cells. Herbal extracts that have antiangiogenic activity and low toxicity can increase the focus on using natural sources an important therapeutic agent for treatment of different diseases including tumors. The present research was designed to evaluate the possible antiangiogenic activity of Iraqi Scabiosa palaestina L.

Methods: Plants materials were extracted by 85% methanol using soxhlet apparatus for 12 hours. Aortic rings of rats were seeded in the growth medium and were loaded with a concentration of 100 µg/mL of each fraction (petroleum ether, chloroform, ethyl acetate, and n-butanol) from the aerial parts and roots of the plant. Moreover, six concentrations of the most active fraction (200, 100, 50, 25, 12.5 and 6.25 µg/mL) were tested to assess the dose-response relationship.

Results: The results showed that the petroleum ether, chloroform and the ethyl acetate fractions from the roots in addition to the ethyl acetate fraction from the aerial parts significantly inhibited the growth of blood vessels on the fifth day of the experiment; there was a significant difference in blood vessels growth inhibition from the negative control ($p<0.05$).

Conclusion: The anti- angiogenic activity showed by ethyl acetate fraction may be attributed to the presence of antioxidant compounds. The presence of phenolic compounds like flavonoids correlated with different health benefits in addition to their antiangiogenic properties.

KEYWORDS:
angiogenesis; VEGF; natural products; Scabiosa palaestina L.

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INTRODUCTION

Scabiosa palaestina L. (F. Caprifoliaceae) is one of species included in the genus Scabiosa which is considered a large and taxonomically complex genus with many species distributed in different areas like Asia, Mediterranean Basin and southern Africa. Scabiosa palaestina L. is annual plant with leaf rosettes and leafy stems. It is shrubs with 60 cm high and their flowers have crowded small heads with white to purple colour.

Plant materials have been used as important therapeutic sources against numerous diseases like cancer for a long time [1] and the identification of herbal extracts with antiangiogenic activity and low toxicity can increase the focus on natural products with similar activity to discover the most important compounds responsible for this effect which may be subjected for further investigation and analysis to develop new treatments for different diseases coupled with excessive angiogenesis.

.Typically, angiogenesis starts within hypoxic tissues where high blood is needed to maintain supply of oxygen and nutrition [2]. In hypoxic tissues, cellular oxygen sensing mechanisms are stimulated and consequently activate gene expression of different pro-angiogenic mediators. The most important factors that are activated are HIFs (hypoxia inducible factors) which act by up-regulation of multiple pro-angiogenic genes by

direct or indirect pathways [3]. These genes include VEGF-A (vascular endothelial growth factor- A) which is the major protein responsible for the proliferation and migration of cells during this process. The process is followed by vasculogenesis and involves the differentiation and organization of endothelial cells into capillary tubes and the interplay between growth factors and cytokines [4].

Over seventy disorders are highly related to angiogenesis. In disease states such as ocular inflammatory disorders and cancer, excessive angiogenic stimuli are produced with an imbalance between the inducing and inhibiting agents, resulting in angiogenesis switch [5]. Angiogenesis mediators were described four decades ago and include growth factors, cytokines, chemokines, adhesion molecules, proteinases, and extracellular matrix (ECM) components. Prominent among these are VEGF, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), angiogenin, angiopoietins (Ang-1, Ang-2), metalloproteinases, adhesion molecules (CD146), transforming growth factor-b (TGF-b), integrins, and chemokines [6]. Large attention is directed toward antiangiogenic therapies, and they are widely administered in numerous types of cancers. This type of treatment may induce different signaling pathways in cytotoxic drugs and lead to worsen the outcomes in terms of resistance, invasion, and metastasis. Cancer cells need to have access to blood vessels for growth, development and metastasis. The discovery of angiogenic inhibitors expand the hope for reducing the mortality and morbidity from carcinomas [7].

MATERIALS AND METHODS

Ethical considerations

This research was carried out with the approval of the Ethics Committee of Al-Nahrain university college of pharmacy, Khadimia city, Baghdad, Iraq, with the code of ethics Coph.AEC3.

Chemicals

Methanol 98% (BDH), dimethyl sufoxide (DMSO) Romil, UK, foetal bovine serum (Sigma-Aldrich, USA), aprotinin (Sigma-Aldrich, USA), fibrinogen (Sigma-Aldrich, USA), thrombin 100 IU vial (Sigma-Aldrich, USA), aminocaproic acid (Sigma-Aldrich, USA), L-glutamine (Sigma-Aldrich, USA), gentamicin 80 mg/2 mL vial (Al-Hikma, Jordan), amphotericin B vial (Bristol Myers Squibb, England), Earls salt M199 solution (Sigma-Aldrich, USA).

Plant material

The whole plant of Scabiosa palaestina L., which grows as a wild plant in Iraq, was collected during the March-April 2020 from Kalobazian, north of Iraq. The plant was authenticated at the Herbarium of the Department of Biology, College of Science, University of Baghdad, Iraq (No. 1197). The plant parts were left to dry in shade, ground with an electric blender, weighted and subsequently subjected to extraction procedures.

Extraction and fractionation

Aerial parts and roots (250 g of each) were subjected to extraction using a Soxhlet apparatus in which each part of plant was extracted separately with 600 ml of 85% methanol for 12 hours, then the crude methanol extract of each part was filtered, concentrated under reduced pressure and suspended in distilled water then partitioned successively using Liquid-Liquid partitioning (fractionation) technique by the separatory funnel with petroleum ether, chloroform, ethyl acetate and n-butanol (500 mL for each fraction). This process was repeated three times. Later on, the first three fractions for each plant part were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure using rotary evaporator, weighted and assigned for further analysis.

Preparation of rat aorta rings from albino rats

The Preparation of rat aorta rings was done according to Brown and his colleagues protocol [8].

Animals

Ten albino male rats of 12 to 14 weeks-old (weighed 150-200 g) were obtained from the animal house of the college of pharmacy, Baghdad University. These animals were sacrificed humanely by cervical dislocation using diethyl ether as anesthetic. After excision of thoracic aorta, it was rinsed with serum free media and cleaned from the fibro-adipose tissue to prepare it for cross sectioning into 1 mm thickness rings.

Seeding the rat aorta ring in the culture media

Fibrinogen and aprotinin (3 mg/mL and 5 µg/mL, respectively) were added to the M199 medium to prepare the lower layer; 300 µL of M199 medium was dropped in each well of 48-well plate, and one aortic ring was placed in each well. Then, 10 µL thrombin (prepared at 50 NIH U/mL in 0.15 M NaCl) was added. The plates were incubated in a CO2 incubator for solidification at 37 °C in 5% CO2 for 50 min. The preparation of top layer medium was conducted by addition of 20% of heat-inactivated fetal bovine serum, 1% L-glutamine, 0.1% aminocaproic acid, 0.6% gentamicin and 1% amphotericin B to M199 medium.

Stock solutions (10 mg/mL) of aerial parts and roots fractions (petroleum ether, chloroform, ethyl acetate and n-butanol) were prepared and adjusted by solubilizing each sample in dimethyl sulfoxide (DMSO) then diluting in M199 medium to create the final concentration of 1%. One hundred µg/mL of plant extracts were added to the top layer of the medium, and each addition was performed six times. Using a humidified incubator, the seeded rings were incubated at 37 °C, 5% CO2. At the fourth day, the upper layer medium was changed with freshly prepared medium as mentioned previously. Negative and positive controls were DMSO (1% v/v) and acetylsalicylic acid "aspirin" (100 µg/mL), respectively. Aspirin was used as the appositive control due to its antiangiogenic activity that is mediated through cyclooxygenase (COX) dependent pathway

and COX independent pathway by blocking the NF-κB which is closely related to inflammation and angiogenesis [9]. On day five, the consequences were examined using microscope. The scope of blood vessel growth was quantified using 10X magnification by the camera and software. The degree of inhibition of blood vessels was measured according to the developed technique by Nicosia and colleagues [10]. The results are presented as mean percent inhibition compared to the negative control. The procedure was repeated three times through six replicates per sample. The percentage of inhibition was calculated according to the formula [11]:

Blood vessel growth inhibition (%) = [(Distance of growth in the control (μm) – Distance of growth in the sample (μm)) / Distance of growth in the control in μm] × 100

Dose-response study on the most active fraction seeds with rat aorta ring assay

After detection of the most active fraction of Scabiosa palaestina, a stock solution was prepared in DMSO and serial dilutions of 200, 100, 50, 25, 12.5 and 6.25 μg/mL were prepared in M199 medium (1% DMSO in media) [12]. The wells treated as negative controls received medium with 1% DMSO.

STATISTICAL ANALYSIS

The results were reported as mean±SD. The statistical analysis was carried out using of one-way ANOVA. The IC50 (the concentration that inhibits 50% of blood vessels growth, cell proliferation and tube formation) of the most active fraction was measured through by logarithmic equation that has been resulted from the draw of fraction concentration in μg/ml vesus the percentage of inhibition, Where Y= the percentage of inhibition, and X= concentration [13].

RESULTS AND DISCUSSION

Aortic rings seeded in the growth medium were overloaded with the concentration of 100 μg/mL of all aerial parts and roots fractions (Table 1).

Table 1. The percentage inhibition of blood vessel growth by fractions

Samples	**% Of Inhibition (mean ± SD)**
Aerial parts	
Petroleum ether fraction	6.4 + 0.64
Chloroform fraction	23
Ethyl acetate fraction	96.15 + 0.45 **
n-Butanol fraction	12.8 + 0.97
Roots	
Petroleum ether fraction	62 + 1.16
Chloroform fraction	69 + 1.35
Ethyl acetate fraction	85 + 1.57 *
n-Butanol fraction	28.2 + 0.83
Control	
Negative control (DMSO 1%)	0
Positive control (aspirin)	95.8 + 1.39

The screening showed that petroleum ether, chloroform and ethyl acetate fractions from the roots and the ethyl acetate fraction of the aerial parts significantly inhibited blood vessels growth at day five of the experiment as compared to the negative control ($p<0.05$). Among these four fractions, the ethyl acetate fraction of the aerial parts showed the highest antiangiogenic activity (in term of blood vessels growth inhibition) by 96.15 % followed by ethyl acetate fraction of the roots which showed 85% inhibition. Also, there was a comparable effect for both ethyl acetate fraction of the aerial parts and the positive control (acetylsalicylic acid), as shown in Figures 1-3. Ethyl acetate fraction of the aerial parts was significantly differing from the negative control and showed no difference from that of aspirin.

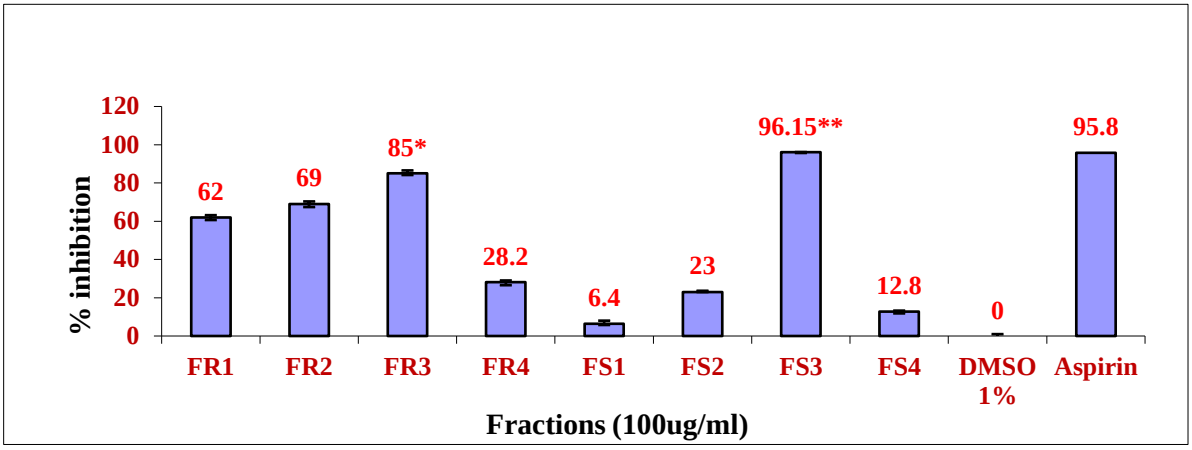

Fig.1: Anti-angiogenesis activity of Scabiosaa palaestina fractions (100 µg/mL); FR1: root petroleum ether fraction; FR2: root chloroform fraction; FR3: root ethyl acetate fraction; FR4: root n-butanol fraction; FS1: aerial parts petroleum ether fraction; FS2: aerial parts chloroform fraction; FS3: aerial parts ethyl acetate fraction; FS4: aerial parts n-butanol fraction; * significant; ** highly significant

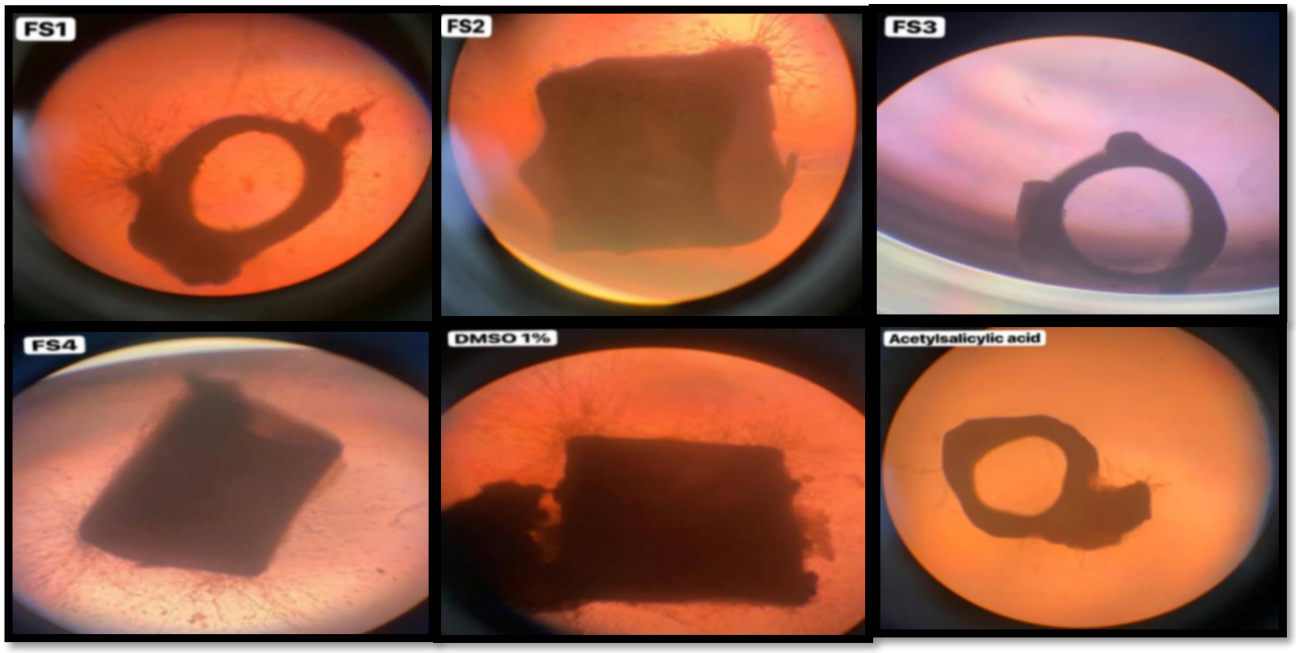

Fig.2: Anti-angiogenesis effect of 100 µg/mL of Scabiosaa palaestina aerial parts fractions; FS1: aerial parts petroleum ether fraction; FS2: aerial parts chloroform fraction; FS3: aerial parts ethyl acetate fraction; FS4: aerial parts n-butanol fraction. DMSO1% (negative control) and acetylsalicylic acid (positive control) in ex vivo aortic ring model.

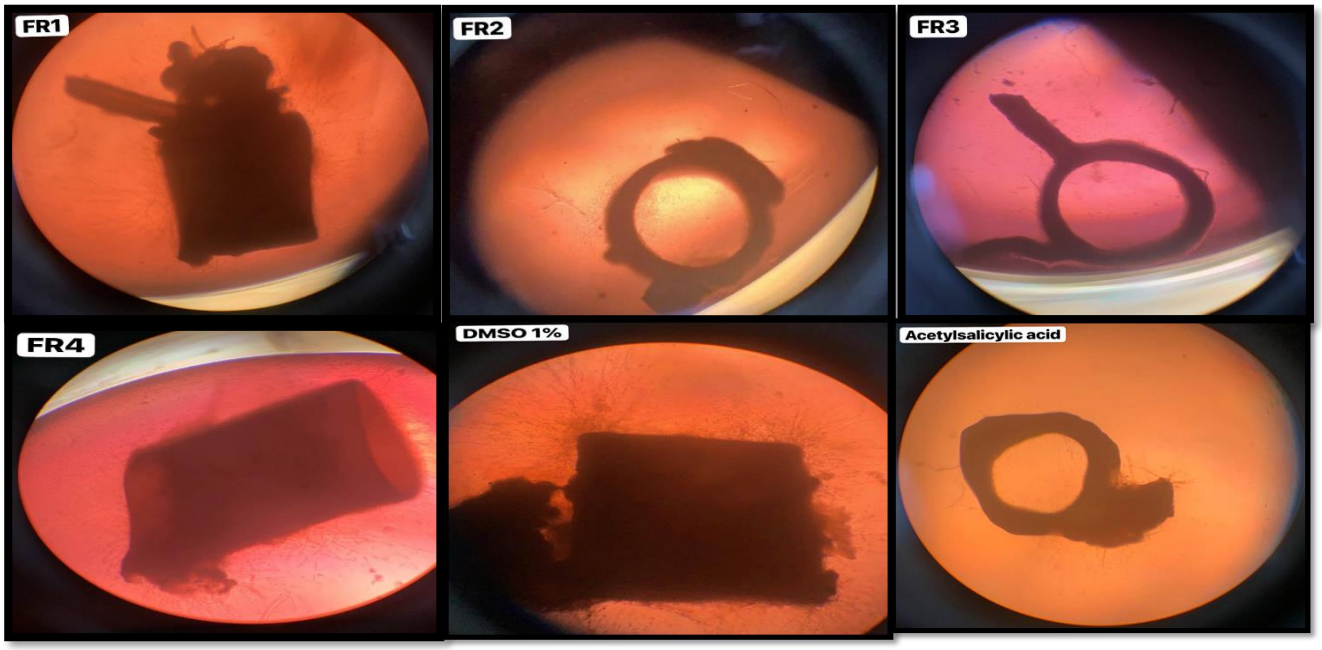

Fig.3: Anti-angiogenesis effect of 100 µg/ml of Scabiosaa palaestina roots fractions; FR1: root petroleum ether fraction; FR2: root chloroform fraction; FR3: root ethyl acetate fraction; FR4: root n-butanol fraction; DMSO1% (negative control) and acetylsalicylic acid (positive control) in ex vivo aortic ring model

Literature survey indicate the presence of different flavonoids in Scabiosa species like quercetin, kaempferol, apigenin, astragalin, luteolin and others. These compounds have different health benefits in addition to their anti- angiogenic properties. Preliminary phytochemical investigation of the extracted fractions confirm that ethyl acetate fractions contain much higher concentration of poly phenolic compound and flavonoids than the other fractions of both plant parts.

Several researches document the antiangiogenic activity of flavonoids by inhibition the proliferation and migration of endothelial cells by interfering with signaling cascades of angiogenesis like phosphoinositide 3-kinase (PI3K) pathways and the mitogen activated protein kinase (MAPK). They can inhibit the expression of proangiogenic factors such as VEGF and matrix metalloproteinases (MMPs) [14].

Different types of polyphenols present exhibit different polarities, thus the type of solvent and the temperature applied during the extraction highly affect polyphenols in the extracts [15,16]. The inhibition of micro vessels outgrowth produced by FS3 and FR3 in this screening assay may be attributed to the presence of different phenolic compounds with different polarities in both extracts. Petroleum ether and chloroform fractions of the roots also appears to contain other types of phytochemicals like unsaturated fatty acids, and some phytosterols, which also may be responsible for the anti-angiogenic activity [17]. Dose response assessment for ethyl acetate fraction (FS3), the most active fraction, was performed to detect the concentration that inhibited blood vessels growth by 50%.

Dose response curve was determined by loading six dilutions of ethyl acetate fraction of the aerial parts (the most active fraction) to the embedded rat aortic rings. FS3 fraction gave a significant dose dependent suppression of blood vessels growth compared to the results given by the negative control ($p<0.001$) at day five of the (Table 2 and Figure 4, respectively).

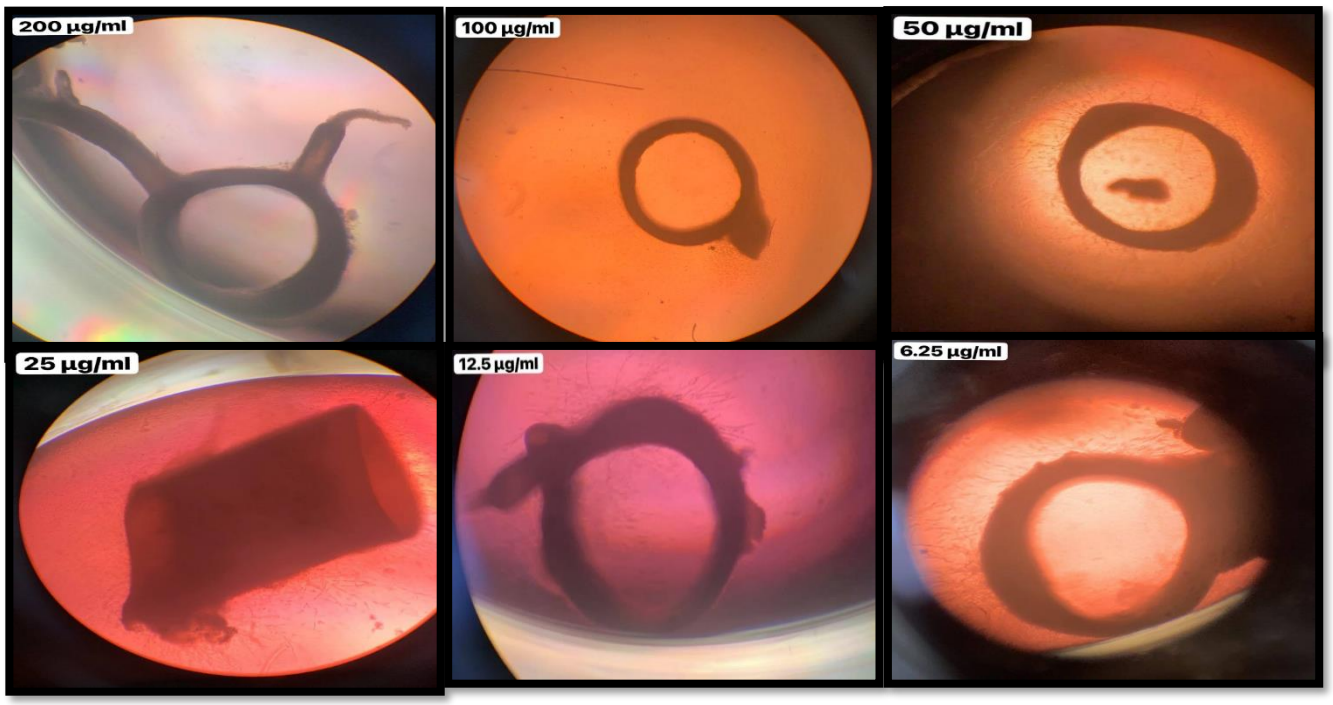

Fig.4: The dose -response effect to the serial concentrations of ethyl acetate fraction (FS3) of Scabiosa palaestina aerial parts in rat aortic rings model

The IC50 was detected from the logarithmic equation (y=27.617ln(x) - 38.468), that is shown in figure 5 and it was found to be 24.61 µg/mL. Where Y= the inhibition percentage and X= the concentration (Figure5).

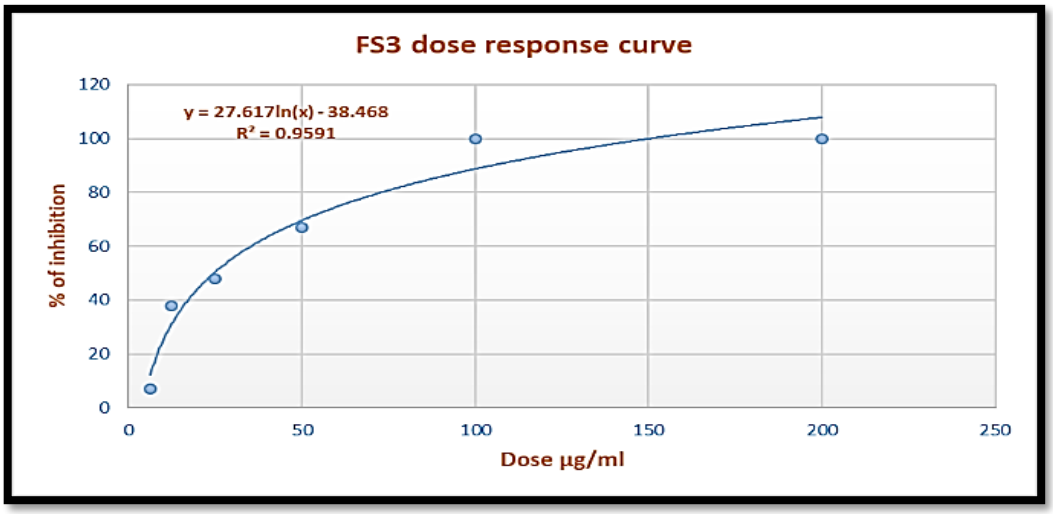

Fig.5: Dose-response curve of ethyl acetate fraction (FS3) of Scabiosa palaestina aerial parts in rat aortic rings model

CONCLUSION

Imbalance of angiogenesis and oxidative stress result in disorders including cancer, diabetes exacerbations especially diabetic retinopathy, and ischemic heart diseases. Therefore, chemicals that neutralize oxidative stress or have anti-angiogenic activity can be suggested as modulatory therapy to diminish the risk of these diseases. Scabiosa palaestina is characterized by the presence of several constitutions including glycosides, flavonoids, terpenoids, and steroids. The ethyl acetate fraction of the aerial parts demonstrated significant anti-angiogenic activity in the rat aortic rings model. The presence of phenolic compounds in ethyl acetate fraction is much higher than other fractions in both plant parts. These compounds have different health benefits in addition to their anti-angiogenic properties by interfering with the major keys of signaling cascades that inhibit endothelial cells proliferation, migration and tube formation like PI3K, MAPK, VEGF, MMPs.

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AUTHOR CONTRIBUTIONS

The present study was designed by Amjed haseeb khamees and Hayder Bahaa Sahib.The plant were collected by Enas J. khadim from Kirkuk province. All authors performed experiments and handled the research data. Amjed haseeb khamees contributed to data collection, writing and editing the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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