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Evaluation of the Anti-angiogenic and Antioxidant Effects of Lawsonia inermis Leaves Ethanolic Extract: Ex Vivo and In Vivo Study

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ABSTRACT

Objective: Angiogenesis is an essential process in tumor growth and progression, and thus it represents a promising therapeutic target. Lawsonia inermis (henna) is a widely-used traditional medicine with different biological applications, and its bioactive components, especially lawsone, showed anticancer activity. The objective of this research was to measure the anti-angiogenic and antioxidant properties of Lawsonia inermis leaf ethanolic extract in ex vivo and in vivo systems.

Methods: Soxhlet was used to prepare the ethanolic extract of the Lawsonia inermis leaf. The ex vivo rat aorta ring assay was used to test the anti-angiogenic activity at the concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL. The in vivo chick chorioallantoic membrane (CAM) assay was employed to confirm the anti-angiogenic effect at a concentration of 10 mg/mL. DPPH radical scavenging assay was used to determine the antioxidant activity with a concentration range of 3.125 to 100 µg/mL.

Results: The ethanolic extract demonstrated high anti-angiogenic activity in the rat aorta ring with 65.82% inhibition at 100 µg/mL and dose-dependent inhibition with an IC 50 of 54.2 µg/mL. In the CAM assay, acetylsalicylic acid (positive control) resulted in complete suppression of vascularization, validating the assay system. The extract exhibited a concentration-dependent radical scavenging ability of DPPH radical with an IC 50 value of 0.05 µg/mL.

Conclusions: Lawsonia inermis ethanolic extract has strong anti-angiogenic and antioxidant properties, which implies its possible application as a treatment of angiogenesis-related disorders, such as cancer. The anti-angiogenic effect was confirmed in both ex vivo and in vivo models.

Keywords: Lawsonia inermis, anti-angiogenesis, CAM assay, DPPH, rat aorta ring assa

INTRODUCTION

Angiogenesis is a complicated biological mechanism of forming new blood vessels on the basis of the existing capillaries, which includes the degradation of the basement membrane, the activation of endothelial cells, growth and movement (1,2). The balance between the pro-angiogenic (VEGF, FGF and angiopoietins) and the anti-angiogenic (angiostatin, endostatin and thrombospondins) factors strictly controls this process (3,4). Angiogenesis takes place mainly in embryonic development, wound repair, and the ovarian cycle under physiological conditions. Nevertheless, when the balance is disrupted, pathological angiogenesis occurs which is essential in numerous diseases such as cancer, rheumatoid arthritis, diabetic retinopathy and cardiovascular diseases (4,5). Angiogenesis of tumors is particularly noteworthy because a solid tumor cannot grow more than 1-2 mm 3 without the formation of a blood supply to deliver oxygen and nutrients. The microenvironment of the hypoxic tumor leads to the production of pro-angiogenic factors, especially, the release of vascular endothelial

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growth factor (VEGF), which stimulates endothelial cells and causes the angiogenic switch (6,7). This has promoted angiogenesis as a therapeutic target thereby giving rise to a number of anti-angiogenic drugs, such as bevacizumab, sunitinib and sorafenib. Nevertheless, these synthetic agents have serious restrictions, such as cardiovascular toxicity, hypertension, proteinuria, drug resistance, and high cost, leading to the need to find safer and less expensive alternatives (8,9). Medicinal plants have received a lot of interest as potential sources of bioactive compounds with anti-angiogenic effects. Traditional medicine is used as the primary healthcare system by a large part of the world population, estimated to be between 65-80 percent of the world population and plant-based compounds have played a significant role in the discovery of modern drugs; among them are the anticancer drugs vinblastine and paclitaxel. Natural products have a number of benefits, such as reduced toxicity, multi-modality of action, and cost(10,11)*Lawsonia inermis L.* (henna), which is a member of the Lythraceae family, is a small shrub that is extensively grown in the tropical and subtropical areas of Asia and North Africa (12). Historically, henna has been used to dye the hair, skin and nails and in medicine. Phytochemical investigations have found *L. inermis* to have several bioactive compounds, namely, naphthoquinones (mainly lawsone), flavonoids, phenolic compounds, tannins, terpenoids and alkaloids (13). These compounds are involved in its various pharmacological functions such as antidiabetic, antibacterial, antifungal, antioxidant, anti-inflammatory, wound healing, and anticancer (14). The anticancer effects of *L. inermis* extracts have been shown on numerous cancer cell lines in the past with some of the mechanisms being cell cycle arrest effects, apoptosis stimulating effects and antioxidant modulating effects. The major naphthoquinone constituent, lawsone has been shown to act antiproliferative against colon cancer cells with S-phase arrest. Also, a report on the endophytic fungus Alternaria alternata growing on the *L. inermis* leaves showed anti-angiogenic effects in the chick chorioallantoic membrane assay and indicated that the bioactive compounds of *L. inermis* that have been associated with it could have angiogenesis- inhibitory activity (15,16). In spite of these encouraging results, direct anti-angiogenic action of *Lawsonia inermis* leaf extracts has not been studied in a systematic manner. Moreover, the molecular pathways of its possible anti-angiogenic effect, especially in modulating VEGF pathways, and interaction with VEGFR-2 are not studied.

MATERIALS AND METHODS

Experimental Animals

The experiments were done on adult albino male rats of age 12-14 weeks. Animals got at the animal house of College of Pharmacy, Al-Nahrain University and held at 23 +/-2 o C with water and food. The animal-related experimental procedures were carried out in line with the ethical guidelines and standards of the Al-Nahrain University, College of Medicine, after being approved by the institution ethics committee.

Preparation of Plant Extract

The specimen of *Lawsonia inermis* was gathered at the Wasit Governorate in Iraq. The leaves were rinsed adequately under the running water and left to dry at the room temperature within 7-10 days. The drying process was followed by grinding the leaves into fine powder in an electric grinder, and then they were stored in a dark, hermetically sealed bottle at 4 ° C. Dried powdered leaf (100 gm), was extracted with the help of a Soxhlet apparatus with the help of ethanol (99.9% analytical grade). The powder (100 grams) was soaked in 600 mL of ethanol for 6-8 hours. Upon the extraction, the resulting filtrate was concentrated and filtered by low pressure using a rotary evaporator. The corresponding crude extract was further dried, then put in amber colored bottles and kept at 4 ° C until used.

Rat Aorta Ring Anti-Angiogenesis Assay

The ex vivo rat aortic ring assay of *Lawsonia inermis* ethanol extract was done based on the conventional procedure of Brown, with modifications (17,18). Humanely euthanized adult albino male rats were of 12-14 weeks old, and were euthanized by inhalation of diethyl ether. A thoracic aorta was removed with a dissecting microscope and put directly into chilled Hank Balanced Salt Solution (HBSS). The peri-aortic connective and adipose tissue was also trimmed and the aorta cut into regular rings of about 1 mm thickness with a sterile scalpel. Rings that had mechanical damage or luminal clot were not used in the experiment. Aortic rings were seeded on 48-well tissue culture plates (0.30 ml of M199 medium with 20 percent heat-inactivated fetal bovine serum (HIFBS), 0.1

percent 0 5 M 8 -aminocaproic acid (0.1%), L-glutamine, and gentamicin (0.6%). The event of the fibrin clot formation was triggered by 10 µL addition of thrombin solution (50 NIH U/mL) with 37C incubation. Then, 300 µL of fibrinogen mixture (3mg/mL), a protein solution that had been prepared in serum-free M199 medium and aprotinin (5 mg/mL) were introduced to reinforce the fibrin matrix and to promote angiogenic sprouting. An ethanol extract of stock solution of Lawsonia inermis was made under sterile conditions and a concentration of 10 mg/mL in dimethyl sulfoxide (DMSO) and subsequently diluted in the M199 culture medium to give the desired working concentrations. A control vehicle was aortic rings that were treated with 1% DMSO. A humidified incubator containing 5% CO_2 at 37 ° C was used to maintain cultures over a period of five days. The culture medium was changed very carefully on the fourth day to maintain the viability of the tissues and sufficient supply of nutrients. Microvessel outgrowth on the fifth day was assessed under an inverted microscope under 40X magnification, and some typical images were recorded with the help of a digital imaging system. The degree of expansion of blood vessels was calculated and the degree of suppression was established through the following formula:

$$\textit{Blood vessel growth inhibition \%} = [(L0 - L) / L0] \times 100$$

Where:

- L: Distance of blood vessels growth in treated rings (mm)
- L0: Distance of blood vessel growth in vehicle control rings (mm)

Dose Response Study of Lawsonia inermis Ethanol Extract with Rat Aorta Ring Assay

A stock solution of the ethanol extract of *Lawsonia inermis* in DMSO was prepared at a concentration of 10 mg/mL. From this stock solution, serial dilutions were prepared by dissolving the appropriate volume in M199 media (with 1% DMSO) to obtain final concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL. Wells containing culture medium supplemented with 1% DMSO and devoid of plant extract served as the vehicle control. All experimental treatments were conducted in replicates, and the results were expressed as mean ± standard deviation (SD). The IC_{50}, which is the concentration that inhibits blood vessel growth by 50%, was calculated using a logarithmic dose-response regression model (19,20).

Chick Chorioallantoic Membrane (CAM) Assay

The antiangiogenic effect of *Lawsonia inermis* ethanolic extract was tested by using chick chorioallantoic membrane (CAM) assay. Chicken eggs were fertilized and received at a local hatchery in Baghdad. Surface-disinfection of the eggs was done with 70% ethanol followed by incubation and the eggs were placed that way in an incubator at 37 °C with about 60% relative humidity in aseptic conditions to allow initial development of CAM. After incubation, about 2 mL of albumin was aspirated using a small opening in the lateral of the eggshell after a period of incubation of 72 hours. The cut was immediately covered to decrease the pressure within the shell and allow CAM to be separated off the shell. The eggs were reincubated once again in the incubator which lasted 24 hours. Four days later, the circular window about 3-4 cm in diameter was cut in the eggshell taking the CAM into the circle. The ethanolic extract of *Lawsonia inermis* at a concentration of 10 mg/mL was put on sterile filter paper discs that were dried in a sterile environment before use. The discs were then carefully put on the CAM surface in these wet solutions. Acetylsalicylic acid served as a control sample. The sterile adhesive taping was used to seal the window and the eggs were reintroduced back into the incubator so as to further evaluate the angiogenic response under the same conditions (21).

DPPH Radical Scavenging Activity Assay

The scavenging ability of Lawsonia inermis ethanol extract against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was estimated based on the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging test(22,23) . The DPPH solution, 0.1 mM, was freshly prepared by dissolving 39.4 mg of DPPH in 1L of Methanol in amber volumetric flask. The solution was gently mixed until completely dissolved and protected from light throughout the experiment. Serial dilutions of the ethanol extract were prepared in methanol to obtain concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. The assay was carried out in 96-well microplates by adding 100 µL of each extract concentration to 200 µL of the DPPH solution. Methanol alone served as the blank, while a mixture of methanol and DPPH solution served as the negative control. All samples were tested in triplicate. Plates were

gently mixed and incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 517 nm using an ELISA microplate reader. The percentage of DPPH radical scavenging activity was calculated according to the following equation:

$$Scavenging\ activity\ (\%) = [(A_0 - A_1) / A_0] \times 100$$

Where:

- A_0 is the absorbance of the control (DPPH solution without sample)
- A_1 is the absorbance of the sample after reaction with DPPH

Plotting the sample concentration against the matching DPPH scavenging activity on the dose inhibition curve allowed for the calculation of the IC50, or the concentration needed to scavenge 50% of the DPPH free radical (24).

RESULTS

Screening of the Anti-Angiogenic Activity of Lawsonia inermis Extracts

Screening of *Lawsonia inermis* extracts using the rat aortic ring assay (Figure 1) revealed significant inhibition of microvessel outgrowth compared to the negative control ($p < 0.05$). Among the tested extracts, the ethanolic extract showed the highest anti-angiogenic activity (65.82% inhibition at 100 µg/mL), followed by the aqueous extract (48.72%) and the chloroform extract (12.82%), as shown in **Table 1.**

Table 1: Screening of the anti-angiogenic activity of *Lawsonia inermis* extracts using the rat aortic ring assay.

Compound (Extract)	Concentration (µg/mL)	Microvessel growth (mm) Mean ± SD	Inhibition (%)
Chloroform extract	100	10.2 ± 6.99	12.82
Ethanolic extract	100	4 ± 1.52	65.82
Aqueous extract	100	6 ± 1.38	48.72
Negative control	---	11.7 ± 5.02	---

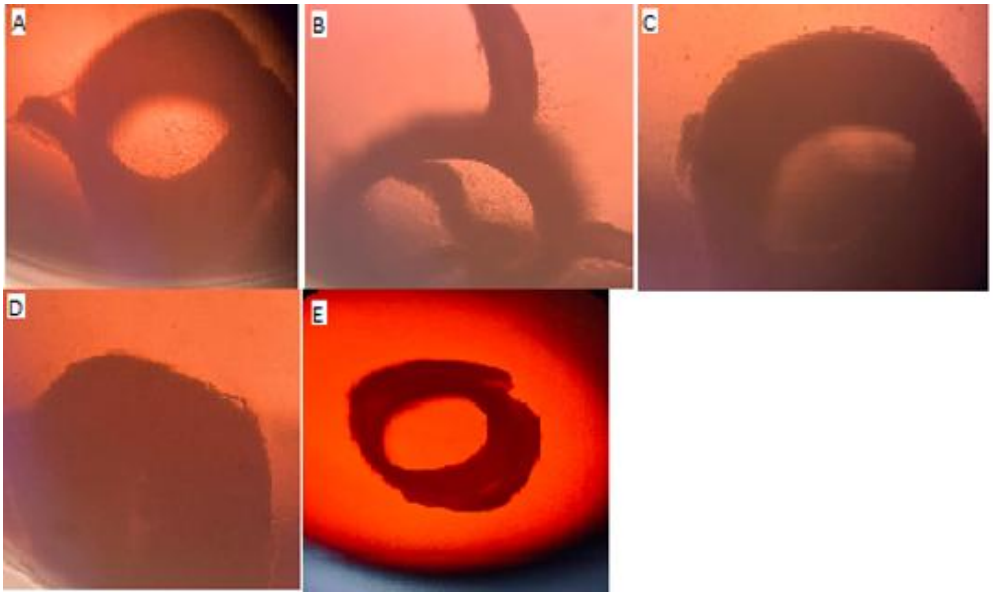

Figure 1: Representative microscopic images of rat aortic rings showing microvessel outgrowth after 5 days of incubation. Rings were treated with (A) negative control (1% DMSO), (B) chloroform extract, (C) ethanolic extract, (D) aqueous extract of Lawsonia Inermis extract , and (E) suramin (100 µM) as a positive control.

Dose-Response Study of Lawsonia inermis Ethanolic Extract

The ethanolic extract of *Lawsonia inermis* exhibited a clear dose-dependent anti-angiogenic effect in the *ex vivo* rat aortic ring assay. The extract induced 61.00 ± 0.72%, 45.00 ± 0.50%, 41.00 ± 2.21%, 30.00 ± 3.61%, and 18.00 ± 4.04% inhibition of microvessel outgrowth at concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL, respectively, compared with the negative control ($p < 0.05$). The estimated IC_{50} value was approximately 54.2 µg/mL, as shown in **Table 2 and Figure 2.**

Table 2: Dose-dependent inhibition of angiogenesis by *Lawsonia inermis* ethanolic extract in the rat aortic ring assay.

Conc. (µg/ml)	Blood vessel inhibition (%) ± SD
100	61 ± 0.72
50	45 ± 0.50
25	41 ± 2.21
12.5	30 ± 3.61
6.25	18 ± 4.04

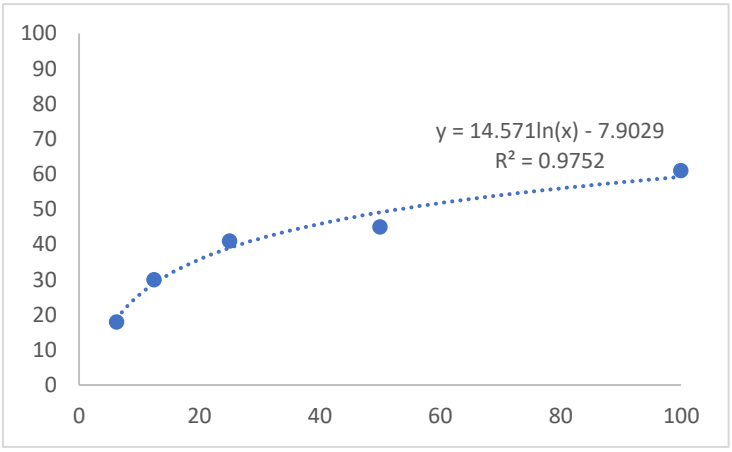

Figure 2: Dose response curve of *Lawsonia inermis* leaves ethanol extract on rat aorta rings.

Chick Chorioallantoic Membrane (CAM) Assay

The acetylsalicylic acid which was used as a reference control caused a full visual suppression of the CAM vascularization and no blood vessels were observed in the treated region. This anti-angiogenic effect was highly detected and it was qualitative and ascertained the effectiveness of the CAM assay system in testing the anti-angiogenic impact of *Lawsonia inermis* ethanolic extract with the same experimental conditions (Figure 3). Representative CAM images: (A) negative control and (B) lawsoni Inermis ethanolic extract–treated CAM.

Figure 3: Representative CAM images: (A) negative control and (B) lawsoni Inermis ethanolic extract–treated CAM.

DPPH Radical Scavenging Activity

The *Lawsonia inermis* ethanolic extract exhibited high DPPH radical scavenging activity in a concentration-dependent fashion ($P \leq 0.05$). The activity of the scavenging was the most effective at 100 µg/mL (42.69%), and the lower concentrations exhibited a progressive drop-in antioxidant activity as indicated in **Table 3 and Figure 4**. The IC_{50} value was approximately 0.05 µg/mL.

Table 3: DPPH radical scavenging activity of the ethanolic extract.

Concentration (µg/mL)	Absorbance (Mean ± SD)	DPPH Scavenging (%)
100	1.66 ± 0.04	42.69%
50	2.19 ± 0.08	24.24%
25	2.64 ± 0.18	9.02%
12.5	2.72 ± 0.15	6.18%
6.25	2.80 ± 0.03	3.40%
3.125	2.91 ± 0.01	0.38%

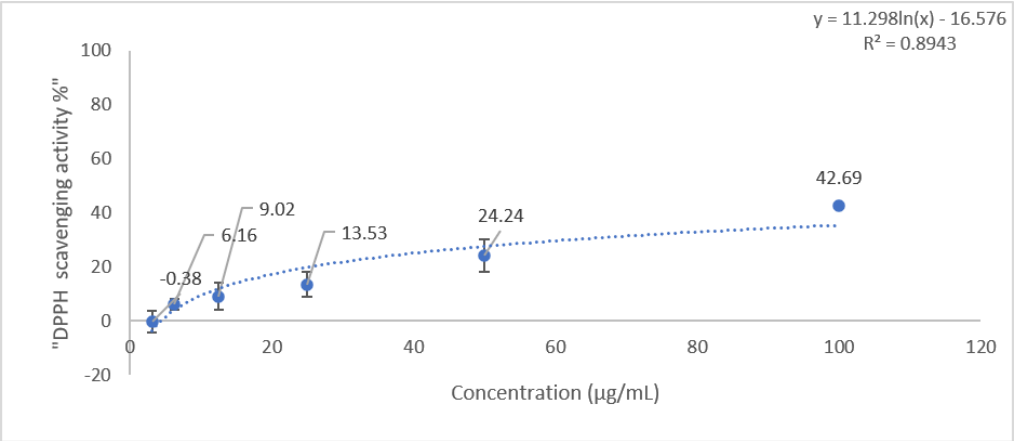

Figure 4: A dose–response curve illustrating the DPPH radical scavenging activity of the ethanolic extract of the green husk of *Lawsonia inermis*.

DISCUSSION

Screening and Dose-Response Anti-Angiogenic Activity

The *ex vivo* rat aorta ring assay is commonly chosen for analyzing blood vessel formation due to its reproducibility, cost-effectiveness, and accurate representation of *in vivo* findings. In the present study, all extracts were screened with the rat aorta ring assay, and it was found that all of them significantly inhibited blood vessel growth compared to the negative control. However, the ethanol extract showed the highest biological activity (65.82% inhibition) compared to other extracts. This may be due to the presence of a higher concentration of biologically active compounds, particularly phenolic compounds and naphthoquinones, in the ethanol extract. The dose-response study on the ethanol extract showed significant dose-dependent inhibition with an IC_{50} value of 54.2 µg/mL. The blood vessel growth inhibition was quantified after five days of aortic ring culture, as the blood vessel growth reaches its maximum at day five. These findings are consistent with previous studies on medicinal plants with anti-angiogenic properties(25,26) .

Anti-angiogenic Activity of Lawsonia inermis Ethanolic Extract in Chick Chorioallantoic Membrane In Vivo Assay

Chorioallantoic membrane (CAM) is a well-known in vivo model of assessing anti-angiogenic agents because it is highly vascularized and sensitive (27). Acetylsalicylic acid (ASA) was selected as a reference control in this study and it was found that it entirely suppressed the vascularization, which validates its appropriateness

as a positive control. ASA has an anti-angiogenic effect ascribed to the inhibition of cyclooxygenase as well as the down-regulation of VEGF (28). The CAM has been extensively employed to show that plant-derived compounds have anti-angiogenic activity (29). In the present experiment, the CAM assay was used to confirm the anti-angiogenic effect of the *ex vivo* rat aortic ring assay *in vivo* to support the experimental system in assessing the *Lawsonia inermis* extracts under the same conditions.

Free Radical Scavenging Activity

The antioxidant potential of the ethanolic extract was assessed by the DPPH free radical scavenging assay. The results showed that the ethanolic extract exhibited significant DPPH radical scavenging activity in a concentration-dependent manner, with an IC_{50} value of 0.05 µg/mL. The intense radical scavenging activity may be explained by the presence of phenolic compounds, flavonoids, and naphthoquinones, especially lawsone, in the leaves of *Lawsonia inermis*(30). Phenolic compounds possess hydroxyl groups capable of donating hydrogen atoms or electrons to neutralize free radicals, while their conjugated aromatic structures allow stabilization of the resulting radicals (31). The observed antioxidant potential may contribute to the reported anti-angiogenic effects, as antioxidants are known to suppress ROS-mediated VEGF signaling pathways involved in angiogenesis. There are several reports that implicated ROS as an inducer of angiogenesis by mediating signaling cascade initiated by VEGF receptor-2, activation of hypoxia inducible factor-1 (HIF-1), or by up-regulation of VEGF gene expression (32).

CONCLUSIONS

The present study demonstrated that all extracts of *Lawsonia inermis* leaves (chloroform, ethanol, and aqueous) exhibited significant anti-angiogenic activity in the *ex vivo* rat aorta ring assay, with the ethanol extract showing the most potent effect. The ethanol extract induced dose-dependent inhibition of micro vessel outgrowth with an IC_{50} value of 54.2 µg/mL, confirming its anti-angiogenic potential. Furthermore, the ethanol extract exhibited significant free radical scavenging activity in the DPPH assay with an IC_{50} value of 0.05 µg/mL, suggesting that its anti-angiogenic effect may be mediated through antioxidant mechanisms. The *in vivo* CAM assay further confirmed the anti-angiogenic activity, with acetylsalicylic acid showing complete suppression of vascularization. These findings indicate that *Lawsonia inermis* ethanolic extract possesses promising anti-angiogenic and antioxidant properties that could be beneficial in the management of angiogenesis-related diseases.

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CONFLICTS OF INTEREST

The author declares that they have no conflicts of interest.

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ETHICS STATEMENTS

Ethical approval was obtained from The Ethics Committee of the college of pharmacy, Al-Nahrain University, (Approval No. SY/2/1/1843, dated 21 December 2025).

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تقييم التأثيرات المضادة لتكوين الأوعية الدموية والمضادة للأكسدة لمستخلص الإيثانول من أوراق الحناء :(Lawsonia inermis) دراسة خارج الجسم الحي وداخل الجسم الحي

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الخلاصة

الهدف: تُعدّ عملية تكوّن الأوعية الدموية عملية أساسية في نمو الأورام وتطورها، ولذلك فهي تمثل هدفًا علاجيًا واعدًا. تُستخدم الحنّاء على نطاق واسع في الطب التقليدي لما لها من تطبيقات حيوية متعددة، وقد أظهرت مكوناتها الفعّالة حيويًا، ولا سيما مركب اللوسون، نشاطًا مضادًا للسرطان. هدفت هذه الدراسة إلى تقييم الخصائص المضادة لتكوّن الأوعية الدموية والمضادة للأكسدة لمستخلص الإيثانول لأوراق الحناء باستخدام نماذج خارج الجسم الحي وداخل الجسم الحي.

الطرائق: تم استخدام جهاز السوكسليت لتحضير مستخلص الإيثانول لأوراق نبات الحناء. جرى تقييم النشاط المضاد لتكوّن الأوعية الدموية خارج الجسم الحي باستخدام اختبار حلقة الأبهر للجرذ عند تراكيز 100، 50، 25، 12.5، و6.25 ميكروغرام لكل ملليلتر. كما استُخدم اختبار الغشاء الكوريون والألنتويسي لجنين الدجاج داخل الجسم الحي لتأكيد التأثير المضاد لتكوّن الأوعية الدموية عند تركيز عشرة ملليغرامات لكل ملليلتر. وتم تقييم النشاط المضاد للأكسدة باستخدام اختبار اقتناص الجذور الحرة ضمن مدى تراكيز تراوح بين 3.125 و100 ميكروغرام لكل ملليلتر.

النتائج: أظهر مستخلص الإيثانول فعالية عالية مضادة لتكوّن الأوعية الدموية في اختبار حلقة الأبهر للجرذ، حيث بلغت نسبة التثبيط 65.82 بالمئة عند تركيز 100 ميكروغرام لكل ملليلتر، مع وجود تثبيط يعتمد على الجرعة وقيمة تركيز نصف التثبيط بلغت 54.2 ميكروغرام لكل ملليلتر. وفي اختبار الغشاء الكوريون والألنتويسي لجنين الدجاج، أدى حمض الأسيتيل ساليسيليك بوصفه مجموعة ضابطة موجبة إلى تثبيط كامل لتكوّن الأوعية الدموية، مما أكد كفاءة نظام الاختبار. كما أظهر المستخلص قدرة عالية على اقتناص الجذور الحرة بطريقة تعتمد على التركيز، إذ بلغت قيمة تركيز نصف التثبيط 0.05 ميكروغرام لكل ملليلتر.

الاستنتاج: يمتلك مستخلص الإيثانول لنبات الحناء خصائص قوية مضادة لتكوّن الأوعية الدموية ومضادة للأكسدة، مما يشير إلى إمكانية استخدامه كخيار علاجي محتمل للأمراض المرتبطة بتكوّن الأوعية الدموية مثل السرطان. وقد تم تأكيد التأثير المضاد لتكوّن الأوعية الدموية باستخدام نماذج خارج الجسم الحي وداخل الجسم الحي.

الكلمات المفتاحية: Lawsonia inermis، مضاد تكوين الأوعية، فحص DPPH ،CAM، فحص حلقة الشريان الأورطي للفئران