

Antiangiogenic potential of endophytic fungi *Alternaria alternata* isolated from *Lawsonia inermis* Linn

Neha N. Bendre* and Ghanshyam R. Gonjari

Department of Zoology and Fisheries, Yashwantrao Chavan Institute of Science, Satara-415001, India

ABSTRACT

Antiangiogenesis is the complex mechanism used for the inhibition of growth of blood vessels from the pre-existing vasculature. Blood vessel is known to perform a crucial role in development of tumor. Blocking of angiogenesis through the control over the action of the cytokine VEGF could be possible mechanism in cancer therapy. This is very important mechanism used to prevent the proliferation of blood vessels towards growth of tumors. It has proved to be potentially attractive approach in case of controlling dreadful disease like Cancer. Knowing the importance of this process, for the first time, the study was aimed to investigate the antiangiogenic potential of endophytic fungi from the medicinal plant *Lawsonia inermis* linn. We successfully isolated *Alternaria alternata* from the leaves of *Lawsonia inermis* Linn and identified microscopically as well as by 18srRNA and ITS sequence analysis. The GC-MS analysis revealed the presence of wide range of bioactive secondary metabolites showing significant antimicrobial property against four human bacterial pathogens. The ethyl acetate extract of *Alternaria alternata* showed maximum zone of inhibition of 21.8 ± 0.8 mm against *Pseudomonas aeruginosa*. After these analysis, antiangiogenic property of *Alternaria alternata* ethyl acetate extract was studied by Chick Chorioallantoic membrane assay (CAM) which showed the treated CAM has (84 ± 2.60) less no. of tertiary blood vessels as compared to the control (124 ± 2.64). Therefore the results suggest that *Alternaria alternata* can be considered as a potential source of antiangiogenic agent. Further investigation of characterization and structure elucidation of active compounds from this extract is needed to know the exact antiangiogenic component.

KEY WORDS: ANGIOGENESIS, ANGIOGENESIS, VEGF, ENDOPHYTIC FUNGI, SECONDARY METABOLITES CHORIOALLANTOIC MEMBRANE (CAM) ASSAY, LAWSONIA

Article Information:*Corresponding Author: patilneha1227@yahoo.in

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INTRODUCTION

Angiogenesis is the dynamically regulated process where new blood vessels are escalated from the previously formed blood vasculature. It is known to be essential for normal physiological process like embryonic development, organ formation, reproductive system, cyclical ovarian function, tissue renewal, and wound healing. Also it also play a vital role in pathological conditions like rheumatoid arthritis, arteriosclerosis, myocardial infarction, ischaemic diseases, diabetic retinopathy and cancer. The later pathological disease called Cancer has drawn more attention of researchers some few year back. The reason behind the cancer being the most dreadful disease is the unending requirement of oxygen and nutrients supply from the growing blood vessels which later on make it critical, metastatic and leads to death of the person.

According to Folkman (1971) tumor growth is known to depend on angiogenesis where it is triggered by chemical signals from tumor cells in a phase of rapid growth. Tumors do not grow progressively unless they induce a blood supply from the surrounding stroma. Cancers that lack angiogenesis remain dormant. Therefore in order to get control over the tumor cells blood vessels supply to cancer cells should be inhibited through process called antiangiogenesis. Since in the absence of vascular support tumors become necrotic, or even apoptotic, (Holmgren *et al* 1995). As per Judah Folkman, the main reason behind the excessive formation of blood vessels around the tumor is due to the Vascular Endothelial Growth Factor-A (VEGF-A) is supposed to be a potent angiogenic mitogen inducing migration and proliferation of endothelial cells. Blockade of VEGF has proven to be effective way of inhibiting tumor angiogenesis. Therefore Folkman and his colleagues were the first to propose using inhibition of tumor vasculature formation as anticancer therapy (Folkman 2003). Of late researchers are now actively involved in the development of novel anti cancerous drugs throughout the world. According to some reports, many of the available anticancer drugs are exhibiting toxicity to proliferating normal cells along with the cancer cells also the repetitive dosage is enhancing its resistance which results in increasing the the need for bioactive components from natural products, (Remesh 2017).

Natural products are naturally derived metabolites and by products obtained from microorganisms, fungi, plants or animals, (Baker *et al* 2000). According to Schulz (2001), novel secondary metabolites are derived from organisms that occupy unique biotopes with unusual environment are known to be prolific producers of bioactive metabolites. Endophytes are one of those microbes that have the ability to thrive in the specific

biotopes like higher plants which are traditionally been exploited for medical, industrial and agricultural use. Endophytic fungi are the microbe that colonize the internal tissues of plant without causing any infection or harm to them, (Bacon and White 2000, Remesh 2017).

In recent advances of research, endophytes are viewed as a excellent source of bioactive natural product as they are known to possess therapeutic value for the prevention and treatment of various types and stages of cancer. As per the estimation Dreyfuss and Chapela Rossman, there are as many as 1 million species of endophytic fungi unexplored and undescribed. There are very few research has been reported on the study of angiogenesis and antiangiogenesis property of endophytic fungi using Chick chorioallantoic membrane assay. it was found that Hulikere *et al*, (2016) studied the antiangiogenic and antioxidant activity of endophytic fungus *Penicillium citrinum* and *Cladosporium Cladosporoides* from seaweed (*Sargassum wightii*). This research work has created impetus in us to investigate the antiangiogenic potential of bioactive secondary metabolites of endophytic fungi isolated from medicinal plant *Lawsonia inermis* Linn (Mehndi) located in Satara District, (Maharashtra), India, Using chick Chorioallantoic membrane assay (CAM).

Chick embryo chorioallantoic membrane in vivo assay is one of the best established and most popularly used model for angiogenesis and cancer studies. As this method is Cost effective, less time consuming, easy to perform, reproducible and has visible vasculature which make this useful for both intravascular and topical administration of study agent, relatively rapid assay method and can be adopted very easily to study tumor growth, (Ribatti *et al*, 2001). Therefore in order to discover alternative treatment for cancer chemoprevention, the chorioallantoic membrane assay is used to analyse various natural compounds that could reduce or inhibit several pathways involving malignancies and excessive angiogenesis related diseases.

MATERIAL AND METHODS

Collection of plant material: In order to obtain endophytes, *L. inermis* has been selected on the basis of their unique biology, age, endemism, ethnobotanical history, or environmental setting. It seems that endemic plants growing in moist, warm climates or in areas of great biodiversity are among the first choices for study, so accordingly *L. inermis* is collected from Satara district in Northern Western Ghats. The properly identified and authenticated plant was used in the current study. Healthy and mature leaves were collected from the field grown plants were brought to laboratory and processed within 24 hrs of collection or stored in icebox.

Isolation of endophytic fungi from *Lawsonia inermis*:

To isolate endophytic fungi from leaves of *Lawsonia inermis* Linn, Collected leaves were washed thoroughly with water to expel the unwanted material from the surface. Each leaf was surface sterilized by using the method described by Arnold et al (2007) with minor changes. For sterilization of surface of leaves, leave were immersed in 70 % ethanol for 1 min, further dipped in 0.1% HgCl_2 for 1 min and later washed in autoclaved distilled water, (Bisht et al 2016) and blot dried on filter paper. Finally the leaves were cut aseptically into 1 cm long segments and placed on Potato dextrose agar plate supplied with 50ug for /ml of streptomycin to avoid any bacterial contamination .later on the plates were sealed with parafilm and incubated at $28\pm 2^\circ\text{C}$ for 8-10 days in incubator. After the respective days, the hyphal tips of fungi emerging from leaves were transferred aseptically to fresh PDA slants to get pure culture for fungi.

Identification of Isolated fungi- The fungal isolates mounted on the sterile slides then it was stained with lactophenol cotton blue and then examined in 100x light microscopy. Morphological identification of the isolates was carried out on the basis of surface texture, pigmentation, and spores at the hyphal tips which helped to identify the endophytic fungi at the species level. The authorized identification of endophytic fungi was done from National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, and Pune, India. The identified fungal strains were isolated and then sub-cultured in a Petri dish which contains sterile PDA media. To preserve as a pure culture, the endophytic fungi were inoculated in PDA slant.

Molecular identification: Fungal genomic DNA was isolated using Qiagen DNeasy kit. Fungal ITS region gene was amplified using standard PCR reaction. The primer pair ITS1 and ITS4 was used in a PCR reaction with an annealing temperature of 54°C . After amplification, products were purified by using Invitrogen PCR product purification kit (Life technologies, USA) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at Blast N site at NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>).

Fermentation and extraction of secondary metabolites: Erlenmeyer flasks (250 ml) containing 100 mL potato dextrose broth were used for cultivation of fungal isolates. The broth was inoculated with two loops of fungal isolates and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The extraction was carried out according to the method described by Radji *et al* (2011). The broth culture was filtered by double-layered muslin cloth to separate the mycelia and

filtrate. To the filtrate equal volume of ethyl acetate (1:1) was added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. Then the upper layer of ethyl acetate was collected using separating funnel as it contained the extracted compounds. The mycelium was ground properly in a pestle and mortar using ethyl acetate as a solvent and then it was filtered using cheesecloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in the hot air oven. The extract residue was dissolved in Dimethyl Sulfoxide (DMSO) and stored at 4°C to be used as a stock solution for spectroscopic characterization and antimicrobial assay.

Antimicrobial screening by Agar Well diffusion assay:

Agar well diffusion assay method described by Magaldi *et al* with little modification has been used for the evaluation of antimicrobial property. The crude extracts of fungal endophytes were used and dissolved in DMSO. The test organisms viz. *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* were obtained from the Department of Microbiology, Yashavantrao Chavan Institute of Science, Satara, India. The wells of 1cm in diameter were bored in the media with the help of sterile cork borer. The well was filled with 40 μl of crude extracts of different fungi. For negative control, one well was filled with only 40 μl DMSO. These plates were then refrigerated at 4°C for 4-6 hrs for antimicrobial diffusion and then incubated at 37°C for 24 hrs. The clear zones of inhibition formed around the wells were measured by counting the diameter of a circle in millimeters. The test was performed in triplicate with each bacterial strain and mean zone of inhibition was recorded.

Gas Chromatographic Mass Spectroscopic profile:

To identify the compounds from the fungal extract, GC-MS analysis was carried out using Shimadzu model QP-2010 with a nonpolar 60 M RTX 5MS column. The carrier gas used was Helium and the temperature programme was adjusted to initial oven temperature at 40°C for 3 min and the final temperature of oven was 480°C with rate at 10°C . One microliters (1 μl) sample was injected using a split less mode. Mass spectra was recorded over 35-650 amu range with electron impact ionization energy 70 eV. The total time required to run the sample was 45 min. Relative quantitative determinations were made by relating respective peak areas to total ion chromatogram (TIC). Unknown components compared with known mass spectra of National Institute of Standards (NIST) for molecular identification of compounds. Name, molecular weight, retention time and peak area percentage of the test material was tentatively determined.

Chick Chorioallantoic membrane (CAM) *In vivo* assay:

The antiangiogenic potential was detected by using CAM

assay model as described by Domenico R. *et al* (1996). Fertilized eggs of *Gallus gallus* were purchased from Satara Poultry hatchery, then were cleaned and disinfected with 70% alcohol and divided in three groups for 48 hrs, 72 hrs and 96 hrs. The eggs were incubated in an aseptic incubator in vertical position such as blunt end of egg faced upward and was maintained at 37°C temperature and humidity at 70%.

Dose administration - For the dose administration Hanks Balanced Salt Solution (HBBS) was used as saline. Endophytic fungal extract was prepared in ethyl acetate, of which 20 ug/ml of extract was dissolved in 1ml DMSO. All treatments were given in final volume of HBBS (mg/ml). At different developmental stages (48 hrs 72 hrs 96 hrs) dose was initiated by making a small window at the blunt end of egg. With the help of insulin syringe of 0.2 ml of dose was injected on to the chorioallantoic membrane and then sealed with surgical adhesive tape. The development was continued up to 144 hrs. (Korn & Cramer, 2009). After 144 hrs The eggs were then opened for morphometric evaluation. Later the CAM samples were fixed in CAF (Calcium Acetate

Formalin) fixative to carry out further histopathological process and finally stained with Haematoxylin and eosin for microscopic evaluation.

RESULTS AND DISCUSSION

From the present study, after following the standard isolation procedure of endophytic fungi from the leaves of medicinal plant *Lawsonia inermis* Linn, *Alternaria spp.* was isolated successfully. Pure culture was maintained by sub culturing it at least three times.

From the preliminary identification of fungi it was observed that the fungal colony had olivaceous black or dark gray color with white margin with woolly texture (Fig:1) while features like elongated chains of septate brownish conidia with short beak and smooth surface were observed in the microscopic examinations which were confirmed from National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute (ARI) Pune. Therefore as per the morphological and microscopic observations the isolate was identified as *Alternaria spp.*, further confirmation up to species level was

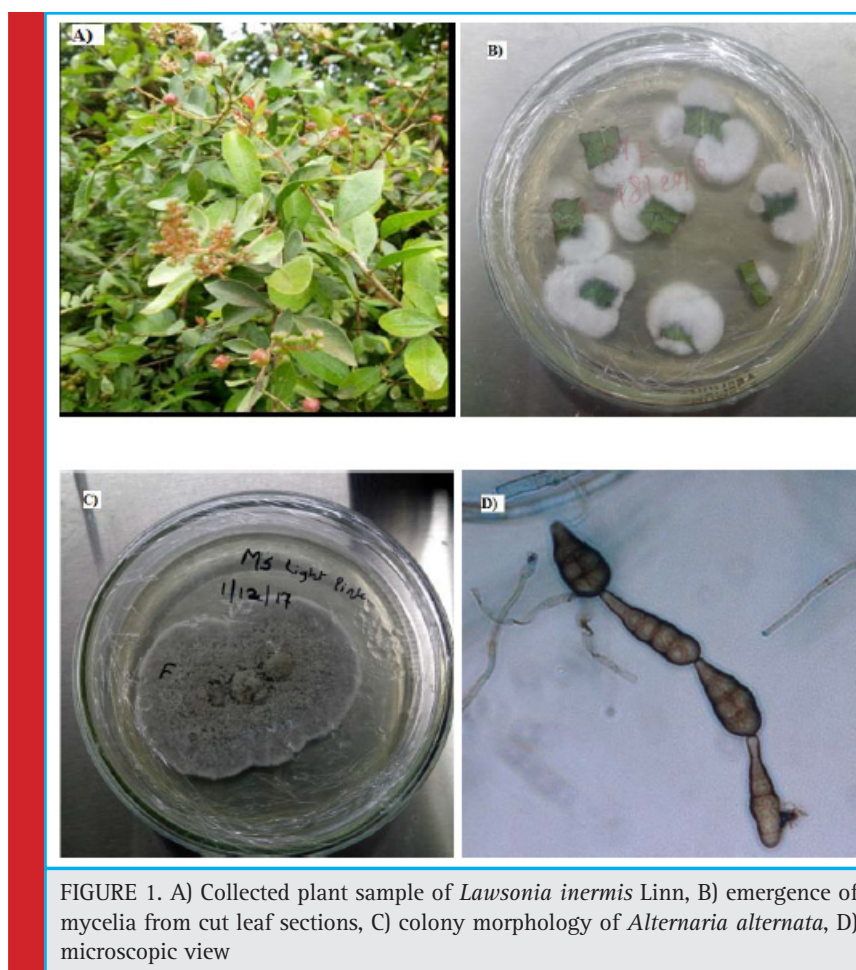
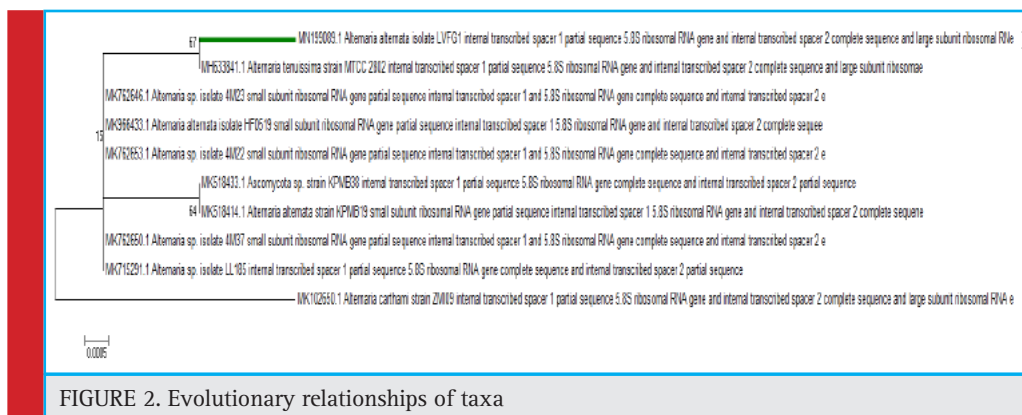


FIGURE 1. A) Collected plant sample of *Lawsonia inermis* Linn, B) emergence of mycelia from cut leaf sections, C) colony morphology of *Alternaria alternata*, D) microscopic view



confirmed by molecular analysis. Molecular identification was done with the help of 18s rRNA gene and Internal transcribed Spacer (ITS) region which identified the *Alternaria* spp as *Alternaria alternata* based on BLAST results showing 100 percent identity, 100 % query coverage with nucleotide homology and phylogenetics analysis. The information regarding close homologs is represented in the alignment view (fig: 2).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei (1987). The optimal tree with the sum of branch length = 0.01238757 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches, (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura M. 1980) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 486 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al* 2013).

The Agar well diffusion assay was performed to evaluate the antimicrobial property of the *Alternaria alternata* ethyl acetate extract. In this, it showed promising inhibition of four human bacterial pathogens (table 1). In this assay 10 μ l of extract was dissolved in DMSO which also was used as a negative control to state whether the inhibition was not occurred due to solvent. The evaluation was carried out on the basis of classification as (0-6mm)- no activity, (7-9mm)-not significant, (10-12mm)- poor activity (13-15mm)- low activity (16-18 mm)- good activity, and above 18 mm significant activity. From this it was observed that the extract showed significant activity with maximum zone of inhibition against *Pseudomonas aeruginosa* (21.8mm), while *Bacil-*

lus subtilis showed good activity with (17mm) inhibition while *S.aureus* showed poor activity with (13.2mm) inhibition and *E.coli* showed low activity with 9.4 mm inhibition (table no.1). Thus the above results indicates that the endophytic fungi *Alternaria alternata* possess significant antimicrobial property which was then further characterized for secondary metabolite profile and investigation of antiangiogenic potential.

Name of organism	Diameter of zone of inhibition (mm)
<i>P. aerogenusa</i>	21.8±0.8
<i>B . subtilis</i>	17±0.6
<i>E.coli</i>	9.4±0.2
<i>S.aureus</i>	13.2±1.3
DMSO	--
(--)	No inhibition, DMSO - Dimethyl Sulfoxide

Antiangiogenic activity was studied by Chick chorio-allantoic Membrane assay using Window method. After 144 hrs, the eggs were opened for morphometric and histological analysis. For morphometric study, firstly the CAM area was measured as described by Melkonian *et al* (2002), from the (fig :3) and (table no. 2), it can observed that there has been 24.6 % decrease in the total CAM area of eggs treated with *A. alternata* extract than HBSS control eggs. Also the tertiary blood vessels on CAM area were counted on manually on Computer by considering the sprouting points, it was observed that there was significant decrease in the no. of tertiary blood vessels showing 32.7% inhibition while normal and HBSS (Hanks balanced Salt Solution) control showed increase in the number of blood vessel and branches. These suppression of blood vessels were confirmed by histological section stained with Haematoxylin and eosin show-

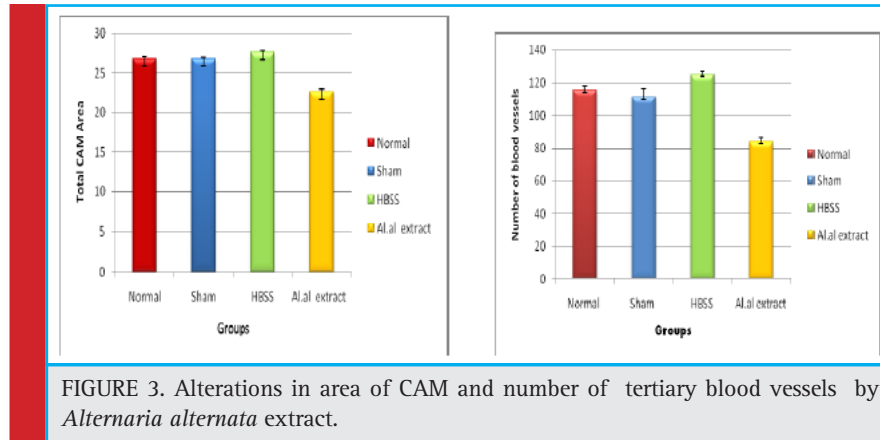


FIGURE 3. Alterations in area of CAM and number of tertiary blood vessels by *Alternaria alternata* extract.

Table 2. Influence of endophytic fungi *Alternaria alternata* methanolic extract on number of blood vessels.

Group	Total Number Tertiary blood vessels	Total CAM area
Normal	115 ±2.9	26.8±0.2
Sham	111±5.5	26.8±0.1
HBSS	124±2.6	27.6±0.1
<i>Al.al</i> extract	84±2.6	22.6±0.4

(HBSS-Hanks Balanced salt Solution, *Al. al* -*Alternaria alternata*)
Results expressed in mean±S.E,P-value <0.05 considered most significant value

ing inhibition of proliferative vessel and reduction in capillary plexus of treated vessels as compared to normal. (Fig:4) For morphometric analysis- CAM area was measured according to method described by Melkonian *et al* (2002) as, CAM area = $(1/2A) \times (1/2B) \times \pi$, where, A=Longest length,B=Longest width, and $\pi=3.14$. For Histological analysis- Normal, controlled and treated CAMs were fixed in CAF Fixative, a section of tertiary blood vessels was cut and processed in alcohol grades for dehydration and then embedded in paraffin. The paraffin blocks were then sectioned of 6 μ size using rotary microtome. these vertical sections were stained with

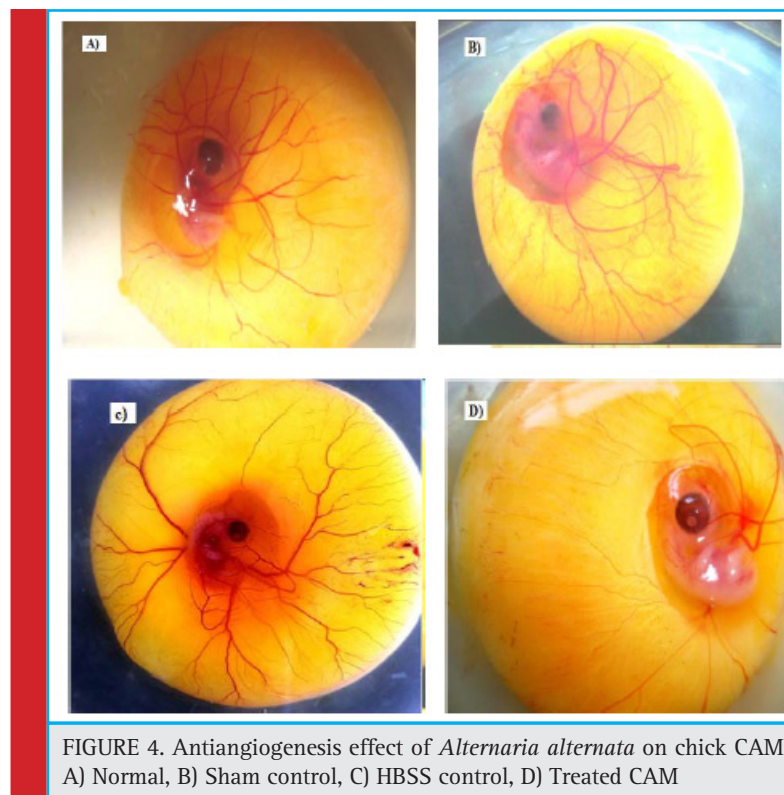
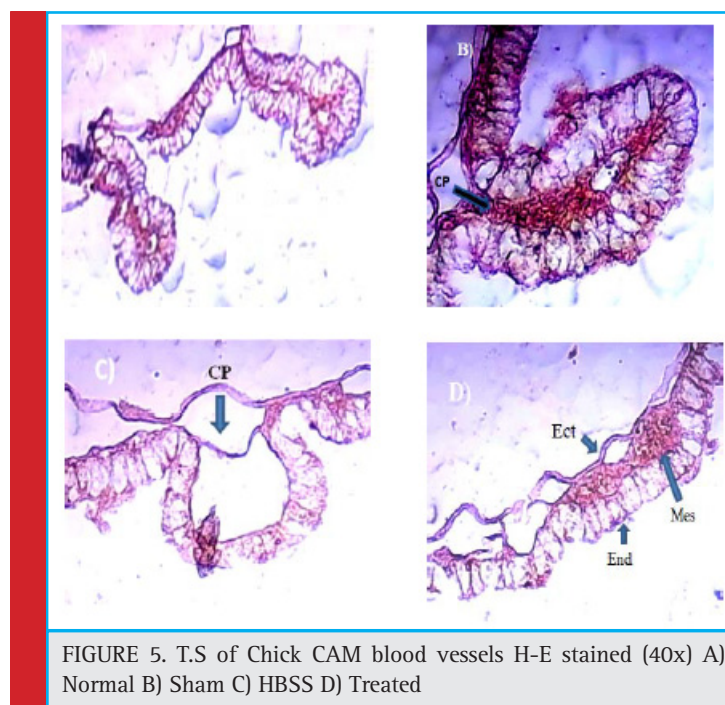


FIGURE 4. Antiangiogenesis effect of *Alternaria alternata* on chick CAM
A) Normal, B) Sham control, C) HBSS control, D) Treated CAM



H-E technique. Finally the slides were observed under light microscope for histological changes in structure of blood vessels.

Gas Chromatographic –Mass Spectroscopic analysis-
The GC-MS analysis was carried out for fungal extract that showed the potential antimicrobial property. Therefore the ethyl acetate fungal crude extract of *Alternaria alternata* showed total twelve peaks indicating the presence of twelve compounds (Fig. 6). Among them Hexadecanoic acid, methyl ester (35.73) showed highest percentage of area followed by 9-Octadecenoic acid, methyl ester(E)- (24.55), 2-Fluorobenzoic acid, Heptadecyl ester (12.79), 9,12-Octadecadienoic acid (Z,Z)-,

methyl ester (3.66), Methyl tetradecanoate (2.19), 2-Ethyl-1hexanol (1.94), Methyl hexadec-9-enoate (1.85), 1,2 Benzenedicarboxylic acid (0.93), Nonanedioic acid, dimethyl ester (0.92), 1-Nonadecene (0.91), and Heneicosane (0.82) (Table 3).

Compounds detected were predominantly derived from the ethyl extract especially hexadecanoic acid methyl ester which is an aliphatic acid ester reported to cause growth inhibition and apoptosis induction in human gastric cancer cells (Yu *et al* 2005). Number of compounds detected including 9-Octadecenoic acid, methyl ester (E)- (24.55) are to possess anti-inflammatory and cancer preventive properties.

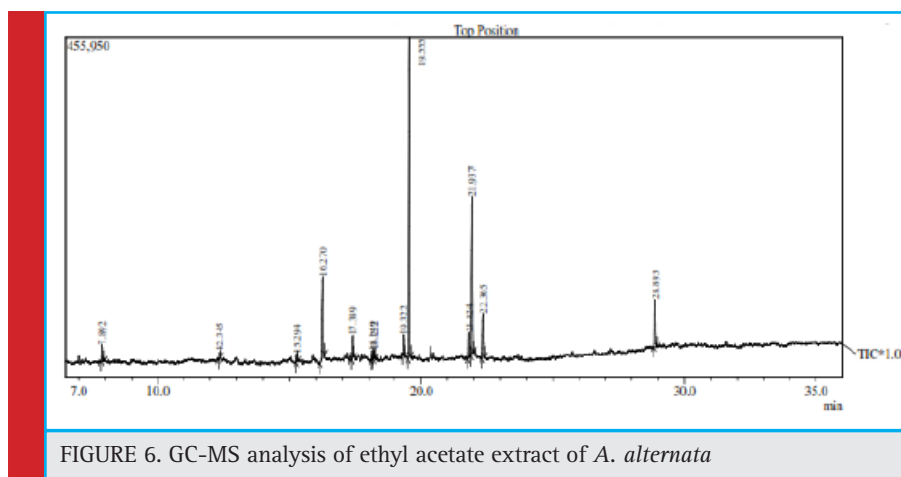


FIGURE 6. GC-MS analysis of ethyl acetate extract of *A. alternata*

Table 3. Phytoconstituents in *Alternaria alternata* ethyl acetate extract

S. No	Name	Peak	R.T	I.T	F.T	Area	Area (%)
1.	2-Ethyl-1-hexanol	1	7.892	7.845	7.955	41804	1.94
2.	1,2-Benzenedicarboxylic acid	2	12.345	12.300	12.395	20046	0.93
3.	Nonanedioic acid, dimethyl ester	3	15.294	15.260	15.325	19804	0.92
4.	2-Fluorobenzoic acid, heptadecyl ester	4	16.270	16.160	16.335	275250	12.79
5.	Methyl tetradecanoate	5	17.389	17.345	17.420	47222	2.19
6.	1-Nonadecene	6	18.149	18.100	18.180	19606	0.91
7.	Heneicosane	7	18.222	18.180	18.255	17585	0.82
8.	Methyl hexadec-9-enoate	8	19.322	19.280	19.360	39844	1.85
9.	Hexadecanoic acid, methyl ester	9	19.555	19.495	19.610	769183	35.73
10.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	10	21.824	21.765	21.870	78770	3.66
11.	9-Octadecenoic acid, methyl ester, (E)	11	21.937	21.885	21.980	528586	24.55
12.	Methyl stearate	12	22.365	22.305	22.415	148763	6.91

CONCLUSION

Many reports suggested the various medicinal properties of endophytic fungi *Alternaria alternata* as antimicrobial, antifungal, antidiabetic, anticancerous but this study has first time reported the antiangiogenic potential of terrestrial endophytic fungi *Alternaria alternata* isolated from leaves of medicinal plant *Lawsonia inermis*. The GC-MS analysis revealed the presence of 12 bioactive compounds which showed significant antimicrobial property against four bacterial pathogens showing significant zone of inhibition (21.8 ± 0.8 mm) against *Ps.aeruginosa*. Due to presence of this bioactive metabolites, we were able to evaluate the antiangiogenic potential of *Alternaria alternata* ethyl acetate extract by observing the maximum inhibition of blood vessels in treated CAM than the control one. This research work defines the very important biological potential which can be considered as potential candidate for antiangiogenic treatment.

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