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ORIGINAL ARTICLE

Ameliorative effect of *Alternanthera sessilis* against Angiogenesis using *In vitro* and *In vivo* methods

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ABSTRACT
Angiogenesis is a characteristic of cancer essential for both tumour growth and metastasis. This study evaluated antiangiogenic activity of Alternanthera sessilis. A crude extract of Alternanthera sessilis was produced, and its antiangiogenic effects were evaluated using the ex vivo rat aortic ring assay and the in vivo chicken embryo chorioallantoic membrane (CAM) assay. They were examined to determine their direct cytotoxic effects on MCF7 cells via the MTT test. The ethanol extract demonstrated complete inhibition of blood vessel development from the basic tissue explants in to the rat aortic ring assay with a dosage of 100µg/mL, whereas the other extracts exhibited negligible antiangiogenic activity. The extract of ethanol was examined at different doses and had a substantial dose-dependent impact. The CAM assay aligned with the findings of the aortic ring assay, as the ethanol extract exhibited a substantial suppression of neovascularisation. The extract demonstrated anti-proliferative action against MCF7 breast cancer lines of cells in the MTT experiment. The results of both experiments indicated that the ethanol extract greatly decreased vascularization. Additional research on the extract from ethanol would be advantageous for separating the active compound accountable for the inhibition.
Key words: *Angiogenesis, CAM assay, Rat aortic ring assay, MTT assay*

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INTRODUCTION

Within current society, coronary heart disease, which is caused by increasing atherosclerosis, continues to be the leading cause of death. The main lipid condition affecting the arteries is atherosclerosis [1]. Numerous clinical processes, including thrombosis, inflammation, atherosclerosis, and ischemia, have been linked to tissue damage through increased intracellular production of reactive oxygen species. Because cancer depends on angiogenesis, any appreciable increase in tumour size must occur concurrently with an increase in blood flow [2]. Most significantly, the new blood arteries help cancer cells spread to other areas by providing the tumour cells with more oxygen and nutrients. Angio-genesis is necessary for the growth & spreading all hard tumours [3]. About 27% of all new malignancies in women are breast cancers, making them the most prevalent type of cancer. It is distinguished by a unique pattern of metastases. The newly created blood vessels serve as a conduit for the cancer cells inside the tumour to spread to other areas [4]. The bulk of fatalities from breast cancer are caused by metastatic tumours, even though fewer than 0.05% of spreading tumour cells have the ability toward develop into constant metastases. One of those characteristics within the cancer, angiogenesis, acts as a role within the development & spread of breast related cancer. Pro-angiogenic growth factors initiate the process, which is closely regulated by the balance of positive & negative regulatory elements [5]. Angiogenesis is necessary for the growth and spread of all solid tumours. About 27% of all new malignancies in women are breast cancers, making them the most prevalent type of cancer. It is distinguished by a unique pattern

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of metastases [6]. The newly created blood vessels serve as a conduit for the cancer cells inside the tumour to spread to other areas. The bulk of fatalities from breast cancer are caused by metastatic tumours, even though not more than 0.05% of circulating cells of tumour have the ability in developing into steady metastases. One of main characteristics of melanoma, angiogenesis, plays a part of development & spread of the breast related cancer. Pro-angiogenic growth factors initiate the process, which is closely regulated by a balance of negative & positive regulatory elements [7]. Over the past three decades, the procedures of angiogenesis in both the pathological & physiological states have become extremely important [8].

The preferred approach for inhibiting tumour angiogenesis has been the obstruction of the vascular endothelial growth factor (VEGF) pathway. VEGF significantly influences the proliferation, survival, migration, and permeability of cancer cells. Multiple categories of antiangiogenic medicines that obstruct VEGF and vascular endothelial growth factor receptor (VEGFR) have been developed, encompassing ribozymes, antibodies, and small molecule inhibitors [9]. The inquiry into antiangiogenic medications that inhibit specific proteins within the VEGF downstream signalling channels captivates medicinal chemists and promotes the utilisation of these agents for this objective. Medicinal plants provide unique pharmacological models for the development of new therapeutic agents for cancer [10]. A variety of plant-derived chemicals are being produced into widely available anticancer treatments. Nonetheless, considerable knowledge has to be acquired regarding the antiangiogenic properties of plant extracts [11].

A. sessilis is herb that can be either annual or perennial and ranges in height from 0.2-1 m. The leaves are roughly lanceolate, simple, opposite and either quickly percolate. Their apex is acutely blunt, with complete glabrous or thin, delicate, articulate edges, while their base is attenuated. The seeds are enclosed by a little, flattened, white piece called an indehiscent fruit. The seeds are glossy, disc-shaped, and dark brown to black [12]. They are light sensitive. The plant *A. sessilis* (Linn.) has been reported to contain Lupeol, α & β-spinosterol, β-sitosterol, stigmasterol and campesterol, ethanol, ethyl acetate, hexane& aqueous excerpts of leaves of *A. sessilis* (Linn.) reported the existence of alkaloids, steroids, glycosides, & terpenoids, flavonoids, tannins, saponins, polyphenols, coumarins, carbohydrates. *A. sessilis* (L.) was reported to have wound healing property. It was used as a medicine with the combination of other medicinal plants to treat different ailments such as, asthma, chest pain, hepatitis, bronchitis, analgesic, dysentery, malarial fever, diarrhoea, urinary tract infections and other curative purposes [12].

MATERIAL AND METHODS

Collection & Authentication

Leaves of Alternanthera sessilis were gathered from Hyderabad, TS, and a botanist from the Department of Botany, verified the authenticity of the plant.

Methodology

Preparation of ethanolic extracts:

Alternanthera sessilis leaves were gathered, cleaned, and allowed to dry in the shade. Using a mortar and pestle, the dried leaves were ground into a coarse powder. After weighing around 300 g of coarsely ground plant material, it was extracted using 99% ethanol as the solvent through a week-long cold maceration procedure that was sometimes stirred. Using muslin cloth, the plant material was separated, then allowed for evaporation at room temperature, filtered, the ethanolic extract of *Alternanthera sessilis* was prepared. The obtained extract was in black colour. It was dissolved in carboxy methyl cellulose during administration [13].

Preliminary Phytochemical screening

Preliminary phytochemical screening was performed for phenolic compounds, flavonoids, carbohydrates, triterpenoids, saponins, proteins, amino acids, & tannins [14].

CELL CULTURE

For cytotoxicity evaluation or the capacity to inhibit cell expansion spectrophotometrically was done using MCF-7 cell lines. With an incubation at 37±1°C and 5±1% CO_2, all cells are seeded at density of 2 × 10^4 cells or well in discrete 96-wellplates with 200 µl of culture average. The test substances were applied to the cells following a 24-hour incubation period. Solution of 100 µl of MTT (0.5 mg/ml) is added to individually well after 48-hour period of incubation was over, the wells were then incubated (3 hours at 37±1°C & 5±1% CO_2). The resultant formazan is dissolved & measured on 570 nm by means of spectrophotometry [15].

MTT ASSAY

Setting up a cell line

From the liquid nitrogen storage, a vial containing each cell line was removed and quickly thawed to ambient temperature. After adding contents of vials to 9 millilitres of whole medium, the mixture was

centrifugated for five minutes at 125 grams. Following centrifugation, pellet are combined by 10 millilitres of full media, suspended in T-25 flask, & incubated at 37°C by means of 5% CO_2 and supernatant was disposed of. The cells were centrifugated for 5 minutes at 125 g once the cell confluence reached around 80%. Pellet was then combined by 15 ml of whole media & put into 2 T-75 flasks. Cells in flask were employed for the test whenever the cell confluence was between 80 and 90 percent [16].

ASSAY PROCEDURE

Without the test agent, 20,000 cells per well of a 200 µl suspension of cell in full media by 10% FBS were planted in 96 well plate and left to breed for 1 day. Following a 24hourdevelopment period, the test chemicals were added in the proper amounts to the wasted media in the 96-well plate wells, & the plate were incubated at37°C for 48 hours with 5% of CO_2. The plates are taken out of the incubator once the incubation period was over, & MTT reagent has added until the 0.5 mg/ml final concentration is obtained (0.2µm filter sterilized). To protect them from light, the plates were coated in aluminium foil before spending three hours in the incubator. Following development, 100 microlitres of dimethyl sulfoxide has added, the MTT reagent was withdrawn. At 570 nm, absorbance was assessed using a Pro spectrophotometer Tecan™ Infinite 200 m [17].

The percentage viability of the cells within the untreated (negative control) sample was established at 100%, and the percentage viability of cells in the groups that were treated was calculated relative to the negative control. The percentage viability was graphed against the concentration & evaluated for dose-response relationships. A suitable model was fit to estimate the Imax and IC_{50} based on the dose-response relationships.

The formula below was used to determine the percentage viability:

$$\% \text{ Viability} = \text{OD } 570\text{e} - \text{OD } 570\text{b}/\text{OD}570\text{b} \times 100$$

OD570e represented an average Optical Density of the test item dilutions, while OD570b denotes the average Optical Density of the negative control.

Rat aortic ring analysis

The process was approved by institutional Animal Ethics Committee, Wistar Male albino rats with in weigh 200–250 g had their thoracic aorta removed after the rodents were put to sleep. Petri plate containing cold & sterile PBS solution was used to hold the aorta. A dissecting microscope was used to remove the surrounding connective tissue. Following thorough washing, the aortae were cut with a surgical blade into rings that were about 1 mm thick, placed in brand-new, cold PBS, and refrigerated. After that, each ring was put in a well on a 48-well plate. Rings got then inserted in middle of 25 µL of low growth factors (LGF), which had been kept on ice the entire time, using pipette tips that had been pre-cooled. Three duplicates of the rings were seeded. A volume of 250 µL (at different amounts of 3.125, 6.25, 12.5, 25, 50, & 100 µg/mL) of each extract, diluted in RPMI1640 medium, got added post 30 minutes of cultivation at 37^0C to guarantee adequate solidification. A 1% v/v concentration of DMSO was utilized as the control. After that, plate got incubated through 37°C. Media got changed to a new unity with extracts on the fourth day. Angiogenic result got assessed by measuring length of vessels that emerged from 1° ring explants by Image J software (National Institute of Health, Bethesda, MD) post aortic rings got imaged with an inverted light microscope (4× magnification) on day five. Minimum35-like assemblies per ring were chosen at consistent breaks around rings, and their growth distances were measured [18].

Inhibition of vessel creation got computed using below formula:

$$\text{Blood vessels inhibition} = [1 - (A_0/A)] \times 100,$$

A_0 = distance of vessels development in treated rings in arbitrary units

A = distance of vessels development in the control in arbitrary units

***In vivo* CAM assay**

Using a modified CAM assess approach, antiangiogenic activity of *Alternanthera sessilis* extract got examined by *in-vivo* methods. In summary, a nearby hatchery provided five-day-old fertilized eggs for the project. The eggshells were meticulously cleaned of dirt by scrubbing with 70% ethanol. After that, the eggs were incubated at 37^0C with a 60%–62% humidity level. To enable the CAM to separate from the shell, the eggs were incubated horizontally while 5 mL of albumin was aspirated using a sterile 5 G-needle syringe. To create discs with 100 µg of extract, the various *Alternanthera sessilis* extracts got liquified in 1.2% agarose. As controls, disc with 1% DMSO were utilized. Agarose discs got placed on CAM after a tiny window aperture was created on the shell's surface. Adhesive tape was used to cover the window opening; it got cautiously cut into tiny fragments that suitable around opening. A digital camera was used to take pictures of the CAMs after the embryos had been cultured for 24 hours [19].

Statistical analysis
GraphPad Prism software (version 7.00) got used to achieve the statistical investigation, and the findings were displayed as mean ± SEM. One-way analysis of variance (ANOVA) got utilized to examine group variances, and P values < 0.05 were deemed substantial.

RESULTS
The phytochemical screening of ethanolic extract of *Alternanthera sessilis* was carried out and it was found that the presence of the phytochemical constituents was revealed and displayed in the Table 1.

Table 1: Phytochemical screening of Ethanolic extract of *Alternanthera sessilis*

Phytochemical Compound	Tests executed	Results
Carbohydrate	Molisch	+
	Fehling's	+
	Benedict's	+
Alkaloid	Dragendroff's	+
	Mayer's	+
	Hager's	+
	Wagner's	+
Steroid	Salkowski's response	+
	Liebermann-Burchard response	+
	Liebermann's response	+
Mucilage	Swelling	+
	Test with Ruthenium Red	+
Flavonoid	Shinoda	+
	Lead acetate	+
	NaOH	+
Tannins	Lead acetate	+
	Bromine water	+
	Dil. Iodine solution	+

Where, + means positive; - means negative.

Table 2: Cytotoxic effects of EEAS on MCF-7 cell lines

Test compound (μg/ml)	EEAS	5-Fluorouracil
	% Mean Viability	% Mean Viability
Blank	-	-
Vehicle+Control	100.00±0.0	100.00±0.0
5.00	96.27±0.58	98.27±0.81
10.00	94.74±1.24	92.29±0.85
20.00	86.44±0.69	80.06±1.24
30.00	66.18±0.29	35.01±0.84
50.00	31.52±0.39	13.85±0.94
100.00	11.01±0.28	9.92±0.24
IC_{50}	36.49	25.49

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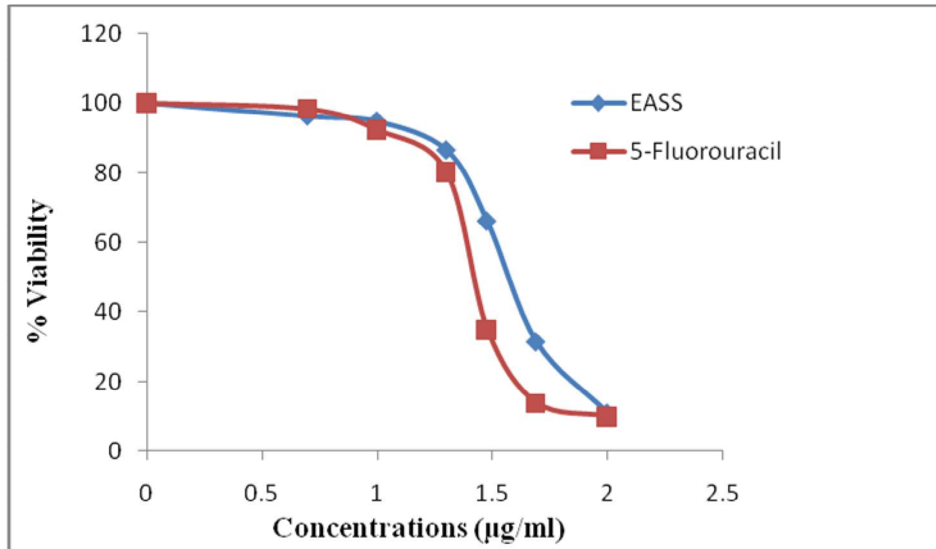

Figure 1: Cytotoxic effect of EEAS on MCF-7 cell lines

Aortic rings preserved with diverse amounts of test excerpt showed a substantial dose reliant on effect that was shown at Table 3 & Figure 2. Half maximal inhibitory concentration (IC_{50}) got examined to be 36.49 µg/mL. Ethanolic extract reserved the development of new vessels as shown at the Figure 3 (A–G).

Table 3: Effect of EEAS on angiogenesis in *in vivo* method

EEAS (µg/mL)	Rat aortic ring assess	
	Inhibition ± SEM(%)	**p-value**
3.12	14.7 ± 15.2	ns
6.26	35.8 ± 8.9	ns
12.4	53.1 ± 16.8	p< 0.001
25.0	98.9.0 ± 0.7	p< 0.001
50.0	99.1 ± 0.9	p< 0.001
100.0	100.0 ± 0.	p< 0.001

Data got characterized as mean±SEM, p<0.001 (*n*=3)

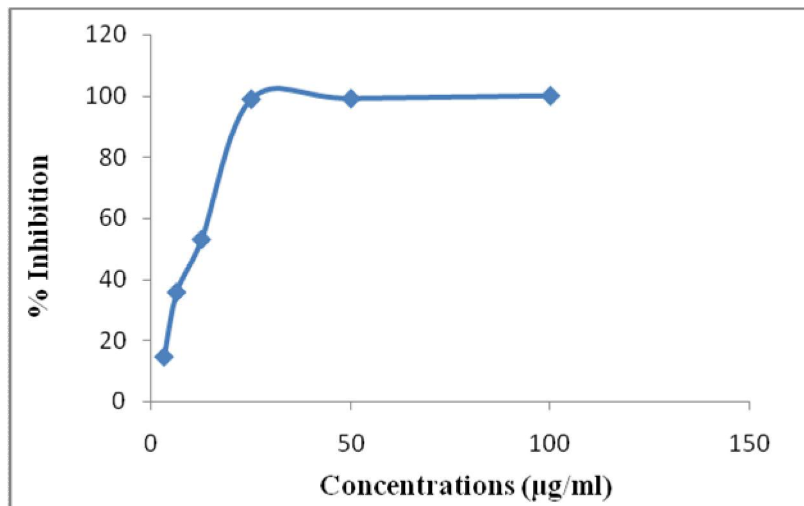

Figure 2: Outcome of EEAS on angiogenesis in *in vivo* method

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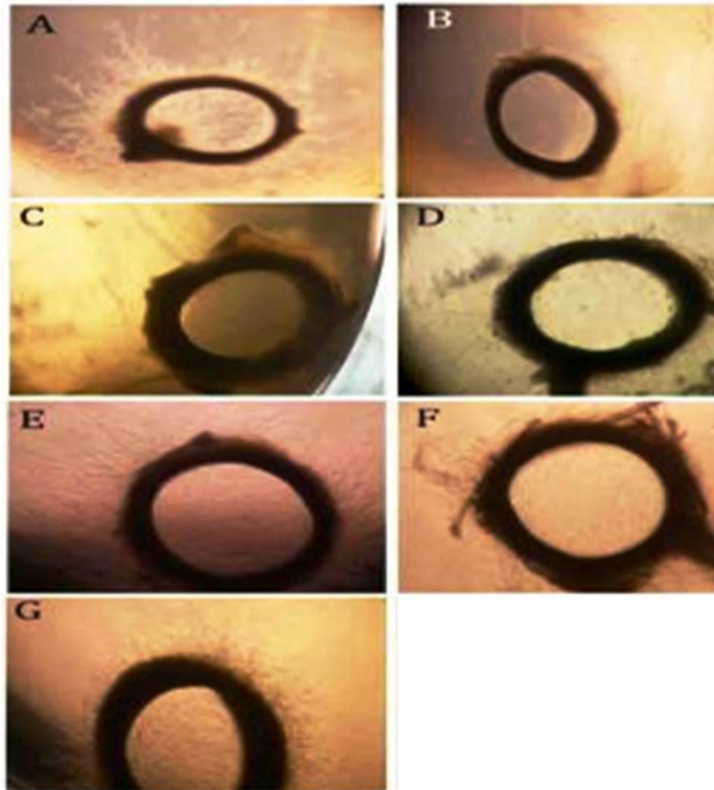

Figure 3: Effect of test extract angiogenesis in *ex vivo* technique

Table 4: Effect of test extract angiogenesis in *ex vivo* technique

EEAS(µg/mL)	CAM assay	
	Inhibition ± SEM (%)	**p-value**
3.125	1.5 ± 7.9	ns
6.25	5.8 ± 8.8	ns
12.5	10.7 ± 4.6	ns
25	15.7 ± 10.8	ns
50	44.5 ± 4.5	$p < 0.001$
100	59.5 ± 6.0	$p < 0.001$

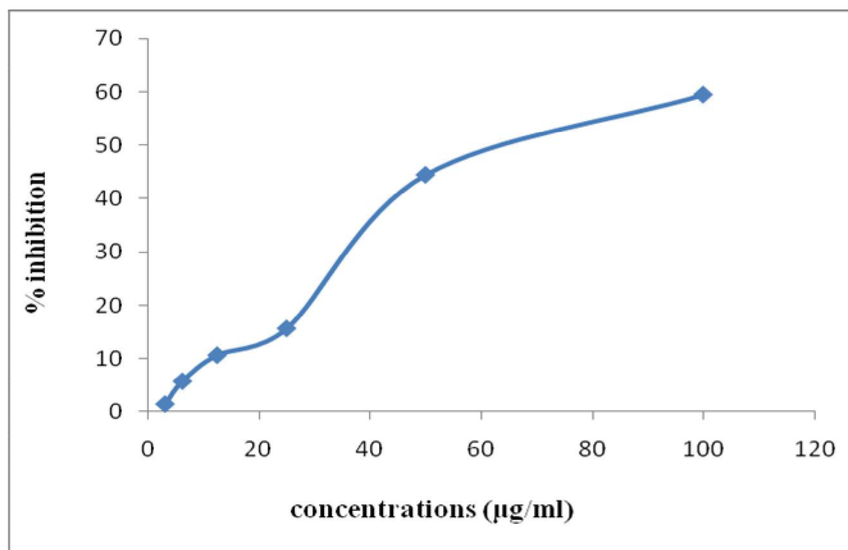

Figure 4: Effect of EEAS on *ex vivo* **angiogenesis**

Alternanthera sessilis extracts showed a characteristic antiangiogenic activity in ***in vivo*** CAM assess. Normal vasculature decoration in control CAMs by1^{o}, 2° and 3° vessels & dendritic branching design could be understood in Figure 4(A).
Vascularization in chorioallantoic membrane got inhibited by 100 & 50µg/ml concentration through 59.5±3.0% (**p**<0.001) & 44.5±4.5% (**p**<0.05), correspondingly when associated by control. Irrelevant inhibition got experiential with other doses (Table 4).

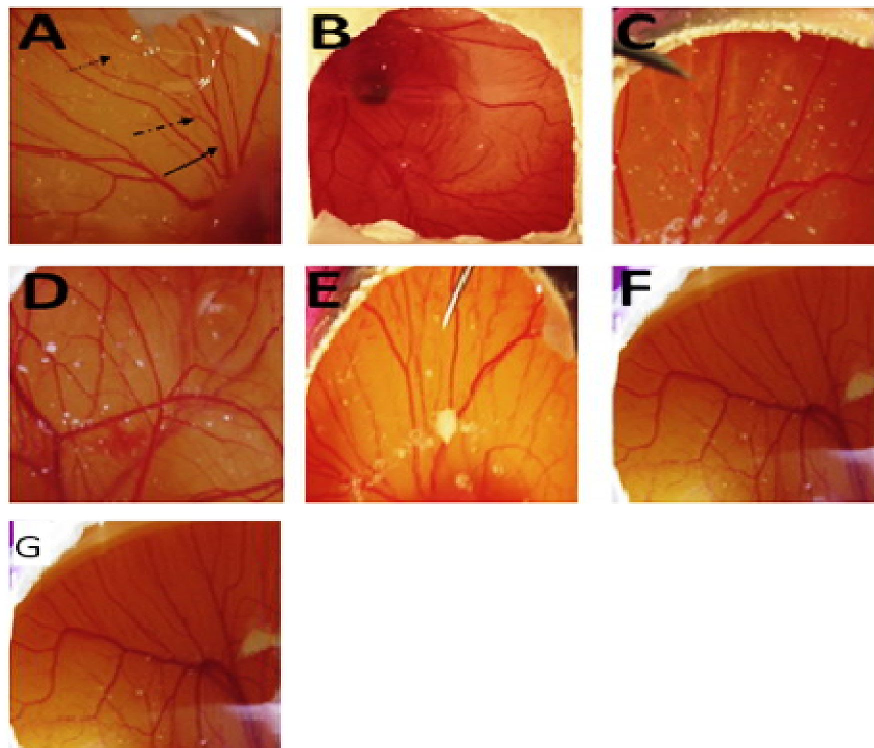

Figure 5: (A) Normal vasculature design in control CAMs by1^{o}, 2°&3°vessels (3 arrows) & dendritic branching design. Membranes 48 h post application of (B) 3.125µg/ml, (C) 6.25µg/ml, (D) 12.5µg/ml, (E) 25µg/ml (F) 50µg/ml (G) 100µg/ml

DISCUSSION

Globally, cardiovascular disease and neoplasms are considered as first and second leading cause of death. About 30 % of the cancer deaths have a cardiovascular disease as a cause. Oxidative stress, inflammation, cell proliferation, apoptosis and angiogenesis play a vital role in the generation of neoplasms and angiogenesis [20]. Early atherogenesis and cardiovascular risk factors primarily operate on the vascular endothelium. The development, invasion, and metastasis of solid tumours depend on angiogenesis. Breast cancer prevention and treatment have been proposed using the anti-angiogenesis method [21]. Direct and indirect channels are the two recognized methods for modifying angiogenesis. Moderating vascular endothelial cells' capacity to divide, move, & react to angiogenic protein like VEGF is direct method. The capacity to modify both the expression and activity of angiogenic protein that trigger angiogenesis is the foundation of the indirect approach. Tumour invasion, metastasis, and progression are all characterized by angiogenesis. The development of anti-angiogenic medicines is necessary goal for treatment of cancer & inhibition due to critical role that angiogenesis plays in the growth of cancer [22]. Controlling the expression of endothelial cell receptors is another aspect of this. The angiogenic potential of *Alternanthera sessilis* crude ethanol extracts has been examined in this work.
Angiogenesis can be studied using a variety of in vitro and in vivo techniques, each with unique benefits and drawbacks. One of the most promising techniques for assessing a compound's capacity to promote or inhibit angiogenesis in endothelial cells is the tube formation assay. Furthermore, *Trigonella foenum-graecum's* effects on angiogenesis are discussed. CAM, which connections nutrients & gases, has solid capillary system& is frequently employed as an *in-vivo* method to investigate angiogenesis [23, 24]. Given the prevalence of numerous common conditions, such as overweightness, atherosclerosis, arthritis, impaired vision, tumour, asthma, psoriasis, & infectious conditions linked to excessive angiogenesis, anti-angiogenic drugs are of great interest.

Conversely, anti-angiogenic drugs could cause unintended consequences such slowed wound healing. There is a lot of interest in natural anti-angiogenic medicines that are more effective and have fewer negative effects. The rat aortic ring test outcomes indicated that the ethanol extract was assessed at six distinct doses, spanning from 3.125 µg/mL to 100 µg/mL. Significant inhibition was achieved at amounts of 12.5, 25, and 50 µg/mL, however at levels of 3.125 and 6.25 µg/mL, inhibition diminished, demonstrating a clear dose-dependent relationship [25]. The findings of this study indicate that the solvents employed are crucial in the processing of plant components. *Alternanthera sessilis* is noted for its abundance of alkaloids, flavonoids, and saponins, which are pivotal to the therapeutic properties of its leaves. *Alternanthera sessilis* contains around 35% alkaloids. The saponin content of *Alternanthera sessilis* is approximately 4.8%, and the leaves include flavonoids. The ingredients significantly influence the pharmacological actions of *Alternanthera sessilis* that have already been previously addressed. Given the dose-dependent effect of ethanol on vascularization in the rat aorta model, it is posited that the key constituent(s) responsible for this action are soluble in ethanol. It is important to note that 70% ethanol may extract a wide range of bioactive compounds from plant materials, suggesting that the action of the extract of ethanol may result from the synergistic effects of a diversity of chemicals. The abundance of saponins in plant materials can enhance the solubility of less polar compounds in 70% ethanol [26]. The CAM assay results confirmed that ethanol extract significantly influences vascularization. In the current investigation, the MTT assay demonstrated a strong anti-proliferative effect on MCF7 cells across six different tested doses.

Researchers have shown that certain flavonoids are potent endothelium inhibitors. Cell division and *in vitro* angiogenesis triggered by VEGF/bFGF (basic fibroblast growth factor).

CONCLUSION

Although *Alternanthera sessilis* has been extensively researched for its several medicinal effects, this work is the inaugural investigation assessing the extract of *Alternanthera sessilis* ex vivo and in vivo through the rat aortic ring test and the CAM test for its antiangiogenic capabilities. The results of both experiments revealed that the ethanol extract greatly decreased vascularization. Future research should focus on bioassay-guided separation, purification, and identifying the contents of the ethanol extract, as well as evaluating their antiangiogenic ability for the suppression of tertiary blood vessels.

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