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Research Article

Anti-angiogenic Activity of Sclareol: In Vivo and Ex Vivo Study

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Abstract

Background: Angiogenesis plays a crucial role in the progression of cancer and other angiogenesis-dependent disorders. Natural products are an important source of novel anti-angiogenic agents. Sclareol, a naturally occurring labdane diterpene, has attracted interest because of its diverse pharmacological properties; however, its anti-angiogenic potential remains poorly understood. ***Objective***: The objective of this study is to investigate the anti-angiogenic activity of sclareol in ex *vivo* and *in vivo* models. ***Methods***: Anti-angiogenic activity was investigated *ex vivo* using the rat aortic ring (RAR) and *in vivo* chick chorioallantoic membrane (CAM) assays. In the RAR assay, thoracic aortic rings from male albino rats were treated with sclareol (6.25–200 µg/mL). Suramin (100 µg/mL) and 1% DMSO served as positive and negative controls, respectively. Microvessel outgrowth inhibition and the half-maximal inhibitory concentration (IC_{50}) were determined from dose-response analysis. In the CAM assay, fertilized chicken eggs were treated with sclareol (0.25 and 0.5 mg/mL), and anti-angiogenic activity was measured. ***Results***: Sclareol inhibited microvessel outgrowths in a concentration-dependent manner, producing 91.28±1.51% inhibition at 100 µg/mL compared with 98.22±1.22% for suramin (IC_{50}=32.31 µg/mL; p<0.05). In the CAM assay, sclareol inhibited neovascularization with avascular inhibition zones of 10.33±1.75 mm and 7.16±0.75 mm at 0.5 and 0.25 mg/mL, respectively (p<0.01). ***Conclusions***: Sclareol demonstrated potent concentration-dependent anti-angiogenic activity in both *ex vivo* and *in vivo* models, indicating its potential as a promising natural anti-angiogenic agent and supporting further investigation for the management of cancer and other angiogenesis-dependent disorders.

Keywords: Angiogenesis; Chick chorioallantoic membrane assay; Rat aortic ring assay; Sclareol.

النشاط المضاد لتكوّن الأوعية الدموية لمركب السكليورول: دراسة داخل وخارج الجسم الحي

الخلاصة

الخلفية: يساهم تكوّن الأوعية الدموية في تطور الأورام واضطرابات أخرى تعتمد على تكوّن الأوعية الدموية الدقيقة ، وتمثل المنتجات الطبيعية مصدراً مهماً للعوامل المحتملة المضادة لتكوّن الأوعية الدموية الدقيقة . السكليورول هو ثنائي تربين من نوع اللابدان، وله تأثيرات دوائية متعددة، إلا أن نشاطه المباشر المضاد لتكوّن الأوعية الدموية ما يزال غير موصوف بصورة كافية. **الأهداف**: تقييم النشاط المضاد لتكوّن الأوعية الدموية الدقيقة لمركب السكليورول باستخدام نموذجي حلقة الأبهر في الجرذان خارج الجسم الحي وغشاء الكوريون-السُّلّوي في بيض الدجاج داخل الجسم الحي. **الطرائق**: عولجت حلقات الأبهر الصدري لذكور الجرذان بالسكليورول بتراكيز 6.25–200 ميكروغرام/مل. استُخدم السورامين بتركيز 100 ميكروغرام/مل وضبط 1 % DMSO كمصدر موجب وسالب، على التوالي. تم تحديد تثبيط نمو الأوعية الدقيقة وقيمة IC50 بواسطة تحليل الاستجابة للجرعة. وفي اختبار غشاء الكوريون-السُّلّوي، عولجت البيوض المخصبة بالسكليورول بتركيزي 0.25 و0.5 ملغم/مل، وقيس قطر مناطق انعدام الأوعية. **النتائج**: ثبط السكليورول نمو الأوعية الدموية الدقيقة بصورة تعتمد على التركيز، وبلغ التثبيط 91.28 ± 1.51% عند 100 ميكروغرام/مل، مقارنة بـ98.22 ± 1.22% للسورامين، وكانت قيمة IC50 تساوي 32.31 ميكروغرام/مل. وفي اختبار غشاء الكوريون-السُّلّوي بلغت مناطق انعدام الأوعية 10.33 ± 1.75 مم و7.16 ± 0.75 مم عند 0.5 و0.25 ملغم/مل، على التوالي. **الاستنتاجات**: أظهر السكليورول نشاطاً مضاداً لتكوّن الأوعية الدموية الدقيقة يعتمد على التركيز في كلا النموذجين، مما يدعم إجراء دراسات إضافية لتقييمه كعامل طبيعي محتمل مضاد لتكوّن الأوعية الدموية الدقيقة ومعالجة السرطان والامراض الاخرى المعتمدة على تكون الاوعية الدموية.

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INTRODUCTION

Angiogenesis—new blood-vessel formation from existing blood vessels—is an important process during physiological normal development and pathological diseases. It is necessary for the healing of wounds and repair of tissue, but it plays a role in the development of tumor growth, diabetic retinopathy, and rheumatoid arthritis when it is dysregulated. Tumors larger than about 2-3 mm in diameter are absolutely reliant on the induction of new blood vessels to grow, making anti-angiogenic therapy a compelling idea for oncological treatment [1,2]. Under hypoxic conditions, the VEGF/VEGFR2 axis is the most important molecular pathway responsible for pathological angiogenesis via HIF-1α-mediated transcription. Endothelial cell (EC) survival, proliferation, and migration are further enhanced by three additional angiogenic cascades: PI3K/AKT/mTOR, RAS/RAF/MAPK/ERK, and JAK/STAT3. Few FDA-approved anti-angiogenic drugs, such as bevacizumab and sorafenib, have proved clinically effective, but resistance has been observed with these drugs, as has toxicity, and these factors place a cap on their long-term utility and underscore the need to find new drugs from natural sources [3,4]. Historically, oncology has been a rich field for natural products, particularly for the development of pharmaceutical scaffolds. An estimate of between 1981 and 2019 suggests that about 40% of all drugs for the treatment of cancer that have been approved were

either derived from, or inspired by, natural products [5]. Within the realm of natural product classes, terpenoids have been particularly studied as anti-angiogenic agents; for instance, tanshinone IIA of *Salvia miltiorrhiza*, triptolide, ursolic acid, and koetjapic acid have been found to inhibit VEGF expression and decrease microvessel density in a variety of experimental systems such as the rat aortic ring, the chick chorioallantoic membrane (CAM), and HUVEC tube formation assays [6,7]. This body of evidence provides a pharmacologically sound basis for the diterpene class as a source of leads for anti-angiogenic drugs. Sclareol (labd-14-ene-8α,13-diol; molecular formula $C_{20}H_{36}O_2$; CAS 515-03-7; PubChem CID 163263) is a bicyclic labdane-type diterpene diol isolated predominantly from the inflorescences of *Salvia sclarea L.* (clary sage, family *Lamiaceae*). The pharmacological profile of sclareol has been progressively characterized over the past two decades. It has possessed characteristic bioactivities, including anti-tumor, anti-inflammation, and anti-pathogenic microbes, as well as anti-diabetes and hypertension. Its anticancer activity was first demonstrated against leukemia cell lines, with IC_{50} values below 20 µg/ml at 48 hours, and subsequent investigations documented anti-proliferative and pro-apoptotic effects across breast, colon, gastric, osteosarcoma, and lung cancer models through mechanisms involving G_0/G_1 cell cycle arrest, mitochondrial membrane potential loss, and modulation of the Bcl-2 family [8,9]. However, limited evidence is available regarding the direct anti-angiogenic activity of sclareol—its capacity to suppress new blood vessel formation in functionally validated experimental models. This study aims to evaluate the anti-angiogenic activity of sclareol using the rat aortic ring *ex vivo* assay and the chorioallantoic membrane (CAM) *in vivo* assay, two complementary and widely validated preclinical angiogenesis models, and to characterize its dose-response relationship and potency relative to the established anti-angiogenic control, Suramin.

METHODS

Experimental animals

Male albino rats aged 12–14 weeks were used in this experiment. All the animals were allowed free access to food and tap water. The animals were housed at 28–30°C after being acquired from the animal house facility, College of Veterinary Medicine, Tikrit University.

Ex vivo anti-angiogenesis assay

The experiment was performed following the standard protocol developed by Brown and colleagues with slight modifications [1]. Male albino rats of 12-14 weeks' age were humanely sacrificed by cervical dislocation after chloroform anesthesia. The thoracic aorta was carefully removed, cleaned of any remaining fibroadipose tissue, washed with serum-free medium, and cut into 1 mm thick rings. For this test a 48-well tissue culture plate was used, and ring tissues were placed in the center of the well. 300 µl of (3 mg/ml) fibrinogen and (5 µg/ml) of aprotinin in serum-free medium (M199) were added to each well to avoid fibrinolysis of the vessel fragments. After adding 10 µl of thrombin (prepared at 50 NIH U/mL in 0.15 M NaCl) to each well, the tissue culture was incubated for 30 minutes at 37°C in a humidified 5% CO_2 incubator to solidify and create fibrin gel. Then, 300 µl of medium M199 containing 20% heat-inactivated fetal bovine serum (HIFBS), 0.1% E-aminocaproic acid, 1% L-glutamine, and 1% penicillin/streptomycin were added in each well. Sclareol was dissolved in DMSO and diluted in M199 to give a final DMSO concentration of 1%, then applied to the whole growth medium at different concentrations. The experiment was performed three times with three replicates for each concentration. Culture plates were incubated in a humidified incubator with 5% CO_2 at 37°C for five days, with new medium replacing the top layer medium on day four. Tissue rings treated with Suramin were used as positive controls, and those treated with only 1% DMSO were used as negative controls. The mean percent inhibition to the negative control ±SD was used to assess vessel growth inhibition. The following formula was used to calculate the percentage of blood vessel inhibition:

$$\text{Inhibition } \% = [1 - (A_0/A)] \times 100$$

Where A_0 = the Sclareol's blood vessel growth distance in millimeters; A = the negative control's blood vessel growth distance in millimeters.

Dose-response anti-angiogenesis study of sclareol

Six serial dilutions of Sclareol were prepared in the following concentrations: 200, 100, 50, 25, 12.5, and 6.25 µg/ml. Sclareol was dissolved in DMSO and diluted in the growth medium to make the final DMSO concentration 1%. Wells with no sample treatment received medium with 1% DMSO, which was used as the negative control. The data was represented as mean ± SD. 100 µg/ml Suramin was used as a positive control. The IC_{50}, which is the concentration that inhibits blood vessel growth by 50%, was calculated using the nonlinear regression analysis.

In vivo chorioallantoic membrane assay

After being cleaned of any dirt or debris using 70% ethanol, fertilized chicken eggs from the University of Baghdad's College of Veterinary Medicine's poultry grounds were incubated for 72 hours at 37°C and 60% relative humidity. The eggs were placed horizontally and turned multiple times. After 72 hours, eggs were incubated for an additional 24 hours after 2 milliliters of albumin were drawn out through a tiny hole poked down by the side and sealed to give a better view of the formed CAM, where the CAM splits from the sack that

is attached to the eggshell. The eggs were subsequently incubated for an additional 72 hours after a small square window (3–4 cm in diameter) of the shell was created; Sclareol of two different concentrations that had previously been soaked in round discs of filter paper was placed on the CAM, and the window was covered with sterile surgical adhesive tape. Lastly, the inhibition zone was measured and photographed. High and low concentrations of Sclareol (0.5 and 0.25 mg/ml) were produced, put on filter paper discs, and allowed to dry before being moved to the CAM. Negative controls were discs that only contained the vehicle (1% DMSO). The entire process was carried out in an aseptic environment. The grades for the responses are represented as + (3–6 mm), ++ (7–9 mm), and +++ (>10 mm). Using an image analyzer, the zone of inhibition's size was calculated [3].

Ethical considerations

The experiment was approved by the Animal Ethics Committee of the College of Pharmacy, Mustansiriyah University (Certificate No. 61, Dated December 1, 2025).

Statistical analysis

The statistical analysis was evaluated by GraphPad Prism 10.4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Results were presented as means ± SD (standard deviation) of three independent experiments. The differences between groups were compared by the one-way ANOVA and considered significant at $p< 0.05$. The concentration that inhibited 50% of blood vessels (IC50) was calculated using a nonlinear regression analysis of a sigmoidal dose-response curve (log[inhibitor] versus response, variable-slope/four-parameter model).

RESULTS

Figure 1 and Image 1 show the effect of Sclareol on blood vessel growth of a rat aorta that is presented as mean percentage ± SD. 100 µg/ml of Sclareol was administered to a rat aorta embedded in a complete growth medium of M199. Sclareol significantly inhibited blood vessel growth at day five by 91.28 ± 1.51% as compared with negative control rings that had received only 1% DMSO. 100 µg/ml of Suramin was used as a positive control and inhibited the blood vessel growth by 98.22 ± 1.22%. Table 1, Figure 2, and Image 2 showed the dose-response relationship of the serially diluted Sclareol that was added to the rat aorta rings. Six serial concentrations were used, ranging from 200, 100, 50, 25, 12.5, and 6.25 µg/ml. These concentrations showed significant dose-dependent inhibition activity ($p< 0.05$). Percentages of inhibition were represented as mean ± SD.

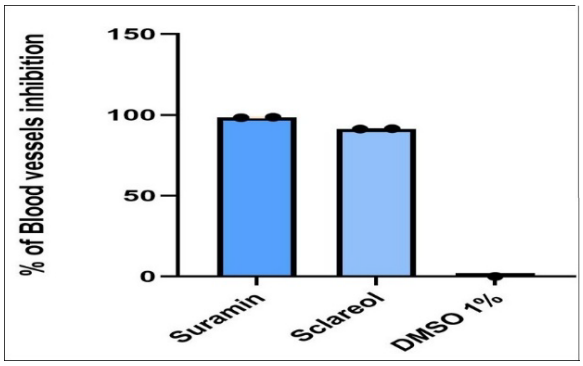

Figure 1: Anti-angiogenesis activity of Sclareol along with the positive and negative controls in ex vivo aortic ring model as a percentage of blood vessels growth inhibition.

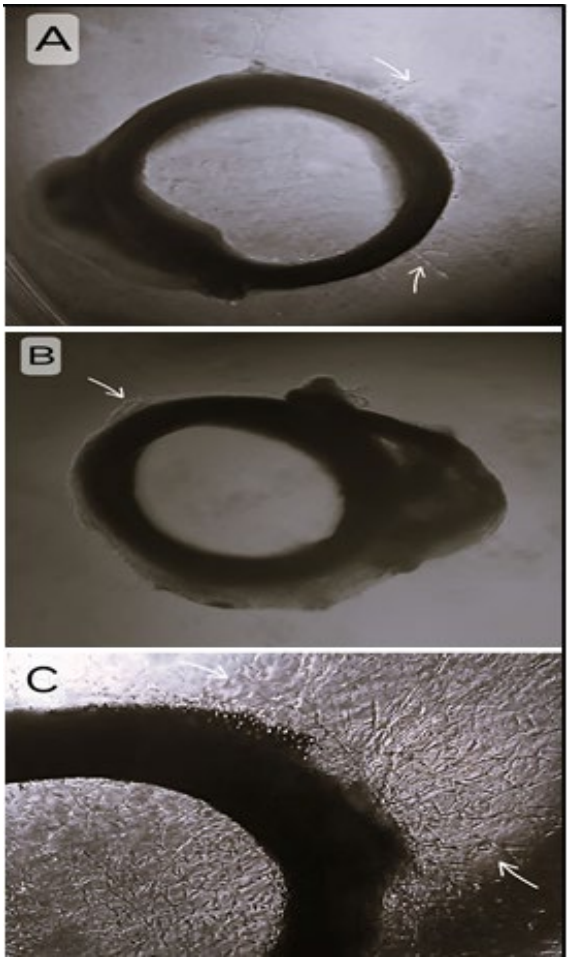

Image 1: Anti-angiogenesis effect of 100 µg/ml of Sclareol (A) in ex vivo aortic ring model along with 100 µg/ml of Suramin as positive control (B) and 1% DMSO as negative control (C). NOTE: white arrows represent the growth of micro-blood vessels.

Table 1: Dose-response data for Sclareol in the rat aortic ring assay.

Concentration (µg/ml)	Blood Vessels Inhibition (%)
200	98.91±0.69
100	91.28±1.51
50	75.63±2.28
25	40.30±1.25
12.5	27.03±1.17
6.25	10.21±1.22

Values are presented as mean±SD.

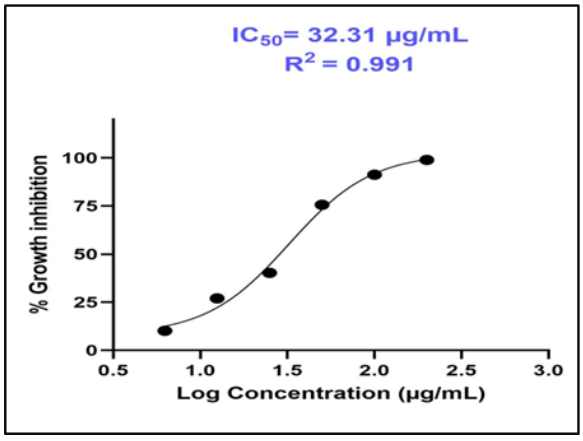

Figure 2: The dose-response curve of serial dilutions of Sclareol in rat aortic rings assay.

Blood vessels were quantified after 5 days of sample addition. The IC_{50} value was calculated by GraphPad Prism 10.4.0 using a nonlinear regression analysis and was equal to 32.31 µg/ml. The result of day 7 of the experiment was used to quantify the zone of inhibition using the previously described scoring system, and it was found to be significant by scoring (+++) and (++) at ($p< 0.01$). The CAM's blood vessels had a pale yellowish color, regression, and disorganization. As seen in Table 2 and Image 3, the inhibition was demonstrated by the development of an avascular zone surrounding the disc that contained 0.5 and 0.25 mg/ml of Sclareol.

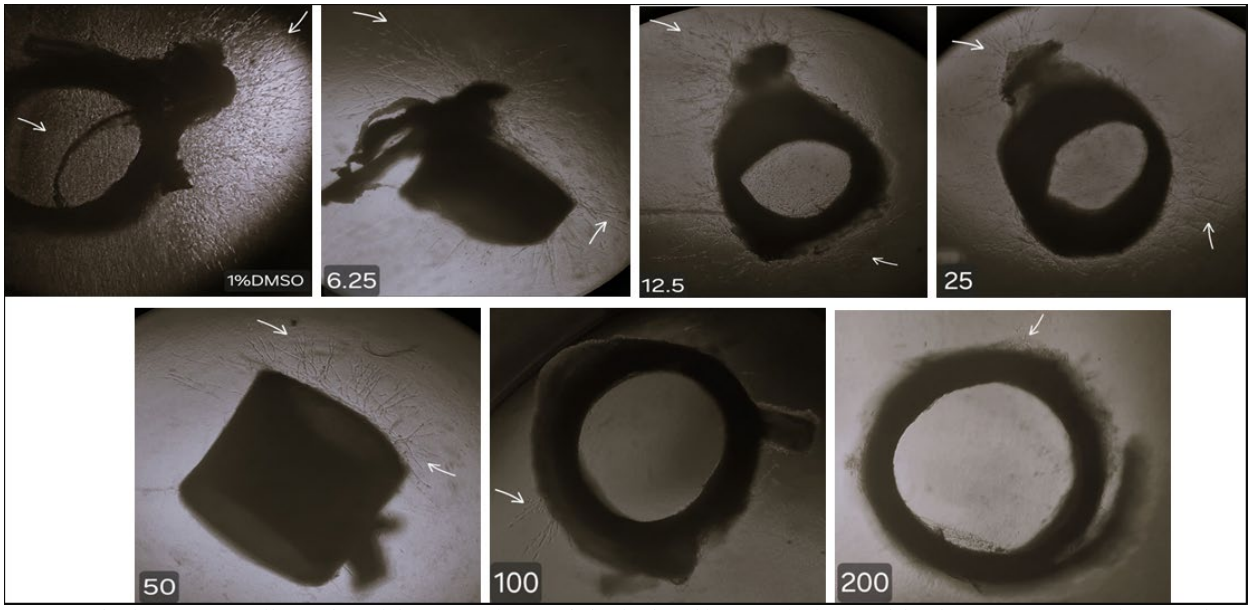

Image 2: The dose response effect to the serial concentrations (µg/ml) of Sclareol in rat aortic rings model and 1% DMSO as negative control. Note: white arrows represent the growth of micro-blood vessels.

Table 2: The results of the inhibitory zone of blood vessel growth in in vivo (CAM) assay for sclareol at two different concentrations.

Egg No.	Inhibition zone (mm) Scoring at 0.5 mg/ml	Inhibition zone (mm) Scoring at 0.25 mg/ml
1	11/ +++	8/ ++
2	10/ +++	7/ ++
3	13/ +++	7/ ++
4	9/ ++	8/ ++
5	11/ +++	6/ +
6	8/ ++	7/ ++
Mean±SD	10.33±1.75/ +++	7.16±0.75/ ++

Score: (+) = 3–6 mm, (++) = 7–9 mm, (+++) = >10 mm (n= 6 for each group)

DISCUSSION

Angiogenesis is a tightly orchestrated process that includes degradation of the basement membrane, proliferation and migration of endothelial cells, tube formation, and vessel stabilization [10]. It is now clear that solid tumors can only grow to a size of 1-2 mm^3 before they start to recruit new vessels and that the angiogenic switch from quiescent to pro-angiogenic phenotype is primarily mediated through transcription of new growth factors, like vascular endothelial growth factor (VEGF) and its receptors, including VEGFR2 [10,11].

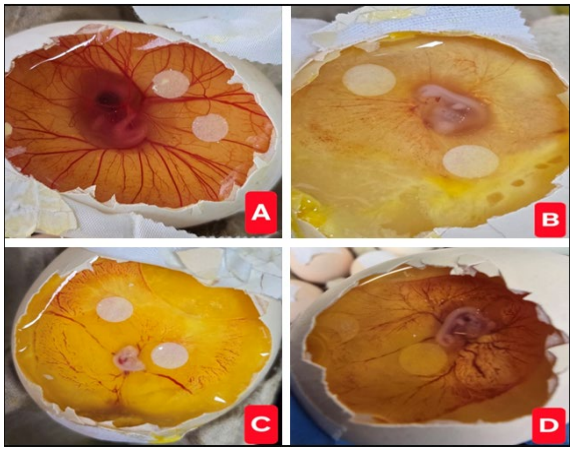

Image 3: The *in vivo* Chick Chorioallantoic Membrane (CAM) assay. (**A**): treated group with DMSO 1% as negative control, (**B**): treated group with 0.5 mg/ml suramin as positive control, (**C**): treated group with 0.5 mg/ml sclareol, and (**D**): treated group with 0.25 mg/ml sclareol.

The unwanted adverse effects, the resistance that is often acquired during therapy, and the limited efficacy with clinically successful anti-VEGF and anti-VEGFR drugs, all of which justify the search for structurally novel anti-angiogenic agents, such as natural origin drugs [12,13]. Of particular interest in this regard are the diterpenes and alkaloids of plant origin that are able to act on more than one step of the angiogenic cascade, including pro-oxidant, pro-apoptotic, and growth factor-signaling pathways, without necessarily acting on a specific receptor, providing a potential benefit over more highly selective synthetic drugs [13,14]. The labdane-type diterpene diol Sclareol was chosen for this purpose because, while the cytotoxic, pro-apoptotic, and ferroptosis-inducing effects of Sclareol have been extensively demonstrated in various cell-based cancer models, including leukemia, breast, colon, gastric, osteosarcoma, lung, and cervical cancer models [15–18], no study had investigated Sclareol's direct effect on angiogenesis in validated *ex vivo* and *in vivo* models. In an *ex vivo* rat aortic ring assay, the magnitude and dose dependence are explored. The rat aortic ring assay was selected because it reflects the sequential events of angiogenesis in a single organotypic system, without requiring the artificial reproduction of cellular and organizational heterogeneity of native vasculature within *in vitro* monolayer assay advantages [19]. The present study showed that Sclareol concentration-dependently inhibited microvessel outgrowth at all 6 concentrations tested (6.25 - 200 µg/ml) on day 5 with 91.28 ± 1.51% inhibition at 100 µg/ml and 98.91 ± 0.69% inhibition at 200 µg/ml and a calculated IC_{50} of 32.31 µg/ml. This positions the anti-angiogenic activity of Sclareol comparable to that observed with the well-known anti-angiogenic reference, Suramin (98.22 ± 1.22%), a prototypic angiogenesis inhibitor already known for many years, which acts mainly by the steric blocking of the binding of heparin-dependent growth factors VEGF, basic fibroblast growth factor, bFGF, and platelet-derived growth factor, PDGF, to cell-surface receptors [20–22]. Given the differential chemical structure, the inhibitory potency of Sclareol compared to a well-studied growth-factor antagonist is comparable, implying that Sclareol could act structurally, if not functionally, on the same growth-factor-dependent signalling nodes in the sprouting endothelium. The anti-angiogenic effects of Sclareol are most likely linked to the fact that it has been demonstrated to inhibit the accumulation of HIF-1α protein in hypoxic cells of cancerous tissues. In A549 human lung cancer cells exposed to $CoCl_2$-induced hypoxia, sclareol significantly reduced the protein level of HIF-1α dose-dependently, while leaving HIF-1α mRNA levels unchanged, thus suggesting a post-translational mechanism of HIF-1α destabilization [23]. This effect of HIF-1α suppression by Sclareol would predictably deplete the concentration of VEGF-A in the aortic ring culture media to bind to VEGFR2 on EC surfaces, thereby dampening pro-angiogenic signaling pathways such as PI3K/AKT/mTOR and RAS/RAF/MAPK/ERK. Afshari et al. (2020) also reported that sclareol inhibits STAT3 phosphorylation in the MCF-7 breast cancer cells, further supporting its anti-angiogenic properties. In both tumor cells and endothelial cells, STAT3 regulates the expression of key genes that promote survival and invasion of endothelial cells in the angiogenic process, such as VEGF-A, MMP-9, Bcl-xL, and Survivin [16]. Inhibition of STAT3-dependent transcription of the VEGF-A gene would be another indirect pathway by which sclareol could inhibit the angiogenic microenvironment in the rat aortic ring model. Furthermore, Sclareol inhibits the nuclear translocation of NF-κB and the subsequent production of inflammatory cytokines (TNF-α, IL-6, and IL-1β), a mechanism that is relevant to its anti-angiogenic effect. Both TNF-α and IL-6 are also known potent angiogenic stimuli, which upregulate E-selectin and VEGF-A via NF-κB in endothelial cells, and whose suppression by Sclareol would create an overall anti-angiogenic milieu in the *ex vivo* system [25]. To further confirm the anti-angiogenic activity of Sclareol, the CAM assay was used. Both concentrations of Sclareol, 0.5 mg/ml and 0.25 mg/ml, produced an avascular zone around the treated filter paper discs, with 10.33 ± 1.75 mm (scored +++) and 7.16 ± 0.75 mm (scored ++) mean inhibition zones, respectively, which were significant and visually striking in the chorioallantoic membrane (CAM) assay. The morphological signs of effective anti-angiogenic activity—the pale yellowish coloring, the regression of vessels, and the disorganization of vessels—are characteristic of the anti-angiogenic activity of the drug, support the disruption of the VEGF pathway, and are consistent with what was observed in treated CAMs [26]. The CAM assay is a model between *in vitro* and mammalian *in vivo* models, with the benefit of an easy-to-develop vascular system in an immunologically privileged environment. It has been confirmed recently with methodological reviews as a valid method for screening anti-angiogenic compounds, which is cost-effective and ethically acceptable [27]. A standardized scoring system is in use with a threshold of +++ (>10 mm), ++ (7–9 mm), and + (3–6 mm) and has been extensively utilized in anti-angiogenic phytochemical research [3]. Several other phytochemicals have been shown to have similar anti-angiogenic properties in the CAM model. *Clerodendrum infortunatum* root extract containing the terpenoid and flavonoid compounds showed dose-dependent vascular regression in the CAM model [28]. Likewise, four medicinal plant extracts were compared in the CAM model, and it was found that terpenoid-rich extracts of *Curcuma longa* had the highest anti-angiogenic score, followed closely by *Zingiber officinale* [29]. A review published in Frontiers in Pharmacology in 2022 summarized the anti-tumor properties of Sclareol in several cancer cell lines, including its ability to induce apoptosis (intrinsic mitochondrial pathway), to arrest the cell cycle at the G1/S juncture, to suppress NF-kB (anti-inflammatory), and to inhibit STAT3 signaling [9]. Furthermore, in 2024, an article in Food Frontiers reported several classes of terpenoids that have been shown to exert anti-angiogenic effects through multiple mechanisms, such as inhibition of VEGF-A secretion, reduction in

VEGFR2 kinase activity, inhibition of the PI3K/AKT signaling pathway, and stabilization of HIF-1α [7]. The present study adds, for the first time, direct experimental evidence for Sclareol's anti-angiogenic potential at both the tissue (*ex vivo*) and organismal (*in vivo*) levels, extending its known pharmacological profile to encompass the vascular compartment.

Limitations and Future Perspects

Even though the current study offers strong *ex vivo* and *in vivo* evidence of anti-angiogenic activity of Sclareol, there are still a number of limitations that need to be resolved in further research. First, precise molecular targets and intracellular signaling pathways should be demonstrated to understand by which mechanism Sclareol inhibits vascular sprouting; therefore, Western blotting, gene expression analysis, and immunohistochemistry analysis are needed to evaluate whether HIF-1α/VEGF-A axis inhibition, direct VEGFR2 kinase inhibition, or STAT3 suppression are the mechanisms of action as hypothesized in previous literature mentioned above in the vascular tissue context. The second limitation is the absence of cellular-level mechanistic data such as *in vitro* endothelial cell assays (HUVEC proliferation, migration, and tube formation). Future studies are advised for several lines of investigation, such as *in vivo* tumor xenograft study to assess anti-tumor activity and safety profile in systemic context, pharmacokinetic studies to evaluate bioavailability, absorption, distribution, metabolic fate, and excretion, and the use of Sclareol combination with established anti-angiogenic agents to evaluate additive or synergistic activity in order to decrease the dose of clinically approved drugs and enhance their anti-angiogenic activity.

Conclusion

This study highlights for the first time the concentration-dependent and potent anti-angiogenic activity of Sclareol in both the rat aortic ring *ex vivo* model and the CAM *in vivo* model. Sclareol at 100 µg/ml showed microvessel outgrowth inhibition of 91.28 ± 1.51%, which is close to the inhibitory activity of the gold standard drug Suramin. In the CAM assay, a high level of avascular zone formation was shown at both concentrations. Based on its well-established pharmacological profile, which includes suppression of HIF-1α, inhibition of STAT3, and NF-κB-mediated anti-inflammatory activity, sclareol is presented herein as a multi-targeted plant-derived anti-angiogenic drug candidate with great potential in further preclinical and translational studies.

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Conflict of interests

The authors declared no conflict of interest.

Funding source

The authors did not receive any source of funds.

Data sharing statement

The data that supports the findings of this study are available from the corresponding author upon a reasonable request.

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