



## Antioxidant and Anti-Angiogenesis Properties of Iraqi Aristolochia Maurorum Seeds Ethanolic Extract Using Ex Vivo, In Vitro, and Alternative In Vivo Model

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### ABSTRACT

**Objective:** This study seeks to examine the anti-angiogenic characteristics of *Aristolochia maurorum* seed extract through various methodologies, including the extraction process, rat aortic ring assay, chorioallantoic membrane (CAM) assay, and DPPH assay for antioxidant assessment.

#### Methods:

We used Soxhlet extraction with chloroform, ethanol, and distilled water to get the seed extract of *Aristolochia maurorum*. The rat aortic ring assay was used to find the extract that had these effects on stopping neovascularization. The RAR results showed that the ethanol extract had the strongest effect, so it was selected for further testing using the chorioallantoic membrane (CAM) assay to confirm its anti-angiogenic properties in a more complex biological model. The DPPH experiment was done to see how well the ethanol extract worked as an antioxidant.

**Results:** The screening through the rat aortic ring assay revealed that the ethanol extract of *Aristolochia maurorum* shows significant inhibition of blood vessels growth. This was followed by confirmation in (CAM) assay, where the ethanol extract continued to show significant inhibition of new blood vessel formation. Furthermore, the ethanol extract demonstrated antioxidant activity in the DPPH assay, confirming its additional therapeutic potential.

**Conclusions:** The ethanolic extract of *Aristolochia maurorum* exhibits potent anti-angiogenic and antioxidant properties. These findings suggest its potential for therapeutic applications in diseases associated with abnormal blood vessel growth, such as cancer and chronic inflammation.

**Keywords:** *Aristolochia maurorum*, DPPH, anti-angiogenesis , phytochemicals .

### INTRODUCTION

Angiogenesis is a physiological process that generates new blood vessels from existing ones and involves the migration, proliferation, and formation of new blood vessels from existing ones by endothelial cells. It is essential for normal growth, wound healing, and numerous pathological conditions (1). It is a highly controlled process that requires the appropriate balance of pro-angiogenic and anti-angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), fibroblast growth factors (FGFs), and angiopoietins(2). When this process occurs improperly, it contributes to the progression of many diseases, including cancer, where tumors cause angiogenesis to happen to ensure they can get enough oxygen and nutrients, allowing them to grow and spread (3). Angiogenesis is also very important in a number of diseases, such as endometriosis, retinopathies, atherosclerosis, and arthritis(4).

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Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor, hypoxia-inducible factor, and Angiopoietins are acknowledged pro-angiogenic factors. VEGF is a very important part of angiogenesis and lymph angiogenesis(4)

Tumor angiogenesis occurs through a series of sequential steps that promote cancer proliferation. The disease occurs due to an imbalance between pro- and antiangiogenic regulators in the tumor microenvironment (5). The tumor itself is what mostly causes angiogenesis. When a malignant tumor grows to a certain size, it makes the cells around it hypoxic(6). Hypoxia is defined by O<sub>2</sub> tension levels falling below 5–10 mmHg, serving as a substantial catalyst for tumor angiogenesis. Hypoxia triggers the excessive expression of angiogenic molecules in hypoxic cells, such as growth factors, cytokines, bioactive lipids, and matrix-degrading enzymes, which interact with receptors on neighboring vascular endothelial cells (ECs) to initiate angiogenesis(7).

Natural products have been important sources of new drugs for treating many diseases, especially cancer because they have different chemical structures and biological activities(8)

One of these plants is *Aristolochia maurorum*(9), The Aristolochiaceae family has the most species-rich genus, Aristolochia. People use the plants in this genus in traditional medicine to treat cholera, abdominal pain, rheumatism, malaria, skin problems, and bites and stings from animals and insects. They are also used as emmenagogues, sedatives, analgesics, anticancer agents, anti-inflammatories, muscle relaxants, antihistamines, and antiparasitic.(10).

Even though it has the potential to be a drug, not much research has been done on its anti-angiogenic effects. This study seeks to fill this void by investigating the capacity of *Aristolochia maurorum* seeds extract to impede blood vessel growth. It accurately examines the effects of the extract on essential genes related to angiogenesis, such as VEGF and CD31, which regulate endothelial cell function and the formation of new blood vessels. Examining the influence of *Aristolochia maurorum* on these genes may deepen our understanding of its capacity to impede angiogenesis and aid in the development of innovative treatments for diseases that exhibit analogous mechanisms.

## MATERIALS AND METHODS

### Plant Materials Preparation

We obtained 1 kg of mature seeds of *Aristolochia maurorum* from a well-known pharmacognosy expert, who collected them from wild plants in northern Iraq during the ripening period. A certified pharmacognosy expert at Al-Razi Center verified the botanical identity of the collected plant sample and confirmed the source's legitimacy. When the seeds were collected, they were visually inspected, and only those that were clean, mature, and free of disease were chosen. The items were cleaned by running tap water over them and then rinsing them with distilled water to get rid of any dirt. In July, the seeds were then air-dried at room temperature, with temperatures between 40 and 45 degrees Celsius and low humidity. This made drying easier while keeping chemicals that are sensitive to heat. The seed coat changed from green to brown during the drying process because of polyphenolic oxidation. The seeds were ground by hand into a fine powder using a home grinder. Then, the powder was sieved to make sure that the particles were all the same size, which made extraction more efficient. The sieved powder, which weighed 1062.72 mg, was put in a plastic bag with silica gel desiccant to keep it from absorbing moisture. It was then kept in a cool, dry place until it was ready to be processed.

### Animals used in this study

- Albino male rats will be used to perform the RAR anti-angiogenic assay. The animals will be housed in the animal facility under controlled environmental conditions, maintaining a temperature of 28-30°C, relative humidity of 50–60%, and a 12-hour light/dark cycle. They will be provided with free access to a standard pellet diet and clean drinking water at all times. The animals were allowed to acclimatize to these conditions for one week before the start of the experiment. All experimental procedures will comply with the ethical regulations approved by the Ethical Committee of the College of Pharmacy, Al-Nahrain University, and the corresponding ethical approval certificate will be secured prior to the initiation of the study.
- Fertilized chicken eggs will be procured from the poultry facilities of the College of Veterinary Medicine, University of Baghdad, and will be utilized for conducting the chorioallantoic membrane (CAM) anti-angiogenic assay.

### Extraction Procedure (Soxhlet / Hot Digestion Method)

The extraction of *Aristolochia maurorum* seed powder was carried out using a Soxhlet apparatus. A total of 150 g of finely powdered, air-dried seed material was placed in a Whatman cellulose extraction thimble. Three solvents of increasing polarity were used: chloroform, ethanol, and distilled water. The extraction process was conducted at the following temperatures: chloroform at 60 °C, ethanol at 65 °C, and water at 100 °C. After each extraction cycle, Whatman filter paper was used to filter the solvent and get rid of plant debris. The filtrates were then kept in amber-colored glass containers to prevent light from reaching them. We used a rotary evaporator under low pressure to concentrate the pooled filtrates from each solvent at the following temperatures: 40 °C for chloroform, 45 °C for ethanol, and 60 °C for the aqueous extract. The extracts were concentrated, then dried in an oven at 40 °C and stored in airtight, amber-colored glass containers at 4 °C to keep them stable and active. It took three weeks to do the whole extraction process.

### Anti-Angiogenic Assessment Using the *Ex Vivo* Rat Aortic Ring Model

This experiment followed the method set out by Brown et al. (1996) with some small changes(11). Following ethical and institutional animal care guidelines, adult male albino rats (12–14 weeks old) were killed by inhaling diethyl ether. The thoracic aorta was carefully taken out and put right away into cold Hank's Balanced Salt Solution (HBSS). Using a sterile scalpel, the connective and adipose tissues next to the aorta were carefully cut away, and the aorta was cut into evenly spaced 1 mm thick rings. Rings that had any kind of mechanical damage or clotting were thrown away.

We put each aortic ring in a 48-well culture plate, with 300 µL of M199 medium in each well. The medium was made with 20% heat-inactivated fetal bovine serum, L-glutamine, gentamicin, and ε-aminocaproic acid. To initiate fibrin clot formation, thrombin solution (50 NIH U/mL) was added, followed by a 15-minute incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Fibrinogen solution (3 mg/mL), supplemented with aprotinin, was then introduced to stabilize the fibrin matrix and support angiogenic sprouting.

The aortic rings were treated with *Aristolochia maurorum* extracts, with 1% dimethyl sulfoxide (DMSO) as the negative control and suramin (100 µM) as the positive control. The cultures were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for five consecutive days. On day four, the culture medium was replaced to ensure continued nutrient supply and tissue viability during the angiogenesis phase.

On day 5, a camera and software package were used to measure the growth of the vessel by hand at 40× magnification (12). The data are presented as the mean percentage of inhibition relative to the negative control, accompanied by the standard deviation (SD). To measure angiogenic inhibition, the percentage of inhibition was determined by comparing the growth of blood vessels in treated rings to the negative control(12).

We used this formula:

$$\text{Blood vessels inhibition (\%)} = 1 - \frac{A_0}{A} \times 100$$

where; A<sub>0</sub> is the length of the vessel growth of the compound being studied (in mm),  
and A is the length of the vessel growth of DMSO (in mm).

### Chick Chorioallantoic Membrane Assay CAM Assay

Fertilized chicken eggs were disinfected using a 70% ethanol solution and incubated at 37°C with 40-60 % humidity. The initial 24 hours of incubation served as day 0, which allowed for embryonic development(13).

On day 4 of incubation, approximately 2 mL of albumin was gently aspirated from the blunt end of each egg using a sterile syringe. This procedure helped relieve internal pressure and made it easier to separate the CAM from the inner shell membrane. The incision was closed with sterile glue, and the incubation continued for another 24 hours under the same conditions.

On the seventh day, a round hole about 3–4 cm in diameter was cut into the eggshell to show the chorioallantoic membrane. To get the right working concentration, a stock solution of *Aristolochia maurorum* Seeds ethanolic extract (10 mg/mL) was made using 1% dimethyl sulfoxide (DMSO). Five millimeter-diameter sterile filter paper discs were soaked in 50 µL of the extract solution and carefully placed on the CAM surface. They were then covered with sterile adhesive tape. The eggs were then put back in the incubator for another 48 hours so that the extract could work.

On day 9 (48 hours after treatment), the CAM was photographed with low magnification using an inverted microscope. To maintain consistency in imaging, the images were captured through the microscope eyepiece using

a smartphone camera. The visible blood vessels in each egg were manually counted under identical magnification conditions. The total vessel count was recorded and input into Microsoft Excel for statistical analysis, where the data were expressed as mean ± standard deviation (SD). The percentage of angiogenesis inhibition was calculated relative to the negative control group using the following formula:

$$\text{Inhibition (\%)} = (1 - (\text{Mean vessel count of treated group} / \text{Mean vessel count of negative control})) \times 100$$

### Free radical scavenging activity assay ( *in vitro* )

We used the DPPH method to assess how effectively the ethanolic extract of *Aristolochia maurorum* seeds scavenged free radicals. In an amber volumetric flask, 39.4 mg of DPPH was dissolved in 1 L of methanol to make a 0.1 mM DPPH solution. The solution was stirred gently until the DPPH was completely dissolved and kept out of the light. We made a stock solution of *Aristolochia maurorum* seeds ethanolic extract by mixing 100 µL of the extract with 990 µL of methanol to make a final volume of 1 mL. After that, serial dilutions were done to get concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. The experiment utilized 96-well plates, where 100 µL of each concentration mixed with 200 µL of DPPH solution. Each concentration was tested in triplicate. After incubating in the dark for 30 minutes, an ELISA reader measured the absorbance at 517 nm. The percentage of activity that scavenges radicals was calculated using the formula(14):

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where; A<sub>0</sub> is the absorbance of the control,  
and A<sub>1</sub> is the absorbance of the sample after reaction with DPPH.

### Statistical analysis:

entirely experimental results are presented as the skilled ± standard deviation (SD). Data were analyzed using one way analysis of variance (ANOVA), followed by Tukey’s post hoc test for multiple comparisons . Differences between groups were considered statistically important at a probability level of P < 0 .05. IC<sub>50</sub> values were calculated using linear or logarithmic regression analysis. Statistical computations were performed using SPSS software version 21.0

## RESULTS

### Extraction Process

The extraction of 150 g of *Aristolochia maurorum* seeds powder was carried out using three solvents: chloroform, methanol, and water. Among the three, the chloroform extract produced the highest yield percentage at 7.69%, whereas the water showed the lowest yield, as shown in Table 1.

**Table1: Weight and yield percentage obtained from *Aristolochia maurorum* crude extracts**

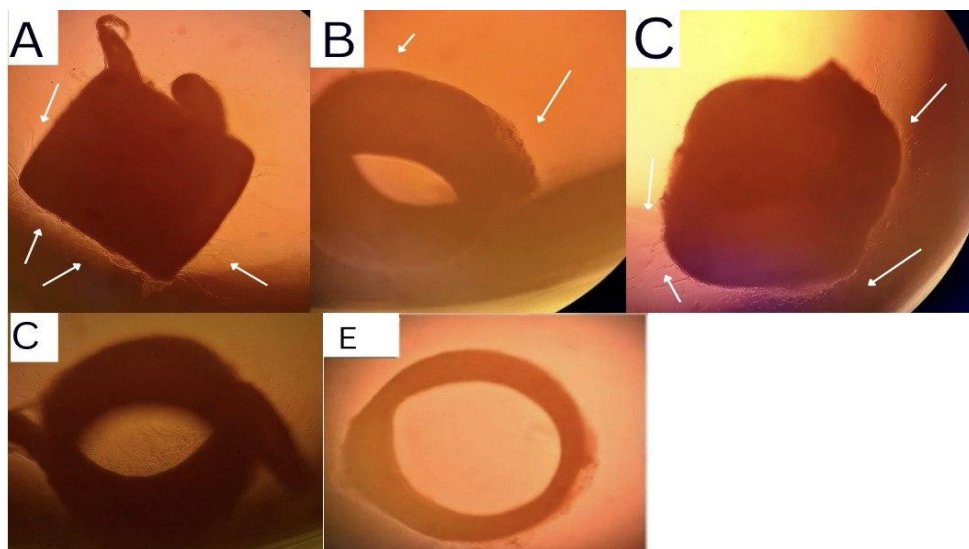
| Type of extract   | Crude extract weight (gm) | Yield (%) |
|-------------------|---------------------------|-----------|
| <b>Chloroform</b> | 11.5                      | 7.69      |
| <b>Ethanol</b>    | 10.6                      | 7.13      |
| <b>Water</b>      | 5.3                       | 3.53      |

### Rat Aortic Ring Assay

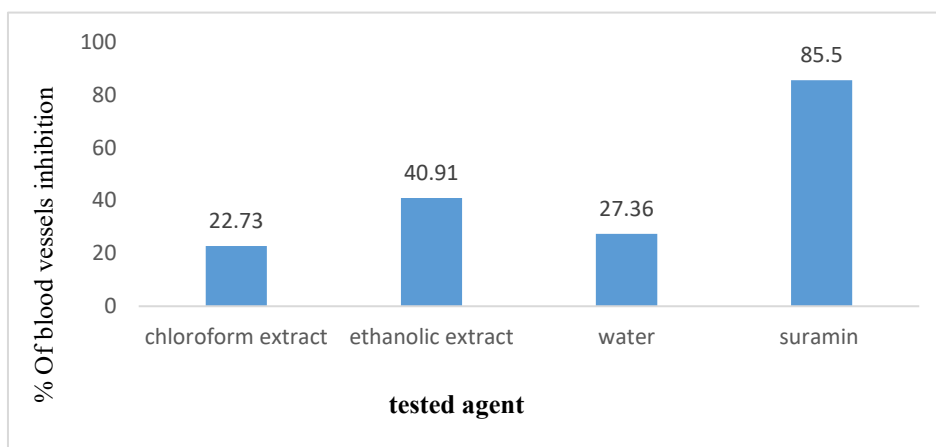
The anti-angiogenic effects of *Aristolochia maurorum* extracts (ethanol, chloroform, and water) were tested at 100 µg/mL using the rat aortic ring assay. The ethanol extract showed significant inhibition of blood vessel growth (P < 0.05), followed by the water extract. As shown in Table 2, the chloroform extract showed the least inhibition. Compared to the 1% DMSO negative control, the ethanol extract significantly reduced angiogenesis, as shown in Table 2, Figure 1, and Figure 2.

**Table 2: Percentage inhibition of blood vessel growth by tested extracts and the control groups**

| Compound                 | ± SD Mean  | %Of inhibition |
|--------------------------|------------|----------------|
| Negative control DMSO 1% | 0          | <b>0.00</b>    |
| Positive control suramin | 1.70±0.20  | <b>85.5%</b>   |
| Ethanol extract          | 6.52± 3.84 | <b>40.91%</b>  |
| Water extract            | 7.95±4.09  | <b>27.36%</b>  |
| Chloroform extract       | 8.57 ±3.87 | <b>22.73%</b>  |



**Figure 1: Effects of Different Extracts of *Aristolochia maurorum* on Blood Vessel Formation in Rat Aorta Ring Assay: A Comparative Study of (A) Chloroform , (B) Ethanol , (C) Water , (D) Negative Control (1% DMSO), and (E) Positive Control (Suramin)**



**Figure 2: Anti-angiogenic activity of *Aristolochia maurorum* extract, along with ethanol, chloroform, and water extracts, compared to the control group, in the ex vivo aortic ring model, expressed as the percentage of blood vessel growth inhibition.**

### Alternative *In Vivo* Chick Chorioallantoic Membrane (CAM) Assay

The CAM assay provided strong evidence supporting the anti-angiogenic effect of the ethanolic extract from *Aristolochia maurorum*. Visual analysis of the treated group (Figure 3.B) revealed a clear reduction in vascular density compared to the negative control group (Figure 3.A). This observation was corroborated by vessel count measurements, which showed a substantial decrease in the mean vessel count, from  $22.5 \pm 11.71$  in the control group to  $6.9 \pm 4.47$  in the treated group (Figure 4). This translates to a 69.19% inhibition of angiogenesis.

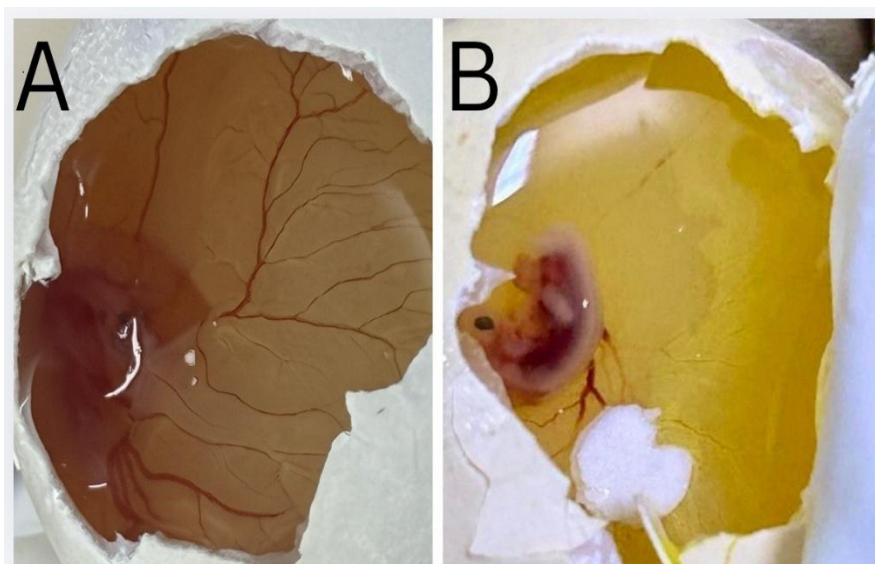


Figure 3: Illustrative CAM Images: (A) Negative Control Group and (B) *Aristolochia maurorum* extract-treated groups

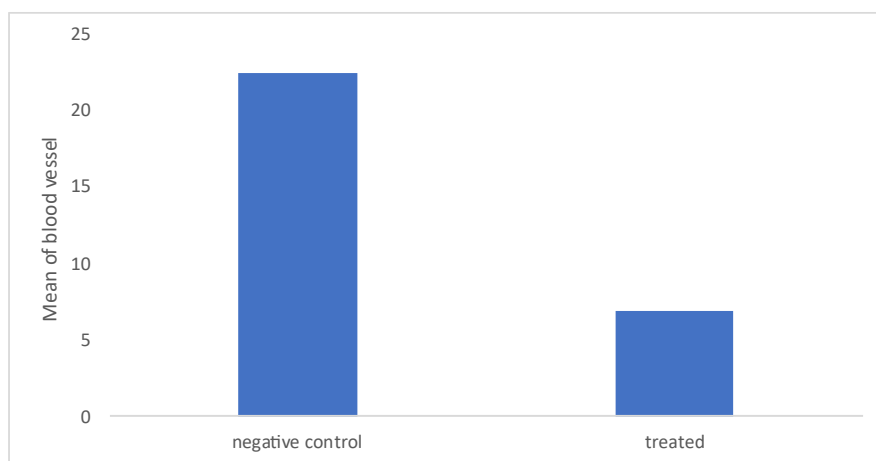


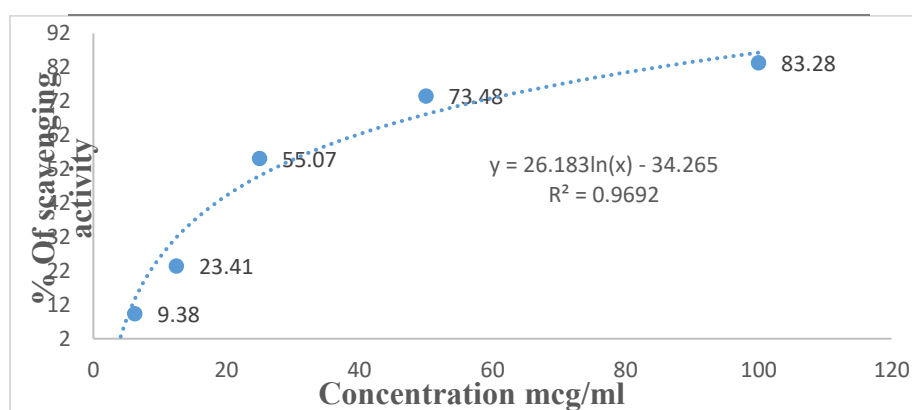
Figure 4: Quantitative analysis of vascular density: comparison between negative control and *Aristolochia maurorum* seeds ethanolic extract-treated groups

### Free Radical Scavenging Activity

The ethanolic extract of *Aristolochia maurorum* showed significant DPPH radical scavenging activity in a concentration-dependent manner ( $P \leq 0.05$ ). As shown in Table 3, the highest scavenging activity was recorded at  $100 \mu\text{g/mL}$  (83.28%), with a gradual decline at lower concentrations, reaching 1.03% at  $3.125 \mu\text{g/mL}$ .  $\text{IC}_{50}$  value was approximately  $25 \mu\text{g/mL}$ , as illustrated by the dose-response curve in Figure 5.

**Table 3: The percentage of free radical scavenging activity of the test agents at varying concentrations, assessed using the DPPH method**

| Concentration (µg/ml) | Mean± SD     | % of scavenging activity |
|-----------------------|--------------|--------------------------|
| 100                   | 0.485± 0.028 | <b>83.28%</b>            |
| 50                    | 0.769±0.015  | <b>73.48%</b>            |
| 25                    | 1.303±0.438  | <b>55.07%</b>            |
| 12.5                  | 2.221±0.045  | <b>23.41%</b>            |
| 6.25                  | 2.628±0.045  | <b>9.38%</b>             |
| 3.125                 | 2.870±0.059  | <b>1.03%</b>             |



**Figure 5: Dose response curve of DPPH radical scavenging activity of the ethanolic extract of *Aristolochia maurorum***

## Discussion

### Extraction Yield

The extraction process used in this study was the hot digestion method by Soxhlet apparatus(15). The varying yields and chemical compositions from different solvents highlight the complex nature of *Aristolochia maurorum* seeds. The high yield from chloroform suggests a predominance of non-polar compounds, particularly aristolochic acids, which are known for their pharmacological properties. Ethanol extracts a wider range of compounds, including flavonoids and polyphenols, which contribute to the plant’s antioxidant activity. Water, being less effective, suggests that the plant’s bioactive compounds are not predominantly water-soluble, reinforcing the idea that *Aristolochia maurorum* is a source of non-polar and semi-polar bioactive compounds (16).

### Rat aortic ring assay

Angiogenesis assays are crucial for identifying potential angiogenic agents and discovering pharmacologic inhibitors. The rat aorta ring assay is one of the most commonly used methods to study angiogenesis and its underlying mechanisms due to its reproducibility, cost-effectiveness, simplicity, and strong correlation with *in vivo* assays (17)(18). In this study, the primary objective was to assess the anti-angiogenic activity of three *Aristolochia maurorum* extracts (ethanol, chloroform, and water) and determine which extract exhibits the highest activity. All extracts were screened using the rat aorta ring model, and results showed that all of them significantly inhibited blood vessel growth compared to the negative control (1% DMSO) ( $P < 0.05$ ). Among the extracts, the ethanol extract demonstrated the highest biological activity, indicating that it may contain a higher concentration of biologically active compounds responsible for its potent antiangiogenic effects.

### **Anti-Angiogenic Activity in Chick Chorioallantoic Membrane (CAM) in vivo Assay**

The CAM assay offers several advantages for studying angiogenesis, including its suitability for both intravascular and topical administration, rapid results, and low cost(19) (20)(13). In this study, the ethanolic extract of *Aristolochia maurorum* demonstrated significant anti-angiogenic effects. Visual analysis and vessel count measurements revealed a substantial reduction in vascular density, with a 69.19% inhibition of angiogenesis compared to the control group. These findings indicate that *Aristolochia maurorum* extract effectively inhibits angiogenesis, a key process in tumor growth and diseases related to abnormal vascularization.

### **Free Radical Scavenging Activity of *Aristolochia maurorum* Seeds Ethanolic Extract**

The DPPH assay is a common way to test how well antioxidants can stop free radicals, which are unstable chemicals that can cause many degenerative diseases, such as mutagenesis, carcinogenesis, cardiovascular problems, and aging(21). Biological systems make free radicals on their own and get them from outside sources. Researchers have shown that antioxidants, such as polyphenols and flavonoids, can stop angiogenesis in many studies(21).

The ethanolic extract of *Aristolochia maurorum* exhibited significant free radical scavenging activity compared to negative. The strong antioxidant effect indicates that the ethanolic extract contains bioactive compounds, such as flavonoids and other polyphenolic compounds, that are very effective at neutralizing free radicals(22) . By scavenging reactive oxygen species (ROS), the ethanolic extract may help restore the redox balance in the body, which is often disrupted during pathological conditions involving excessive angiogenesis(23).

### **CONCLUSION**

The results of this study demonstrate that *Aristolochia maurorum* seed extract possesses notable biological activities. Among the different extracts tested, the ethanolic extract exhibited the most pronounced anti-angiogenic and antioxidant effects, likely due to its high phenolic and polyphenolic content. These findings highlight the ethanolic extract as a promising natural source of bioactive compounds with potential therapeutic applications in diseases associated with abnormal angiogenesis and oxidative stress.

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### **CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest related to this study.

### **FUNDING**

The authors declare that no external funding was received for this study.

### **ETHICS STATEMENTS**

Ethical approval was obtained from the Ethics Committee of the College of Pharmacy, AL-Nahrain University (Approval No. SY /3/2/97, dated 28 January 2026).

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## الخصائص المضادة للاكسدة والمضادة لتكوين الاوعية الدموية لنبات الزراوند باستخدام الدراسات خارج الجسم , ومخبرية وحيوية بتول شامل<sup>1</sup> ، حيدر بهاء صاحب<sup>1</sup>

<sup>1</sup> فرع الادوية والسوموم كلية الصيدلة، جامعة النهرين، بغداد، العراق.

### الخلاصة

**الهدف:** يهدف هذا البحث إلى فحص النشاط المضاد لتكوين الأوعية الدموية لمستخلص بذور الزراوند من خلال عدة أساليب بحثية، بما في ذلك عملية الاستخراج، واختبار حلقة الشريان الأبهر في الجرذان، واختبار الغشاء المشيمي لجنين الدجاج، واختبار (2,2-ثنائي فينيل-1-بيكريل هيدرازيل) للكشف عن النشاط المضاد للأكسدة.

**منهجية العمل:** تم استخلاص بذور النبات باستخدام جهاز "سوكسليت" وباستعمال ثلاثة مذيبات هي: الكلوروفورم، والإيثانول، والماء المقطر. جرى فحص القدرة المضادة لتشكيل الأوعية الدموية لكل مستخلص أولياً عبر اختبار الحلقة الأبهرية للجرذان لتحديد المستخلص الأكثر تثبيطاً لنمو الأوعية الجديدة. وبناءً على النتائج، أظهر المستخلص الإيثانولي الفعالية الأقوى، وبناءً عليه تم اختياره لإجراء اختبار الغشاء المشيمي لجنين الدجاج لتأكيد خصائصه في نموذج بيولوجي أكثر تعقيداً. كما نُفذ فحص (2,2-ثنائي فينيل-1-بيكريل هيدرازيل) لتقييم القدرة الاختزالية والمضادة للأكسدة لهذا المستخلص.

**النتائج:** أظهر الفحص من خلال اختبار حلقة الشريان الأبهر أن المستخلص الإيثانولي من نبات الزراوند يظهر تثبيطاً كبيراً في نمو الأوعية الدموية. تم تأكيد ذلك لاحقاً في اختبار الغشاء المشيمي لجنين الدجاج، حيث استمر المستخلص الإيثانولي في إظهار تثبيط كبير لتكوين الأوعية الدموية الجديدة. علاوة على ذلك، أظهر المستخلص الإيثانولي نشاطاً مضاداً للأكسدة في اختبار (2,2-ثنائي فينيل-1-بيكريل هيدرازيل)، مما يؤكد إمكاناته العلاجية الإضافية.

**الاستنتاجات:** يظهر المستخلص الإيثانولي من خصائص مضادة لتكوين الأوعية الدموية ومضادة للأكسدة قوية. تشير هذه النتائج إلى إمكاناته في التطبيقات العلاجية للأمراض المرتبطة بالنمو غير الطبيعي للأوعية الدموية، مثل السرطان والالتهابات المزمنة.

**الكلمات المفتاحية:** الزراوند , مضاد تكوين الاوعية الدموية , (2,2-ثنائي فينيل-1-بيكريل هيدرازيل)