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RESEARCH ARTICLE

Anti-angiogenic activity of petroleum ether fraction of *Mesembryanthemum cordifolium* extract: *Ex vivo* study

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Abstract

Angiogenesis refers to the formation of new blood vessels from pre-existing ones. This process is essential for growth and tissue repair. However, it also plays a crucial role in supplying nourishment and oxygen to tumours, thereby facilitating their growth and metastasis. Among more than 1800 species in the *Aizoaceae* family, *Mesembryanthemum cordifolium* is one of the most abundant, owing to its pharmacological properties. Although the ethnobotanical use of *M. cordifolium* suggests therapeutic potential, the specific anti-angiogenic activity of its constituent fractions, particularly the non-polar components, has not been thoroughly investigated. The present study aimed to bridge this gap by evaluating the anti-angiogenic effect of the petroleum ether fraction of *M. cordifolium*. The whole plant was sequentially processed by defatting with n-hexane, followed by exhaustive extraction with 85 % methanol using a Soxhlet apparatus. The crude methanolic extract was then subjected to liquid-liquid fractionation to obtain petroleum ether, chloroform and ethyl acetate fractions. The petroleum ether fraction was phytochemically characterized using Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC). The anti-angiogenic potential was assessed using the *ex vivo* rat aortic ring assay. A dose-response relationship was determined by testing the fraction at five concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) against a negative control (1 % DMSO). Compared with the negative control, the results showed that the petroleum ether extract of *M. cordifolium* inhibited the growth of blood vessels in a concentration -dependent manner. The study demonstrates that the petroleum ether fraction of *M. cordifolium* possesses potent anti-angiogenic properties *ex vivo*.

Keywords: angiogenesis; ex vivo study; *M. cordifolium;* petroleum ether fraction; soxhlet

Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. This mechanism is essential for embryogenesis, tissue regeneration and wound healing (1). Although angiogenesis is necessary for normal body function, it is tightly regulated. In diseases such as cancer, this balance is lost and aberrant angiogenesis can become a driving force in disease progression (2). Tumors need their own vasculature in order to grow beyond a small size and they do so by giving off pro-angiogenic signals. Some key mediators of these include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-beta 1 (TGF-β1) (3). These factors stimulate adjacent endothelial cells, initiating a series of events including degradation of the vascular basement membrane, proliferation and migration of endothelial cells into the surrounding matrix, formation of new tubular structures and finally, stabilization of the new vessel with a mature basement membrane and supporting pericytes (4).

Given that angiogenesis is essential for tumor growth, invasion and metastasis, anti-angiogenesis has become a central paradigm in current cancer treatment. The strategy of anti-angiogenic therapy aims to block the blood supply to the tumor, thereby inducing hypoxia and nutrient deprivations to suppress tumor growth or induce cell death (5). This approach has been proved by medications that are approved in clinics. For example, suramin, a polysulfonated naphthyl urea, has demonstrated anti-cancer activity by inhibiting ligand receptor interactions, such as VEGF binding to its receptor (6, 7). Similarly, monoclonal antibodies targeting and neutralizing VEGF, such as Bevacizumab (Avastin®), are now standard components of treatment for several cancers. Nevertheless, due to the development of drug resistance and unfavorable side effects, there remains a need for novel, efficient and safe anti-angiogenic molecules.

Natural products, specifically those derived from medicinal plants constitute an enormous and historically relevant resource for drug discovery, offering extensive chemical diversity and novel molecular architectures. Numerous studies have reported the anti-angiogenic effects of plant-based compounds. *M. cordifolium*, also called the heartleaf ice plant, is a succulent species belonging to the family of Aizoaceae. *M. cordifolium* is a versatile species that thrives occupationally in different challenging climatic conditions, making it a good study area for botanical and environmental research. Found naturally in the coastal deserts of South Africa, it has successfully invaded various

habitats across the globe. This species' adaptability is made even more interesting by the broad scales of temperature and environments in which it has spread from southern and northern Europe to places in the Southern United States like California, Oregon and Florida and even to locations as far away as Australia and Hawaii (8).

The species' remarkable adaptability is paralleled by its reported pharmacological activities. Previous studies on *M. cordifolium* extracts have revealed strong antioxidant and cytotoxic activities against cancer cell lines. These cytotoxic effects indicate the potential existence of strong bioactive agents that can stop cell growth, a basic mechanism in cancer and angiogenesis. However, despite encouraging reports on its cytotoxic and antioxidant effects, a knowledge gap appears to float about the specific anti-angiogenic activity profile of *M. cordifolium* petroleum ether fraction in the scientific literature. In addition, much of the earlier work has been conducted on polar extracts. Investigation of the bioactivity of the non-polar and lipophilic contents of the plant can be achieved by isolation of the organic contents of the plant using a solvent such as petroleum ether. Such compounds may exhibit specific mechanisms of action responsible for the observed biological effects.

Accordingly, the present study aimed to fill this gap and to explore for the first time the anti-angiogenic potential of *M. cordifolium* petroleum ether fraction. The study aimed to demonstrate the effect of this fraction on micro vessel sprouting using the *ex vivo* rat aortic ring assay and establish a foundation for its potential as a new source of bioactive substances possessing anti-angiogenic properties. The *M. cordifolium* plant is represented in Fig. 1.

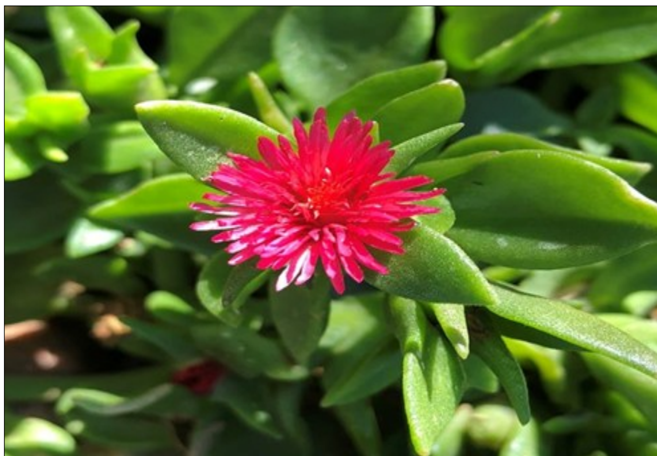

Fig. 1. *M. cordifolium* plant.

Materials and Methods

Collection of plant materials

The taxonomic identification of the plant was carried out by Prof. Assist. Dr. Israa Abdul Razzaq Majeed, Department of Biology, College of Science, University of Baghdad. The plant sample was collected in the summer of 2023 from Al-Diwanyah City. The collected material was air-dried in the shade for sixteen consecutive days, followed by grinding with an electric blender. The powdered material was weighed and subsequently subjected to extraction.

Extraction

Extraction is the first and most important stage in the analysis process to separate and identify the specific chemical components of medicinal plants (9, 10). 100 g of the dried, powdered plant material were first macerated with 1000 mL of n-hexane for one day to eliminate any extra oil and plant debris. Next, it was subjected to Soxhlet extraction using 85 % methanol using 500 mL (11). The resulting extract was filtered through Whatman No.1 filter paper to get a clean extract.

Since raw extract contains a wide variety of chemical components with different polarities, it is recommended to fractionate the extract before chromatographic analysis to isolate the main classes of plant components based on their solubility and polarity differences. This will allow for a comprehensive screening of the plants' phytochemical profile (12). Liquid–liquid partitioning of the crude extract was carried out in a separatory funnel using solvents of increasing polarity: petroleum ether (60 °C-80 °C), chloroform and ethyl acetate. Each fraction was obtained with 250 mL of solvent and the process was repeated several times. The fractions were dried over anhydrous sodium sulfate, filtered and concentrated using a rotary evaporator to remove residual moisture. The dried fractions were weighed and stored for further analysis.

Comprehensive phytochemical analysis of the petroleum ether fraction

A preliminary phytochemical study was conducted to identify primary and secondary metabolites in the petroleum ether fraction. The presence of triterpenoids was determined using Salkowski test, in which a small amount of crude extract was dissolved in 2 mL of chloroform, followed by careful addition of 3 mL concentrated H_2SO_4. Steroids were detected using Liebermann-Burchard test, where a small amount of crude extract was mixed with 5 mL of chloroform. The chloroform layer was then dried with anhydrous sodium sulfate. Finally, 10 drops of acetic anhydride and 2 drops of concentrated H_2SO_4 were added (13-15). The results are summarized in Table 1.

Table 1. Chemical tests used for preliminary phytochemical screening

Constituent class	Chemical Tests	Observations
Terpenoids	**Salkowski test**	Reddish brown colour due to the oxidation reaction
Sterols and steroids	**Liebermann-Burchard test**	The bluish-green solution resulted from the presence of a steroidal nucleus as oxidation occurred in steps

Identification of compounds by HPLC

HPLC analysis was conducted at the Scientific Centre for Chemical Analysis, Baghdad, Hayy Al-Jamia', to identify the active constituents of the petroleum ether fraction. To identify the active components in the fractions, HPLC was used. Under certain circumstances, the retention times of the tested samples were compared to those of reference samples.

HPLC conditions for the petroleum ether fraction

The mobile phase consisted of an isocratic elution with 95 % HPLC-grade acetonitrile and 5 % ultrapure water. The system was operated at a constant flow rate of 1 mL/min and the column oven at 25 °C. The injection volume for all samples was 20 µL and UV detection was performed at a wavelength of 280 nm. The dried petroleum ether fraction was accurately weighed and dissolved in HPLC-grade methanol to a final concentration of 1 mg/mL. The solution was sonicated for 10 min and filtered through a 0.45 µm syringe filter prior to injection. An authentic standard of stigmasterol was prepared under identical conditions. Separation

was performed on a C18 column (5 µm particle size, 250 x 4.6 mm).

Gas chromatography analysis of the petroleum ether fraction from the whole plant of *M. cordifolium*

Secondary metabolites were identified using GC-MS analysis at the Ibn AL-Bitar Centre, Ministry of Industry and Minerals. Compound identification was based on comparison of experimental mass spectra with those in the National Institute of Standards and Technology (NIST) 2017 Mass Spectral Library. The GC–MS software generated chromatograms of intensity versus retention time and compounds were identified from the peak profiles.

Exploring anti- angiogenic effects with the rat aorta ring assay (RAR) *ex vivo* assay

All experimental design and animal handling procedures were conducted in accordance with the "Research Ethical Approval Form" and the approved protocol from the University of Baghdad, College of Pharmacy, Baghdad, Iraq. The ethical approval number RECAUBCP1092023G was issued on 10/9/2023. Male albino rats, aged between 12 and 14 weeks, were used. The RAR assay, performed in the tissue culture laboratory, was employed to assess anti-angiogenic activity, following the method with minor modifications (16).

Preparing RAR from albino rats for laboratory analysis

Aortic rings were prepared according to previously explained methods (17), using male Sprague-Dawley rats. The thoracic aorta was excised, sliced horizontally into 1 mm sections and washed repeatedly in Hanks' balanced salt solution (18). Each aortic ring was placed in a well of a 48-well tissue culture plate (Costar Corning, USA) containing 500 µL of serum-free M199 medium with 3 mg/mL of fibrinogen was added to each well. Fibrinogen is a component that aids in the formation of blood clots. To stop blood clots from breaking down, 5 µg/mL of aprotinin was also administered. 10 µL of thrombin (50 NIH U/mL in 0.15 M sodium chloride) were added to create a gel-like environment around the aorta section. After inserting the blood artery section into the fibrin gels, 0.5 mL of medium M 199 was added to every well. This medium included 20 % heat-inactivated fetal bovine serum (HIFBS), 0.1 % aminocaproic acid, 1 % L-Glutamine and 60 µg/ml gentamicin (to avoid contamination).

The DMSO was used to create three separate solutions of plant extracts. To see how the extracts affected the formation of blood vessels, they were placed into the wells. To make sure each extract was consistent, it was tested six times. Each of the three components (petroleum ether, chloroform and ethyl acetate) had a stock solution that had 10 mg/mL of the respective compound. DMSO was used to dissolve each sample and then the M199 medium was added for dilution until the final concentration reached 1 %. At a concentration of 100 µg/mL, plant extracts were added to the medium. Six times this addition was made. In a humidified incubator, the seeded rings were cultivated at 37 °C with 5 % CO_2, simulating the conditions found inside a rats' body. As mentioned before, after four days, the top layer was replaced with a freshly made medium. As a control, certain wells were designed to contain solvent alone, without any plant extracts. In addition, for comparison, the well-established anti-angiogenic drug Suramin was used as a reference standard.

The findings were examined under a microscope on the sixth day. Using a computer and a digital imaging instrument at a 10x magnification, the proliferation of blood vessels was assessed. A study developed an approach to evaluate the measurement of blood vessel inhibition (19). Inhibition percentages as a mean compared to the negative control indicate the results. With six copies of each sample, the procedure was repeated twice, using the given formula to get the inhibition percentage (20). The percentage of blood vessel inhibition is equal to: -

$$\text{Blood vessels inhibition } \% = 1 - (A0/A) \times 100 \quad \text{(Eqn. 1)}$$

In this case, A0 is the millimeter-measured growth distance of blood vessels in the sample material and A is the millimeter-measured growth distance in the negative control.

Evaluating the effects of petroleum ether fraction through dose-response analysis with the RAR test

A concentrated stock solution in DMSO was serially diluted with M199 medium (containing 1 % DMSO) to yield final concentrations of 200, 100, 50, 25, 12.5 and 6.25 µg/mL. Control wells contained only 1 % DMSO in M199 medium.

Statistical analysis

The mean + standard deviation (SD) is used to show the data. One -way ANOVA and Tukey's post-hoc test (t-test) were used for statistical analysis. Results were considered significant if $p < 0.05$. A logarithmic equation was used to find the half-maximal inhibitory concentration (IC50), which is the concentration needed to stop cell growth, angiogenesis and tube formation by half. The math for this was based on a curve that showed the relationship between the substances' concentration (in µg/mL) and its percentage of inhibition, where Y is the percentage of inhibition and X is the concentration. SPSS software, version 21.0, was used for all statistical studies.

Results

Extraction and fractionation yields

From the initial 100 g of dried, powdered *M. cordifolium*, Soxhlet extraction with 85 % methanol yielded 11.2 g of crude extract, which appeared as a dark green, viscous semi-solid. Subsequent fractionation yielded 4.0 g of petroleum ether fraction, 2.1 g of chloroform fraction and 1.2 g of ethyl acetate fraction. The petroleum ether fraction, being the primary focus of this study, was used for all subsequent biological and chemical analyses.

Comprehensive phytochemical analysis of the petroleum ether fraction

Based on the results, it can be observed that the petroleum ether fraction of *M. cordifolium* has steroids and terpenoids (Table 1).

Identification of compounds by HPLC

HPLC analysis confirmed the presence of stigmasterol in the petroleum ether fraction (Fig. 2 & Table 2).

Gas Chromatography analysis of the petroleum ether fraction from the whole plant of *M. cordifolium*

GC-MS analysis identified eight bioactive phytochemical compounds in the petroleum ether fraction of *M. cordifolium* (Fig. 3 & Table 3).

Exploring anti-angiogenic effects with the rat aorta ring assay *ex vivo* assay

The anti-angiogenic potential of the petroleum ether fraction was

Table 2. Retention time of steroidal compounds and petroleum ether fraction

Compound	The retention time of the standard	The retention time of the sample
stigmasterol	02:09.3	02:03.9

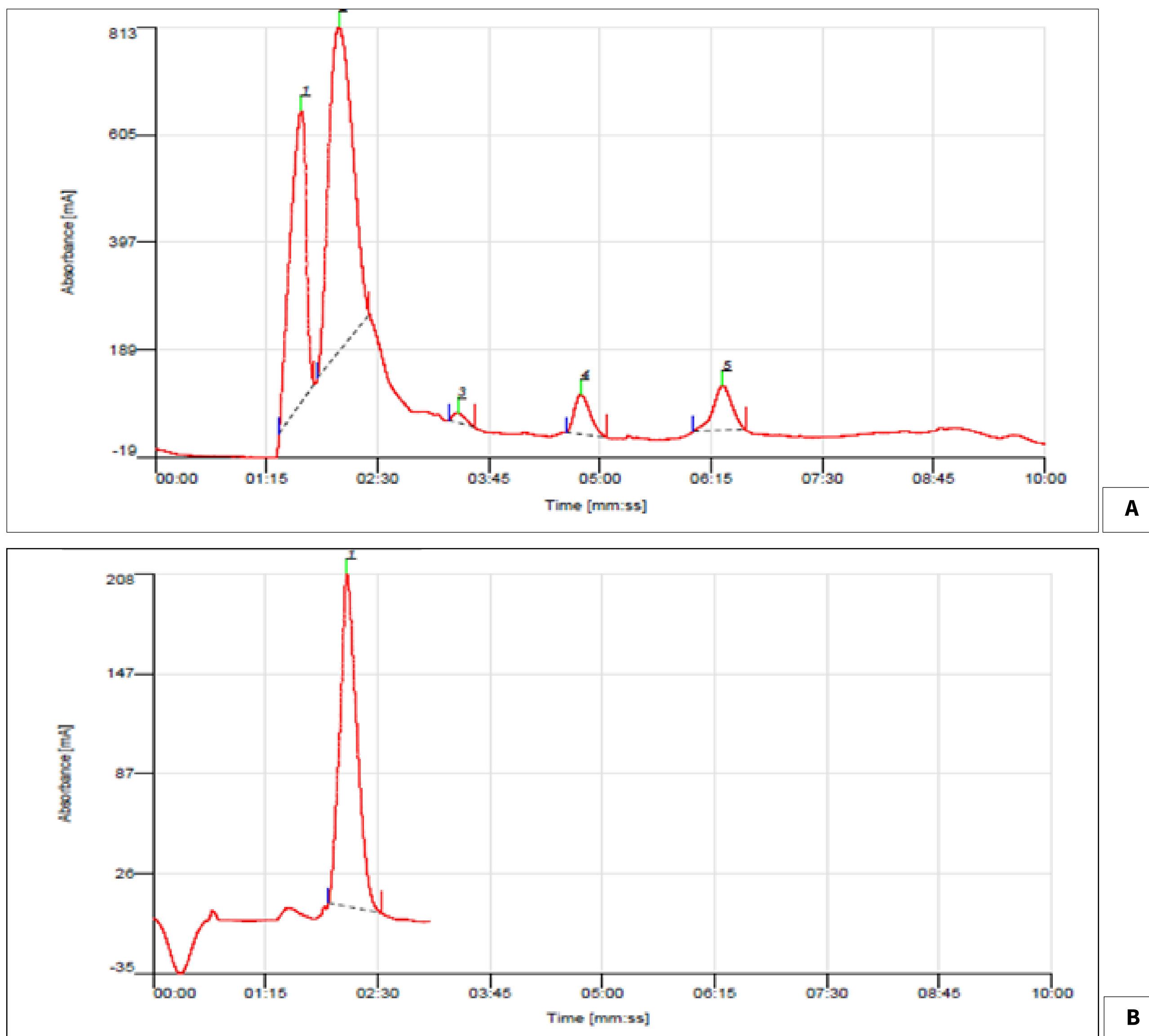

Fig. 2. HPLC chromatogram for A: petroleum ether fraction and B: stigmasterol standard.

Table 3. List of compounds identified by GC-MS analysis of the n-hexane extract of *M. cordifolium*

Srno.	Rt	Compounds	Classes	Chemical formula	Similarity index	Pharmacological activity
1	20.45	Hexadecanoic acid, methyl ester	fatty acid methyl esters	$C_{17}H_{34}O_2$	97	antibacterial (21) antioxidant (22)
2	21.027	Pentadecanoic acid	fatty acid	$C_{15}H_{30}O_2$	90	antibacterial and antifungal activity (23)
3	20.592	4-methyl- Isoamyl cyanide		$C_6H_{11}N$	91	antimicrobial activity
4	11.542	Dimethylamine	simple aliphatic amine	$C_2H_{10}BN$	95	its role in platelet activation, particularly in the context of chronic kidney disease (CKD) (24)
5	17.942	2-Nonanone	ketone	$C_9H_{18}O_2$	95	antibacterial and antifungal activities (25)
6	19.333	Palmitic acid	saturated fatty acid	$C_{16}H_{32}O_2$	90	anti-inflammatory, antioxidant and immune-enhancing effects (26)
7	22.966	Methyl stearate	fatty acid esters	$C_{19}H_{38}O_2$	92	antioxidant, antifungal (27)
8	20.592	omega. -Decenol	aliphatic alcohol	$C_{10}H_{20}O$	96	anti-cancer, anti-oxidant (28)

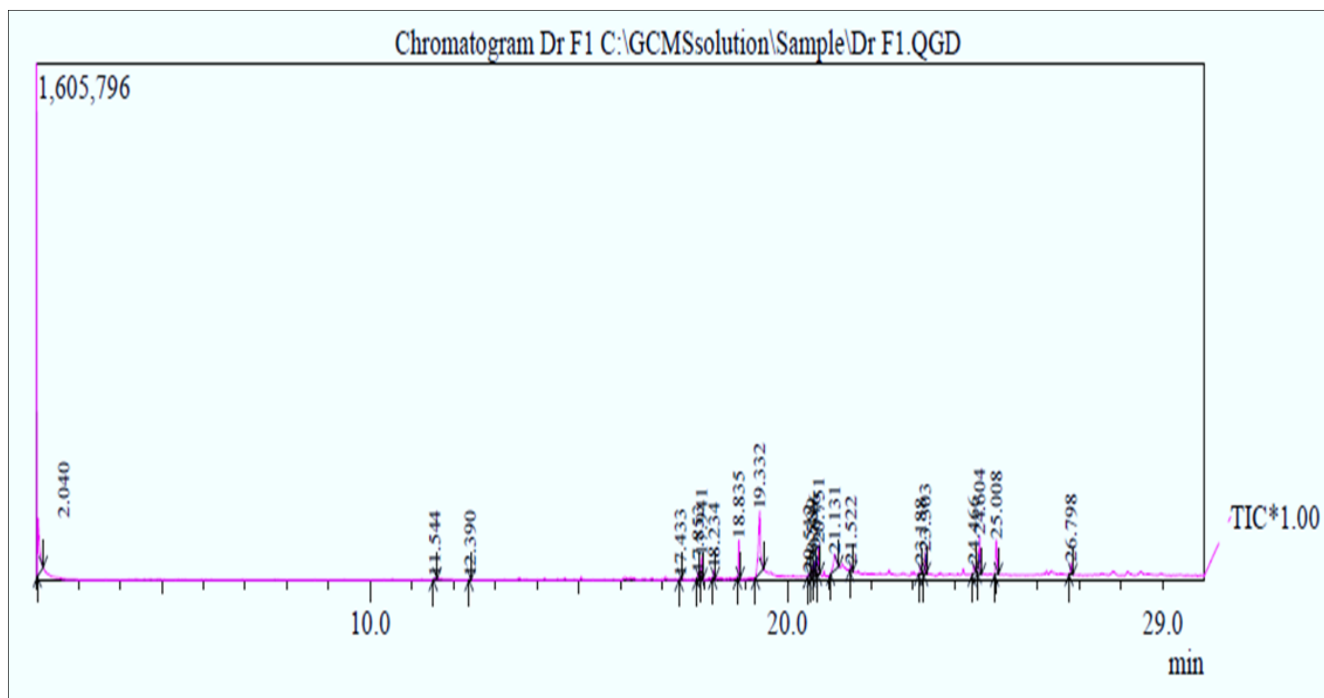

Fig. 3. GC-MS identification of the petroleum ether fraction from the whole plant of *M. cordifolium*.

Table 4. Inhibition percentages (mean ± SD) across serial dilutions of petroleum ether fraction

Concentration µg/mL	% of inhibition (mean ± SD)
200	76 ± 0.6
100	76 ± 0.6
50	45 ± 0.8
25	18 ± 0.2
12.5	10 ± 0.4
6.25	3 ± 0.2

evaluated by constructing a dose-response curve using six concentrations administered to cultured rat aortic rings. The treatment produced a significant, dose-dependent inhibition of micro vessel outgrowth compared with the negative control, with statistical significance observed on day five ($p < 0.001$) (Table 4 & Fig. 4).

The IC_{50} value was determined from the logarithmic equation $y = 1.2602X - 0.4409$, where Y represents the percentage inhibition and X represents the concentration. The calculated IC_{50} was 39.31 µg/mL (Fig. 5).

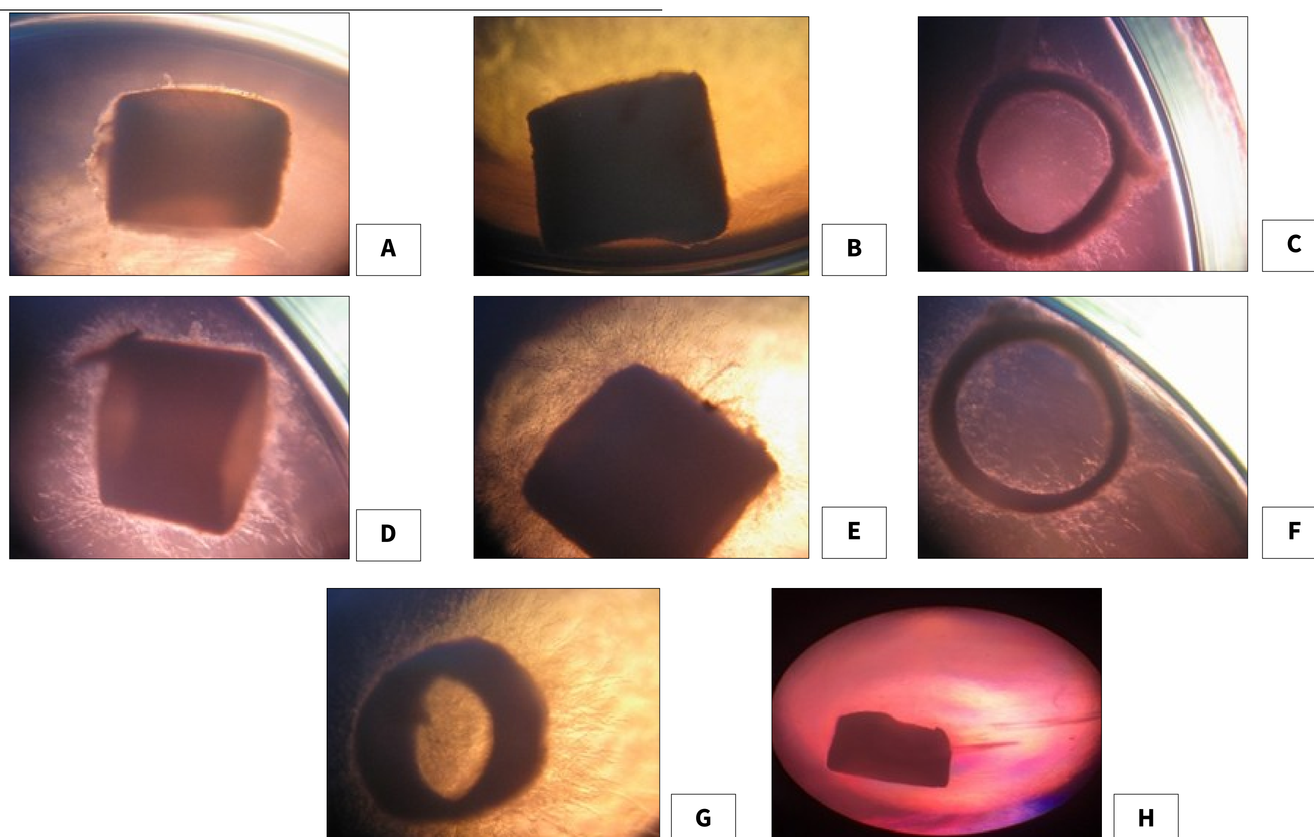

Fig. 4. Anti-angiogenesis effect of petroleum ether fraction in different concentrations (serial dilution) (A)=200 µg/mL, (B)=100 µg/mL, (C)=50 µg/mL, (D)=25 µg/mL, (E)=12.5 µg/mL, (F)=6.25 µg/mL, (G)=negative control, (H)=suramin {positive control}.

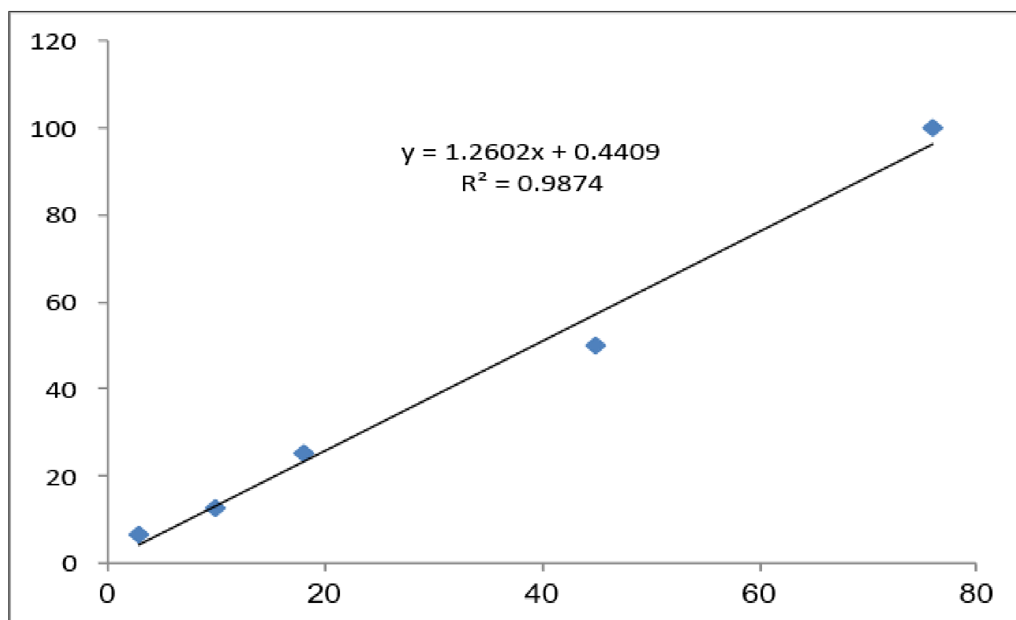

Fig. 5. Dose-response curve of petroleum ether fraction.

Discussion

The global burden of cancer continues to increase, creating an urgent need for the discovery of novel and effective therapeutic strategies. Based on figures from the World Health Organization (WHO), it is projected that the global number of newly diagnosed cancer cases will surpass 27 million by the year 2040 (29). Natural products, particularly those derived from medicinal plants, have long been a fertile source of anti-cancer drug leads due to their inherent bioactivity and chemical diversity (30, 31).

The primary finding of this study demonstrates that the petroleum ether fraction of *M. cordifolium* exerts a significant, dose-dependent inhibitory effect on micro vessel formation in the *ex vivo* rat aortic ring assay. The potency of this fraction was quantified with an IC_{50} value of 39.31 µg/mL. According to the US National Cancer Institute (NCI) guidelines for crude extracts, an IC_{50} value below 50 µg/mL is considered moderately potent and warrants further investigation. This result provides the first direct evidence of the anti-angiogenic properties of *M. cordifolium* and suggests it is a promising candidate for further research (32).

The phytochemical analysis of the active petroleum ether fraction provides critical insight into the potential source of this bioactivity. Preliminary screening confirmed the presence of steroids and terpenoids, which was further substantiated by HPLC and GC-MS analyses. The identification of stigmasterol by HPLC is particularly noteworthy. Stigmasterol, a widely studied phytosterol, has been reported to possess anti-cancer properties through various mechanisms, including the inhibition of key angiogenic pathways like VEGF and Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (Akt) (33). One study demonstrated that stigmasterol could inhibit endothelial cell migration and tube formation, which are critical steps in angiogenesis. Our findings align with this existing literature, suggesting that stigmasterol is likely a major contributor to the anti-angiogenic effect observed in our fraction (34).

The GC-MS analysis identified eight additional compounds. While stigmasterol may play a primary role, the overall potent activity of the fraction could also result from synergistic interactions among these various lipophilic compounds. The fact that significant activity was observed in the non-polar petroleum ether fraction itself is a key finding, as many previous studies on medicinal plants have focused on more polar extracts. This highlights the therapeutic potential of the often-overlooked lipophilic constituents of *M. cordifolium* (35).

The results extend and provide a potential mechanistic basis for previous findings. For instance, a study reported that *M. cordifolium* extracts exhibited cytotoxicity against human cancer cell lines (36). The demonstration of anti-angiogenic activity offers a specific pathway through which this anti-cancer effect might be exerted, by cutting off the tumors' essential blood supply. To our knowledge, this is the first report to specifically validate the anti-angiogenic activity of *M. cordifolium*.

Conclusion

This study provides the first direct evidence that the non-polar petroleum ether fraction of *M. cordifolium* possesses significant anti-angiogenic properties. The extract demonstrated a potent, dose-dependent inhibition of micro vessel formation in the *ex vivo* rat aortic ring assay, with a calculated IC_{50} value of 39.31 µg/mL. This finding is a key step forward, establishing the lipophilic constituents of *M. cordifolium*, such as stigmasterol and other identified terpenoids, as a valuable source for anti-cancer research.

The essential future direction for this work is to proceed with the bioactivity-guided fractionation of this active extract. The goal is to isolate and identify the specific lead compound or compounds responsible for the observed effect. Subsequently, these purified molecules must be validated with *in vivo* models to confirm their therapeutic efficacy and potential for development into new, targeted anti-angiogenic therapies.

The approach of using a non-polar solvent to explore often-neglected lipophilic compounds like steroids and terpenoids marks a deliberate shift from the more common focus on polar extracts. The discovery of potent anti-angiogenic activity in this fraction validates this approach, confirming that valuable therapeutic agents reside in the plant's non-polar constituents.

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Authors' contributions

HIJ conceived and designed the study, performed the formulation experiments and drafted the manuscript. AHA supervised the research work, provided conceptual guidance and critically reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical issues: This article does not contain any studies involving human participants or animals performed by any of the authors. Therefore, no ethical approval was required.

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