



## Anti-angiogenic activity of *Zizyphus spinachristi* Leaves Extracts

Ahmed R Abu-Raghif<sup>1</sup>, Hayder B Sahib<sup>2</sup>, Muneer M Hanoon<sup>1</sup>

<sup>1</sup>College of Medicine, AL- Nahrain University, Pharmacology Department, Iraq.

<sup>2</sup>College of Pharmacy, AL- Nahrain University, Pharmacology Department, Iraq

\*Corresponding author's E-mail: [haider\\_bahaa@yahoo.com](mailto:haider_bahaa@yahoo.com)

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### ABSTRACT

The objective of the experiment is to investigate the possible anti-angiogenic activity of *Zizyphus spinachristi* leaves extracts. The leaves were dried and grounded into fine powder, and extracted successively with Petroleum ether, chloroform, methanol and water. *Ex vivo* rat aorta anti-angiogenesis assay was used to identify the most active anti-angiogenic extract. The most active extract has been chosen for dose response study. Free radical scavenging activity has been tested by 1,1 Diphenyl-2-picrylhydrazyl (DPPH) to detect which extract has the highest free radical scavenging activity to help determine the possible mechanism of action. Methanol extract was the most biologically active extract in terms of blood vessels growth inhibition in comparison with petroleum ether, chloroform and water extracts ( $P < 0.05$ ). Methanol extract serial concentrations showed significant dose dependent inhibition activity ( $P < 0.05$ ) on rat aorta assay and  $IC_{50}$  was (29.08  $\mu$ g/ml). Methanol extract had the highest free radical scavenging activity in comparison with petroleum ether, chloroform and water extracts. The  $IC_{50}$  of DPPH scavenging activity for methanol extract was 33.91  $\mu$ g/ml. The methanol extract of *Zizyphus spinachristi* leaves has potential anti-angiogenic activity and this activity may be due to the high free radical scavenging capacity.

**Keywords:** Anti-angiogenesis, *Zizyphus spinachristi*, free radical scavenging activity.

### INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing one. It is a complex process that involves the proliferation and migration of endothelial cells. It is delicately controlled by a variety of inducers and inhibitors that respectively cause the up- and down-regulation of angiogenesis<sup>1,2</sup>. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the major pro-angiogenic factors both *in vivo* and *in vitro*<sup>3,4</sup>. Many types of anti-angiogenic factors have already been discovered including angiostatin, which decreases bFGF- and VEGF-mediated activation of mitogen-activated protein kinases (MAPK) in endothelial cells leading to inhibition of proliferation and induction of apoptosis<sup>5</sup>. Angiogenesis is a key process in some physiological conditions such as growth, wound healing and action of female reproductive organs. However, disturbances in the mechanisms of physiological angiogenesis play a role in pathogenesis of some diseases in the form of excessive angiogenesis such as cancers, arthritis, psoriasis, retinopathies, asthma, obesity, and atherosclerosis, or impaired angiogenesis in diseases such as heart and brain ischemia, hypertension, neurodegeneration, respiratory distress, osteoporosis, preeclampsia, endometriosis, ovarian hyper-stimulation syndrome and postpartum cardiomyopathy<sup>6</sup>. Agents that inhibit angiogenesis usually do so by interfering with the critical steps of angiogenesis. The major strategies for modulation of angiogenesis include<sup>7</sup>:

1. Intervention with endothelial cell growth.
2. Intervention with endothelial cell Adhesion to and

migration through extracellular matrix.

3. Intervention with Metalloproteinases by using specific inhibitors of proteinases that dissolve the connective tissue, thereby facilitating endothelial cell migration and subsequent vessel formation. *Zizyphus spina-christi*, commonly known as Christ's thorn in English and Sidr or Nabqa in Arabic is a tree belonging to the genus *zizyphs* in *Rhamnaceae* family<sup>8</sup>. The plant has been reported to possess antioxidant, antibacterial, antifungal, antidiabetic and analgesic effects<sup>9</sup>.

### MATERIALS AND METHODS

#### Extraction

The leaves of *Zizyphus spinachristi* were collected from various regions of An nasiriyah city, washed thoroughly under running tap water and oven dried at 40°C. The dried leaves were then grounded in to fine powder using an electric grinder. The dried powdered leaves (500 gm) were extracted by employing successive extraction method using different organic solvent in increasing polarity order. In each of five beakers, 100 gm of the powder was soaked into 600 ml of petroleum ether, left in a shaking water bath at 40°C for eight hours, then filtered through whatman No. 1 filter paper. The residue was further extracted three times with petroleum ether. All the filtrate was pooled together in airtight dark bottle to be concentrated to dryness under reduced pressure and low temperature using rotary evaporator. The dried extract was stored in a refrigerator until used. The resulting residue was air dried and further extracted with chloroform followed by methanol and water similar to



the procedure carried out for the Petroleum ether extraction<sup>10</sup>.

### Experimental animals

Twelve to fourteen weeks old male Sprague dawley rats were used in the experiments. The animals were obtained from the animal house of the institute of embryo researches and infertility treatment, Al-Nahrain University and kept at 25-30°C with water and food *ad libitum*.

### Rat aorta ring anti-angiogenesis assay

This assay was performed according to the standard protocol of Brown with a simple modification<sup>11</sup>. The animals were sacrificed via cervical dislocation under anesthesia with chloroform. Thoracic aorta were rapidly excised, rinsed with serum free media, cleaned as much as possible from peri-adventitial fibro adipose tissue and residual blood clots. These were then cross sectioned into thin rings of 1 mm thickness. The assay was carried out in a 48 well tissue culture plate. The lower layer, consisting of 500 µl Serum free M199 growth medium supplemented with fibrinogen (3mg/ml) and aprotinin (5µg/ml) was added and one aortic ring was seeded in each well. After that, 15 µl of thrombin prepared at 50 NIH U/ml in 0.15 M NaCl was added to each well and the mixture was allowed to solidify at 37°C in 5% CO<sub>2</sub> for 60–90 min. A stock solution of sample in dimethyl sulfoxide (DMSO) (10 mg/ml) was prepared and added to the top layer to obtain a final concentration of 100 µg/ml with a final DMSO concentration of 1% (v/v). After that, the top layer; consisting of 500 µl of M199 supplemented with 20% 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 was added to each well. Dimethyl sulfoxide (1% v/v) and acetyl salicylic acid (ASA) (100 µg/ml) were used as negative and positive controls respectively. The tissue rings were incubated in a humidified incubator at 37°C, 5% CO<sub>2</sub> for five days. On day four of the experiment, the top layer medium was replaced with fresh medium prepared as mentioned above. The experiment was repeated three times. In each experiment, six replicates were performed. The extent of blood vessels growth was quantified under 40X magnification using an inverted microscope (Olympus, Japan) on day five of the procedure with the aid of a camera (Lieca CCD, Japan) and (LiecaQWin) software packages. The magnitude of blood vessels growth inhibition was determined according to the technique developed by Nicosia and coworkers which include measuring the length of the minute blood vessels outgrowths from the primary explants<sup>12</sup>. The percentage of blood vessels growth inhibition was determined using the following formula:

$$\text{Blood vessel growth inhibition\%} = [(L_0 - L) / L_0] \times 100\%$$

Where:

L: Distance of blood vessels growth in µm

L<sub>0</sub>: Distance of blood vessels growth in the control in µm

### Dose response study on the methanol extract of *Zizyphus spinachristi* leaves with rat aorta ring assay

A stock solution of the methanol extract of *Zizyphus spinachristi* in DMSO was prepared. From this stock solution serial dilutions were prepared by dissolving the appropriate volume in the M199 media (1% DMSO in media) and diluting it serially to obtain final concentrations of 200, 100, 50, 25, 12.5 and 6.25µg/ml. Wells with no samples treatment were received medium with 1% DMSO and used as negative controls. The data were represented as mean ± SD. The IC<sub>50</sub>, which is the concentration that inhibit the blood vessels growth by 50%, was calculated by using the logarithmic regression equation<sup>13</sup>.

### 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity assay

The antioxidant activities of *Zizyphus spinachristi* leaves extracts were assessed by measuring free radical scavenging activity using DPPH method. Serial dilutions of each of petroleum ether, chloroform, methanol and water extracts in methanol were prepared with the following concentrations: 7.81, 15.62, 31.25, 62.5, 125, 250 and 500 µg/ml. One milliliter of 0.1 Mm solution of DPPH in methanol was added to 2 ml of each dilution of the extracts. Ascorbic acid a well-known antioxidant; was used as positive control. The DPPH solution (0.1 Mm) in the absence of leaves extract was used as control and methanol was used as blank. All the tests were performed in triplicate. After half hour of incubation in dark, absorbance was measured at 490 nm. The percentage of DPPH scavenging activity (P) was calculated using the following formula:

$$P = [(A_c - A) / A_c] \times 100.$$

Where:

A<sub>c</sub>: Absorbance of the control

A: Absorbance of the sample.

The IC<sub>50</sub>, which is the concentration required to scavenge 50% of DPPH free radical was calculated using dose inhibition curve by plotting the sample concentration versus the corresponding DPPH scavenging activity<sup>14</sup>.

## RESULTS

### Anti-angiogenic activity of *Zizyphus spinachristi* leaves extracts

Aortic rings embedded in complete growth medium were received a concentration of 100 µg/ml of each of the four extracts (Petroleum ether, chloroform, methanol and water). The blood vessels growth inhibition was presented as mean ± SD (table 1). The screening showed that all the extracts significantly inhibited blood vessels growth at day five of the experiment, there was a significant difference in blood vessels growth inhibition among each of the four extracts of *Zizyphus spinachristi*

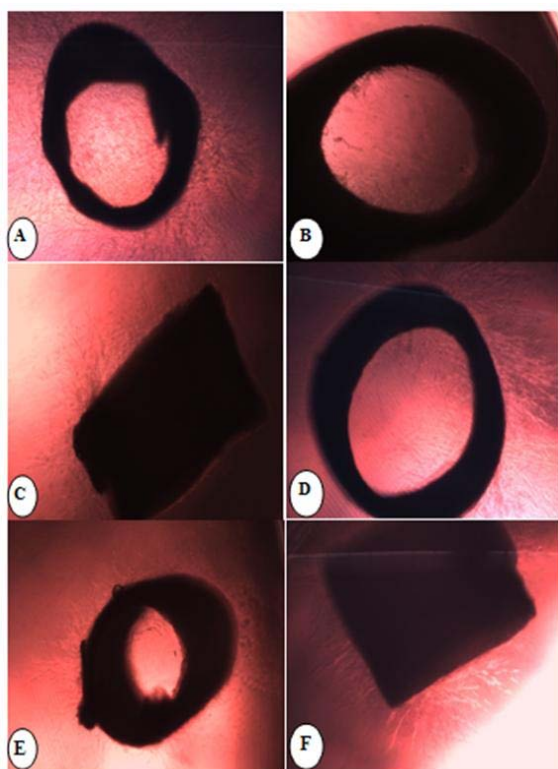


and the negative control (DMSO) ( $P < 0.05$ ). Among the four extracts, the methanol extract showed the highest anti-angiogenic activity (in term of blood vessels growth inhibition) in comparison with the other extracts. Also, there was a significant difference between the methanol extract and the positive control (acetylsalicylic acid) and there were a significant differences among each of petroleum ether, chloroform, methanol and water extracts ( $P < 0.05$ ).

**Table 1:** Blood vessels growth inhibition induced by the tested samples.

Tested samples	Mean Inhibition (%)±SD
Acetyl salicylic acid	84.05±2.36
Petroleum ether extract	58.39±0.58
Chloroform extract	23.79±4.26
Methanol extract	75.95±3.46
Water extract	60.45±0.64

Figure (1) show the effects of the four extract of *Zizyphus spinachristi* leaves on blood vessels growth in rat aorta rings.



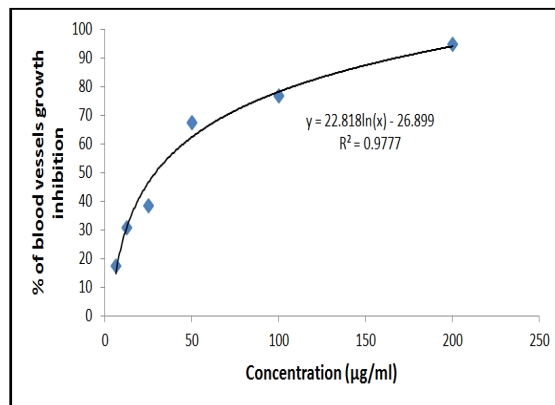
**Figure 1:** Images showing the effects of *Zizyphus spinachristi* leaves extracts on blood vessels growth in rat aorta rings, where A, B, C, D, E and F represent the activity of Dimethyl sulfoxide (negative control), Acetylsalicylic acid (positive control), petroleum ether, chloroform, methanol and water extracts, respectively.

**Dose response curve of *Zizyphus spinachristi* leaves methanol extract on rat aorta rings**

The serial dilutions of the methanol extract of *Zizyphus spinachristi* were added to the rat aorta rings. Six

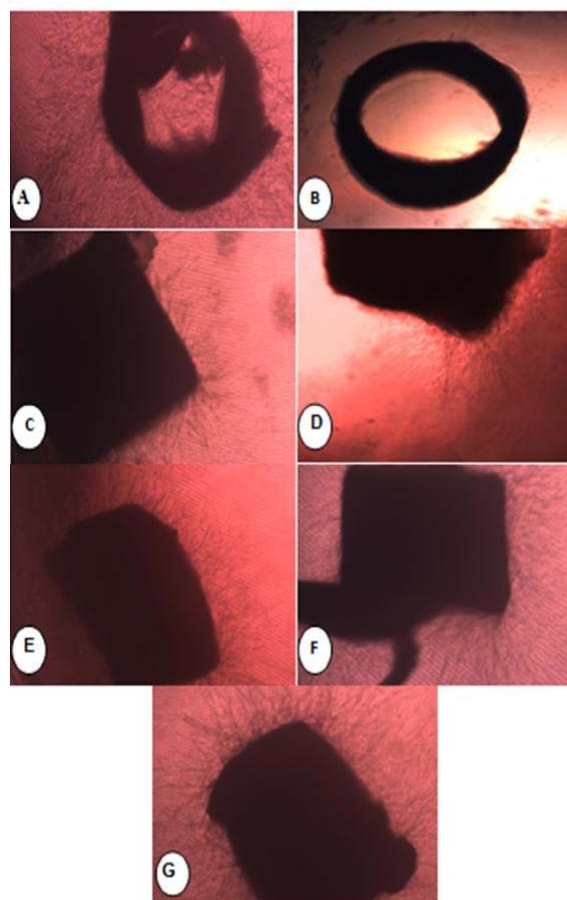
concentrations were used (6.25, 12.5, 25, 50, 100, 200 µg/ml). At these concentrations, the methanol extract showed significant dose dependent inhibitory activity ( $P < 0.05$ ). The  $IC_{50}$  value was calculated from the logarithmic regression equation:

$y = 22.818 \ln(x) - 26.899$ , where:  $y$  = the percentage of inhibition,  $x$  = concentration, and was equal to 29.08 µg/ml (Figure 2).



**Figure 2:** Dose response curve of *Zizyphus spinachristi* leaves methanol extract on rat aorta rings.

Figure (3) shows the effects of serial concentrations of the methanol extract of *Zizyphus spinachristi* leaves on blood vessels growth in rat aorta rings.



**Figure 3:** Images showing the effects of serial concentrations of *Zizyphus spinachristi* leaves methanol



extract. A, B, C, D, E, F, G represented the activity of the negative control and serial concentrations (200, 100, 50, 25, 12.5 and 6.25 µg/ml) respectively.

### 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of *Zizyphus spinachristi* leaves extracts

The results showed that DPPH scavenging activities of ascorbic acid and the four extracts of *Zizyphus spinachristi* leaves were dose dependent (Figure 4). The IC<sub>50</sub> of DPPH scavenging activities of the tested samples were calculated by the logarithmic regression equations as follows:

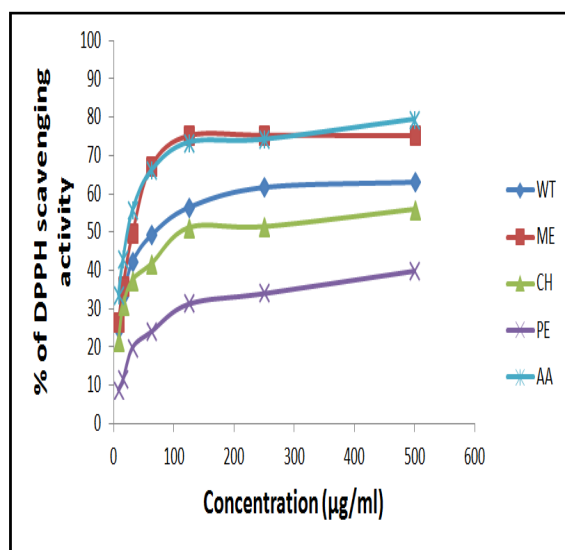
For ascorbic acid:  $y = 11.221\ln(x) + 14.508$ , the IC<sub>50</sub> was 23.64 µg/ml.

For petroleum ether extract:  $y = 7.7477\ln(x) - 7.8838$ , the IC<sub>50</sub> was 1756.52 µg/ml.

For chloroform extract:  $y = 8.1296\ln(x) + 7.8148$ , the IC<sub>50</sub> was 179.3 µg/ml.

For methanol extract:  $y = 12.913\ln(x) + 4.4977$ , the IC<sub>50</sub> was 33.91 µg/ml.

For water extract:  $y = 9.585\ln(x) + 7.7324$ , the IC<sub>50</sub> was 82.25 µg/ml.



**Figure 4:** DPPH radical scavenging activity of ascorbic acid (positive control) and petroleum ether, chloroform, methanol and water extracts of *Zizyphus spinachristi* leaves.

## DISCUSSION

### *Zizyphus spinachristi* leaves extraction

The extraction process used in this study was cold maceration method, this method is suitable for extraction of thermo-labile compounds since high temperature may cause destruction of these compounds<sup>15</sup>. The leaves of *Zizyphus spinachristi* were extracted successively with increasing polarity order of solvents, from petroleum ether (non polar) to water (highly polar); to ensure that a wide range of bioactive compounds have been extracted and separated according to their polarity<sup>16</sup>.

### Rat aorta anti-angiogenesis assay

Angiogenesis assays are important for discovery of potential angiogenic agents and seeking for pharmacologic inhibitors<sup>17</sup>. Rat aorta ring assay is one of the most widely used assays to study angiogenesis and its mechanisms owing to its reproducibility, cost effectiveness, easiness and good correlation with *in vivo* assays<sup>18</sup>. In this study the main objective was to identify the anti-angiogenic activity of the four extracts of *Zizyphus spinachristi* leaves and which one has the highest activity. All the extracts were screened on rat aorta rings and it was found that all of them significantly inhibited blood vessels growth compared to negative control. However, the methanol extract showed the highest biological activity compared to the other extracts; this may be due to presence of higher concentration of biologically active compounds or other compounds with biological activity in the methanol extract. The dose response study on the methanol extract with rat aorta ring assay showed significant dose dependent inhibition, with IC<sub>50</sub> of 29.08 µg/ml, which is within the safe range according to Nassar who mentioned that in angiogenesis process, the border concentration of a herbal extract to be considered safe is 20 µg/ml<sup>19</sup>.

### Free radical scavenging activity

The DPPH assay is based on the measurement of the scavenging capacity of antioxidants towards the stable free radical, DPPH. Free radicals are chemical species that contain unpaired electrons<sup>20</sup>. They are produced in biological systems and exogenously, and are known to cause various degenerative disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances and aging<sup>21</sup>. Antioxidants have been reported to inhibit angiogenesis in several *in vitro* and *in vivo* assays. These include natural substances (polyphenols, flavonoids, isoflavones, lycopene, catechins, pigment epithelium-derived factor, glutathione and resveratrol); nutritional substances (β-carotene, selenium and vitamins C, D and E); and synthetic and semi-synthetic compounds (N-acetylcysteine, sodium pyruvate, L-NIO, L-NAME, pyrrolidinedithiocarbamate and organoselenium compounds)<sup>22</sup>. The results obtained from this study showed that methanol extract of *Zizyphus spinachristi* leaves has the highest anti-oxidant activity in comparison with the other extracts (petroleum ether, chloroform and water). This may be due to presence of higher phenolic content, particularly flavonoids in the methanol extract<sup>23</sup>. The methanol extract also showed the highest anti-angiogenic activity, as manifested by the rat aortic ring assay. Its effectiveness in inhibiting new blood vessels formation could be attributed to its significant antioxidant activity, as shown in the DPPH scavenging assay. Antioxidants can affect the physiological redox balance that will scavenge reactive oxygen species (ROS)<sup>24</sup>. There are several reports that implicated ROS as an inducer of angiogenesis<sup>25-27</sup>, by mediating signaling cascade initiated by VEGF receptor-2 (Flk1, KDR)<sup>28</sup> and

angiopoietin-1/Tie2 receptor<sup>29,30</sup>, activation of hypoxia inducible factor-1 (HIF-1)<sup>31</sup> or by up-regulation of VEGF gene expression<sup>32</sup>.

Furthermore, it was demonstrated that ROS increased binding of transcription factors NF- $\kappa$ B and activator protein-1 (AP-1) to DNA of endothelial cells, and induced release of IL-8<sup>33</sup>.

## CONCLUSION

Angiogenesis is important process in many diseases, such as cancer, psoriasis and rheumatoid arthritis. *Zizyphus spinachristi* extracts showed potential inhibition activity against this process with highest effect exerted by the methanol extract.

This herb may have promising activity against angiogenesis related diseases.

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