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Research Article
Antiangiogenic and Antioxidant Activity of Endophytic Fungus Isolated from Seaweed (*Sargassum wightii*)

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Abstract

Background and Objective: Angiogenesis plays a vital role in the formation of new blood vessels from preexisting vasculature during embryonic development processes. Excessive angiogenesis leads to several pathogenic conditions such as cancer, arthritis and psoriasis. Marine natural products are gaining the attention of researchers in recent years as they are the rich source of diverse medicinal compounds. Endophytic fungi from marine seaweeds are less studied and presented as a new subject of investigation of novel therapeutic agents. The aim of the present study is to evaluate antioxidant and antiangiogenic activity of ethyl acetate extract of the endophytic fungi, *Penicillium citrinum* isolated from *Sargassum wightii*. **Methodology:** Endophytic fungi were isolated from seaweed *S. wightii*. The isolated fungi was identified by 18S rRNA and ITS sequence. The ethyl acetate extract of endophytic fungi was subjected for antioxidant property using 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging assay. The extract was also analyzed for reducing power, total phenolic content and total flavonoid content. Antiangiogenesis potential of the extract was studied *in vitro* by inhibition of wound healing scratch assay and *in vivo* by CAM assay. **Results:** The endophytic fungus isolated from *S. wightii* was identified as *Penicillium citrinum* based on the analysis of 18S rRNA and ITS sequence. Ehtyl acetate extract of *P. citrium* showed a significant antioxidant and angiosuppresive activity. The antioxidant nature of the extracts was found to be concentration dependent. The ESI-LCMS analysis revealed presence of polyphenols at different range. Further studies are in progress to isolate, characterize and elucidate the structure of active compounds. **Conclusion:** Further studies on the compound present *P. citrium* extract will help to develop an alternative therapy for cancer menace.

Key words: *Penicillium citrinum*, *Sargassum wightii*, antiangiogenesis, antioxidant activity, polyphenols, CAM assay

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Angiogenesis is a complex process of formation of new blood vessels that involves the activation, proliferation and migration of endothelial cells. The balance between the angiogenic and antiangiogenic signals maintain the proper vasculature and physiological functions of our body such as reproduction, healing injured tissues, etc[1]. But, any imbalance between these signals lead to various pathophysiological conditions such as arthritis, diabetes, psoriasis and cancer. Inhibition of angiogenesis was suggested by Folkman[2] as a means for the effective control of cancer. Of late, there has been a growing curiosity among researchers in the modulators of angiogenic processes as they can be exploited in controlling the disorders that involve angiogenesis[3].

More than 300 antiangiogenic drugs have been reported till date. However, most of these therapeutic molecules have low efficacy and high toxicity. So, the demand for therapeutic molecules with high efficacy and low toxicity is escalating. Natural products are used in all traditional and many modern medicine for treating various ailments including cancer[4]. From 1940-2006, 175 anticancer drugs were discovered; among which 14% are natural products and 43% are derived from natural products by semi synthesis. During the past two decades, the development of marine natural products as anticancer drugs has become a promising avenue for research. The first anticancer drug from a marine source, ecteinascidin-743 is now commercially available[5].

Marine microorganisms associated with algae, sponges, corals, tunicates, fishes, etc., have been extensively studied for their natural product content[6,7]. Marine microbes, especially fungi, have newly been utilized as a source of novel bioactive secondary metabolites[8]. The kingdom of fungi is the second largest group after insects and widely distributed in nature[9]. Special ecological niches in the marine environment are responsible for the capability of marine fungi to synthesize novel molecules and they have attracted the attention of many natural product chemists[10].

Marine endophytes are poorly investigated group of microorganisms that produce diverse chemicals having immense implications on conventional chemotherapy[11]. These micro organisms contains different bioactive compounds, such as alkaloids, benzopyranones, chinones, cytochalasines, depsipeptides, enniatines, flavonoids, peptides, polyketones, phenols, quinols, terpenoids, etc., have been reported to elicit a number of pharmacological effects[12].

Even though marine endophytes have been extensively studied for various therapeutic potentials; to our best of knowledge, no report was available on the antiangiogenic activity of endophytes isolated from the seaweeds. Hence, the present study was under taken to study the antiangiogenic and antioxidant activity of the endophytic fungus isolated from seaweed, *Sargassum wightii*.

MATERIALS AND METHODS

Collection of seaweed: The seaweed *S. wightii* was collected from coastal region of Kanyakumari (Latitude 8.0780°N, longitude 77.5410°E), Tamil Nadu, India and brought to laboratory in a polythene bag along with sea water. The sea weed was authenticated by Dr. Lipton, Emeritus Professor (UGC) at CMST, M.S. University, Rajakkamangalam, Tamil Nadu, India.

Isolation of endophytic fungus from *Sargassum wightii*: Endophytic fungi were isolated from marine seaweed according to the protocol outlined by Ariffin *et al.*[13]. The *S. wightii* was washed thoroughly with seawater to expel epiphytic microorgansims, followed by tap water and distilled water to remove the salts and other materials. Seaweeds was surface sterilized with 70% ethyl alcohol for 1 min followed by immersion in 4% sodium hypochlorite solution for 3 min and 70% ethyl alcohol for 1 min. The surface sterilized *S. wightii* was rinsed with deionised sterile distilled water to remove sterilents and blot dried on sterile tissue paper. Finally, the sample was rinsed with sterile distilled water and cut aseptically into 1 cm long segments. The cut segments of *S. wightii* were incubated on potato dextrose agar supplemented with chloramphenicol at 28°C until mycelia were observed. Pure culture of endophytic fungi was isolated, sub cultured on PDA free of antibiotics and incubated for 30 days at 28°C prior to extraction.

Molecualr identification of the endophytic fungus: Total DNA of sea weed endohyte was isolated and Polymerase Chain Reaction (PCR) was carried out using ITS 1 forward primer and ITS 4 reverse primer. About 100 ng of genomic DNA, 100 µM dNTPs and 2.5U Taq DNA polymerase were mixed in a PCR thermocycler. The PCR was performed with 35 cycles of denaturation at 96°C for 45 sec, annealing at 50°C for 45 sec, extension at 72°C for 45 sec and final extension of 72°C for 10 min. The amplified product was subjected to electrophoresis using 0.8% agarose gel. The amplified DNA was purified and sequenced using the same primers used for PCR. The NCBI BLAST N program was used to compare the sequence available in GenBank.

Preparation of fungal submerged fermentation broth: The endophytic fungal isolates were cultured on potato dextrose broth for 21 days at 25-28°C[13].

Extraction of secondary metabolites from the endophytic fungal culture: Extraction of secondary metabolites was carried according to the procedure explained by Ariffin *et al.*[13] with slight modification. Each liquid culture with evident growth of endophytic fungal mycelium was mixed with an equal volume of ethyl acetate (100%). The mixture was blended with the help of pestle and mortar and extracted twice with equal volume of ethyl acetate. The resulting homogenate was filtered with Whatman No. 1 filter paper. The aqueous and organic layers were dried and stored at -20°C.

Determination of total flavanoids: Total flavanoid content was measured according to the method of Kosalec *et al.*[14] with slight modifications. Standard quercetin and ethyl acetate extract of the endophytic fungi (100 µg mL^{-1}) were taken in the range of 0.0-1.0 mL in different test tubes. Volume in each tube was made up to 4.0 mL with distilled water and 0.3 mL of 5% sodium nitrate was added. After 5 min, 0.3 mL of 10% aluminium chloride was added and incubated for 6 min at room temperature. About 2 mL of 1 M NaOH and 3.4 mL of distilled water were added to all the tubes and absorbance was read at 570 nm.

Determination of total phenolic content: Total phenolic content was measured according to the procedure[15] with slight modifications. Standard gallic acid and fungal extract (100 µg mL^{-1}) were taken in the range of 0.0-1.0 mL and volume of each tube was made upto 1.0 mL with distilled water 0.5 mL. About of Folin Ciocalteu reagent (1:1), 2.5 mL of sodium carbonate (20%) and 6.0 mL of distilled water was added to all the tubes. The absorbance of reaction mixture was measured at 760 nm.

Free radical scavenging assay by DPPH method: The DPPH free radical scavenging potential of the extract was determined using the protocol explained in Blois[16] method with slight modifications. The DPPH stock solution was prepared by 0.1 mM DPPH in 99% ethanol and standardized to 1.9±0.02 OD at 517 nm. The fungal extract (1 mg mL^{-1}) and standard ascorbic acid (0.1 mg mL^{-1}) were taken separately in different aliquots and made up to 1 mL using 99% ethanol. To this mixture, 2 mL of the 0.1 mM DPPH was added and incubated for 30 min (Fig. 1). Absorbance of the reaction mixture was read at 517 nm. All the tests were performed in triplicates. Percentage of the DPPH to inhibition was calculated using the equation:

$$\text{Inhibition of DPPH radical (\%)} = \frac{\text{Control absorbance extract absorbance}}{\text{Control absorbance}} \times 100$$

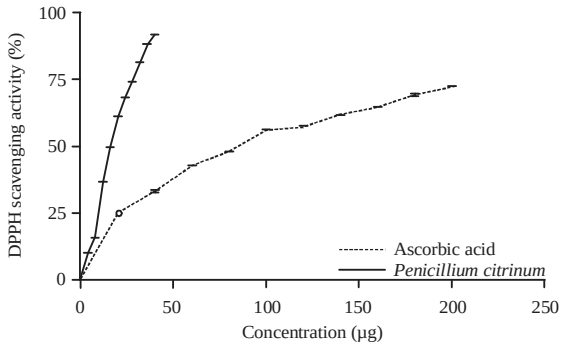

Fig. 1: DPPH scavenging activity of ethyl acetate extract of *P. citrinum*

Total reducing power: The Fe^{3+} reducing power of the extract was determined by the method cited in Oyaizu[17] with slight modification. Different concentrations (0-500 µg mL^{-1}) of the extract (0.5 mL) was mixed with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium hexacyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 mL of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 mL) was mixed with 1 mL distilled water and 0.1 mL ferric chloride solution (0.1%). The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed in triplicates. A higher absorbance of the reaction mixture indicated greater reducing power.

Chick chorioallantoic membrane (CAM assay): The CAM assay was performed as described by Muslim *et al.*[18]. Fertilized eggs were purchased from hatcheries. The eggs were incubated at 37°C in 40-60% humidity for 96 h and the eggs were randomly divided in to four groups. After 7 days, a window (1×1.5 cm^2) was made in the shell to expose a part of the CAM. Different concentration of the fungal extract (100 µg) was loaded on to sterilized whatmans filter paper No. 1 disc (2 mm^2) was applied to the CAM. After 24 h of incubation, number of new vessels in the CAM around the paper disc was photographed with the help camera (Sony). The CAM samples were fixed in 10% formalin, embedded in paraffin, sectioned and finally stained with hemotoxylin and eosin for histopathological evaluation.

Wound healing assay: To evaluate cell mortality affected by the *P. citrinum* extract, a scratching assay of the MCF-7 cells was performed as explained by Liang *et al.*[19]. The MCF-7 cells were seeded in the 6-well plates at a density of 1×10^5. Reference points near the "Scratch" were marked and the

plates were incubated at 37°C. Cells were grown in DMEM having 10% FBS to a confluent monolayer for 2 days and were scraped by 200 µL pipette tips to create a straight line cell-free "Scratch". Each well was washed twice with PBS and treated with extract and standard drug (1 mg mL^{-1}). Migration of cells in the "Scratch" was photographed at the matching reference points with inverted microscope at intervals of 12 h (0, 12 and 24 h). The images were analyzed quantitatively by Micam software; the distances between the two edges of the scratch were measured at the reference points and analyzed statistically.

The cell migration rate was calculated with mean width using following the equation:

$$\text{Cell migration rate (\%)} = \frac{\text{Width}_{0h}\text{-Width}_{xh}}{\text{Width}_{oh}} \times 100\%$$

Statistical analysis: All values were expressed as Mean±SD. Comparison between the control and sample were performed by analysis of variance (ANOVA) with Tukey's multiple comparison tests using Graph pad Prism v3.0 software.

RESULTS

Isolation of endophytic fungi from marine seaweed *S. wightii*: The present study was aimed at isolation of endophytic fungi from *S. wightii* and asses the antiangiogenic and antioxidant activity. Two fungal endophytes were isolated from *S. wightii* (Fig. 2a). One of the fungi was selected for further studies (Fig. 2b). Microscopic observations and the morphological studies revealed that the isolated endophytic fungus is *P. citrinum* (Fig. 2c).

Molecular identification: Molecular identification was achieved by Internal Transcribed Spacer (ITS) region. The identity of *P. citrinum* was further authenticated by 18S rRNA analysis (GenBank accession No. KT384174). Organisms with 99% or more sequence similarity to be considered from the same species and between 93-98% similarity to be considered same genus over the organism in considered to previously unidentified if the ITS sequence similarity[20] is below 93%. The endophyte had 99% similarity to *P. citrinum.*

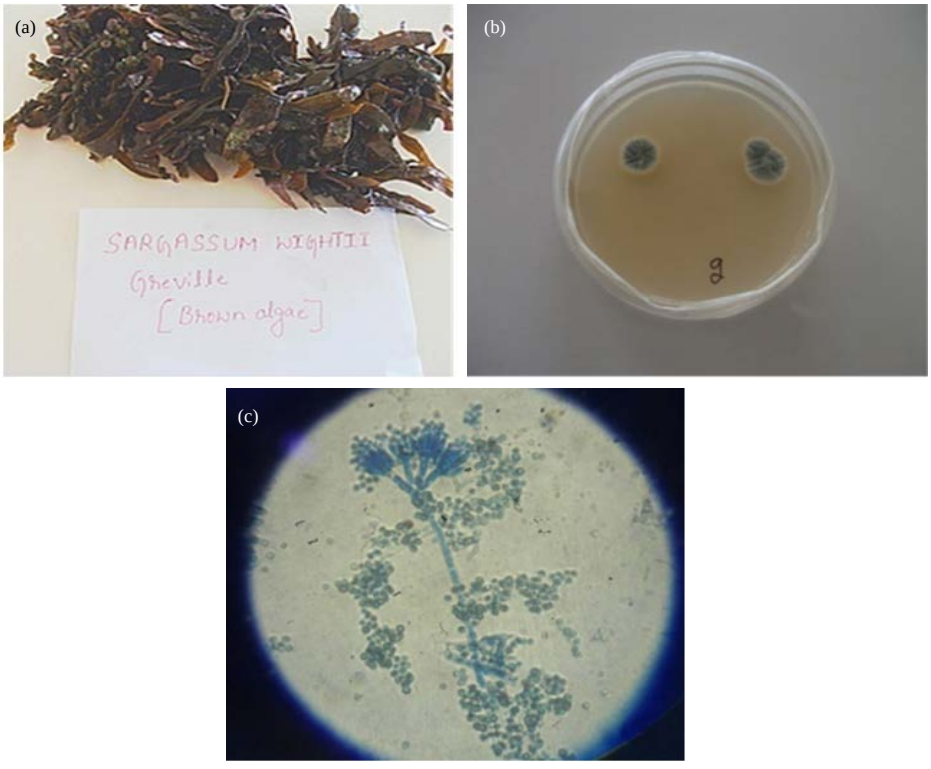

Fig. 2(a-c): (a) Image of seaweed *S. wightii,* (b) *Penicillium citrinum* grown from the segment of *S. wightii* on PDA and (c) Microscopic image of endophytic fungi, *P. citrinum*

Free radical scavenging activity by DPPH method: The free radical scavenging activity was illustrated using DPPH method. Ascorbic acid was taken as standard. *Penicillium citrinum* extract showed a significant antioxidant activity. The antioxidant activity of ethyl acetate extract of the fungus was comparable to that of standard ascorbic acid. The radical scavenging activity was found to be dose dependant.

The ethyl acetate extract was subjected to a quantitative analysis of total phenolics, flavonoids and reducing power assay. Ethyl acetate extract contained 0.79±0.04 mg g^{-1} total phenolics, 0.32±0.05 mg g^{-1} of total flavonoids and 0.70±0.02 mg g^{-1} of the reducing power.

Antiangiogenesis activity by *in vivo* chick chorioallantoic membrane (CAM) assay: *In vivo* antiangiogenic activity was studied by chorioallantoic membrane (CAM) assay. *Penicillium citrinum* extract treated CAM had (12.0±0.86) less number of blood vessels than the control (17.0±1.05). The decrease in the number of blood vessel branches on CAM showed the antiangiogenic activity (Fig. 3). Morphological analysis of the *P. citrinum* extract treated CAM sections were carried out using haematoxylin and eosin staining. Haemetoxylin and eosin staining of the control and treated group of CAM showed a significant variation in the histomarphology control group had a normal organization of CAM layers ectoderm (ET), mesoderm (M) and endoderm (ED), while endophytic extract treated CAM showed the damages to the ectodermal layer compared to control (Fig. 4).

Wound healing assay (*In vitro* scratch assay) by using MCF-7 cells: The MCF-7 cells with and without administration of *P. citrinum* extract were photographed at 0, 12 and 24 h. Scratch assay of MCF-7 cells showed a delayed process of wound healing. Wound healing was defined as the percentage of the starting distance between the two edges of each wound. The MCF-7 cells showed a slower rate of wound closure than the negative control cells. The distance between the two edges of the scratch in the *P. citrinum* treated well was greater than that of negative control and the extent of prevention of wound healing was comparable to the standard drug thalidomide (Fig. 5).

LC-MS analysis: The ethyl acetate extract of *P. citrinum* was subjected to LC-MS analysis to confirm the presence of different secondary metabolites. *Penicillium citrinum* extract showed the presence of various compounds such

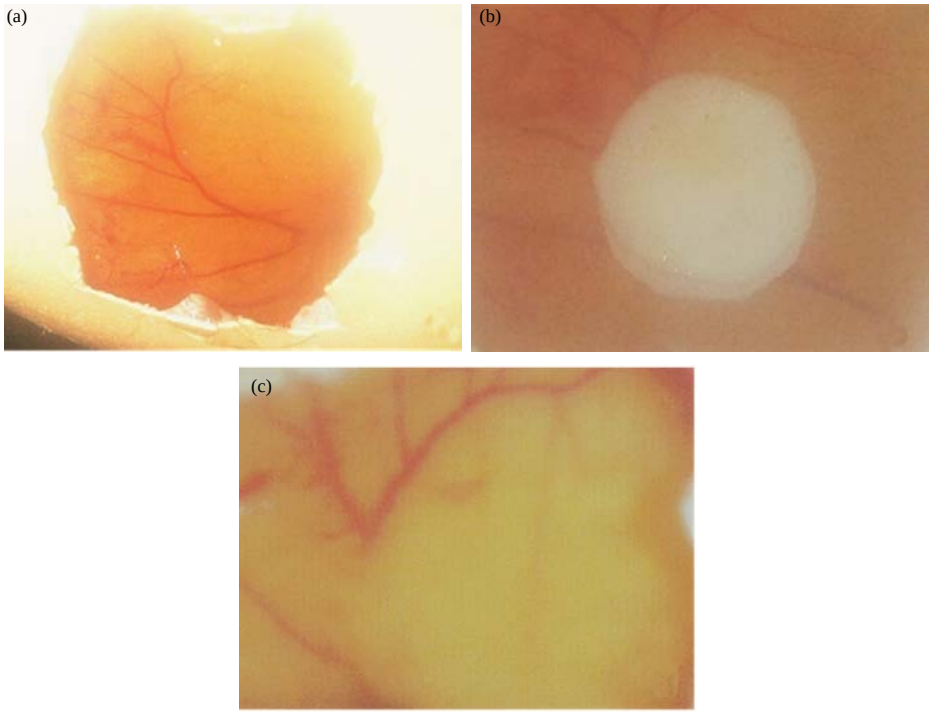

Fig. 3(a-c): Angiogenesis in (a) Control, (b) *Penicillium citrinum* extract on chorioallantoic membrane and (c) CAM with reduced blood vessels

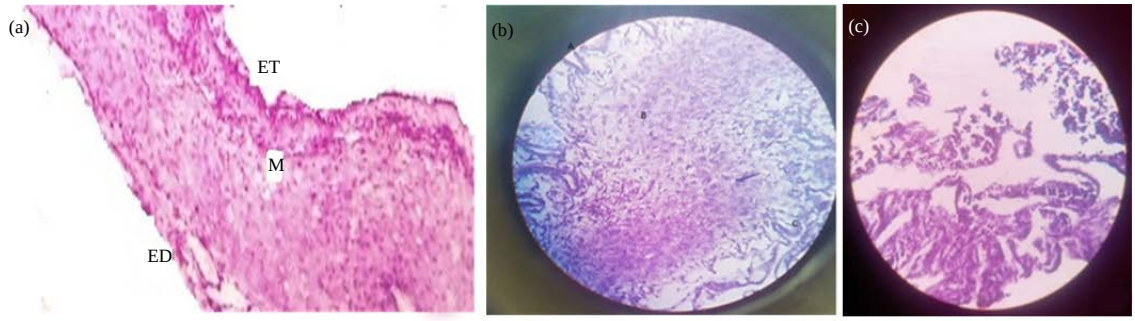

Fig. 4(a-c): (a) Control show the normal organization of the CAM layers; ectoderm (ET), mesoderm (M) and endoderm (ED), (b) Control and (c) Extract treated group showed damage to the ectoderm layer compared to normal (40X magnifications)

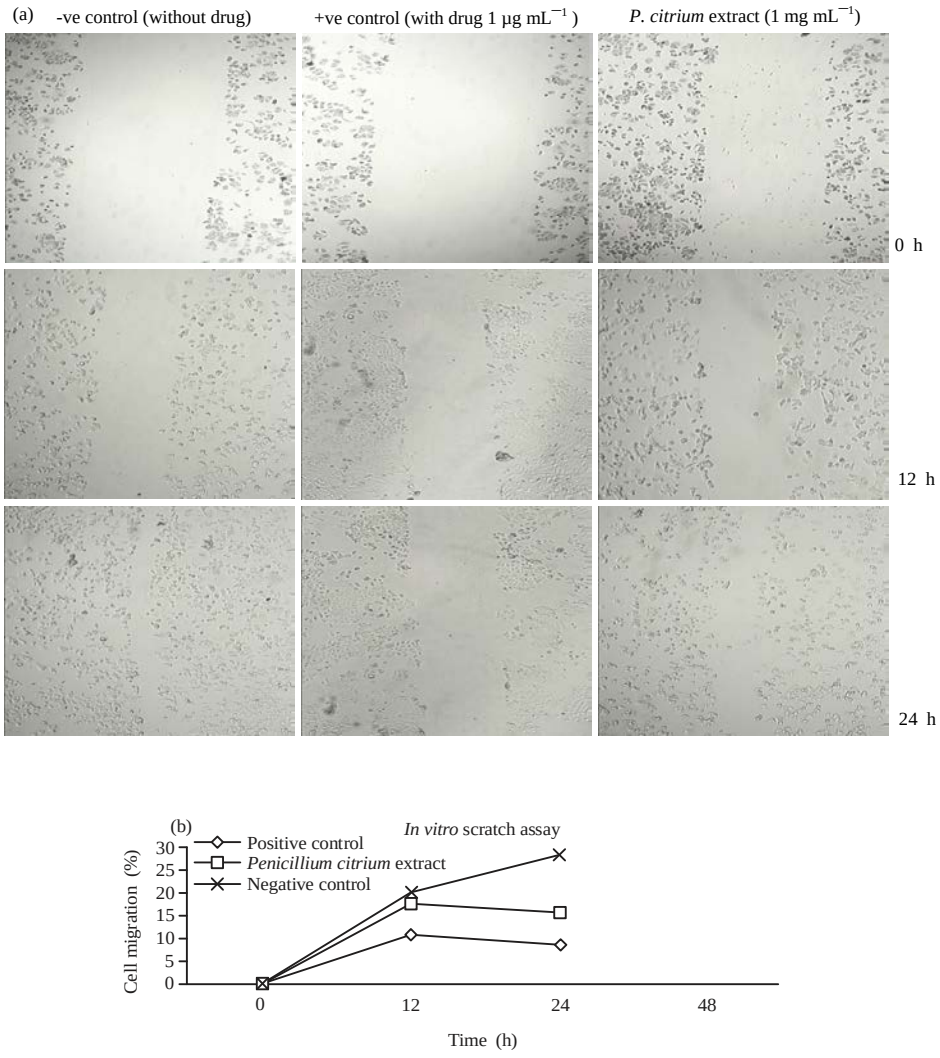

Fig. 5(a-b): Scratch assay of MCF-7 cells. (a) Images of positive control thalidomide 1 µg mL^{-1}, negative control and *P. citrinum* extract 1 mg mL^{-1} treated MCF-7 cells were taken at 0, 12 and 24 h and (b) The distance between the two edges of the scratch in the *P. citrinum* treated well was greater than that of negative control

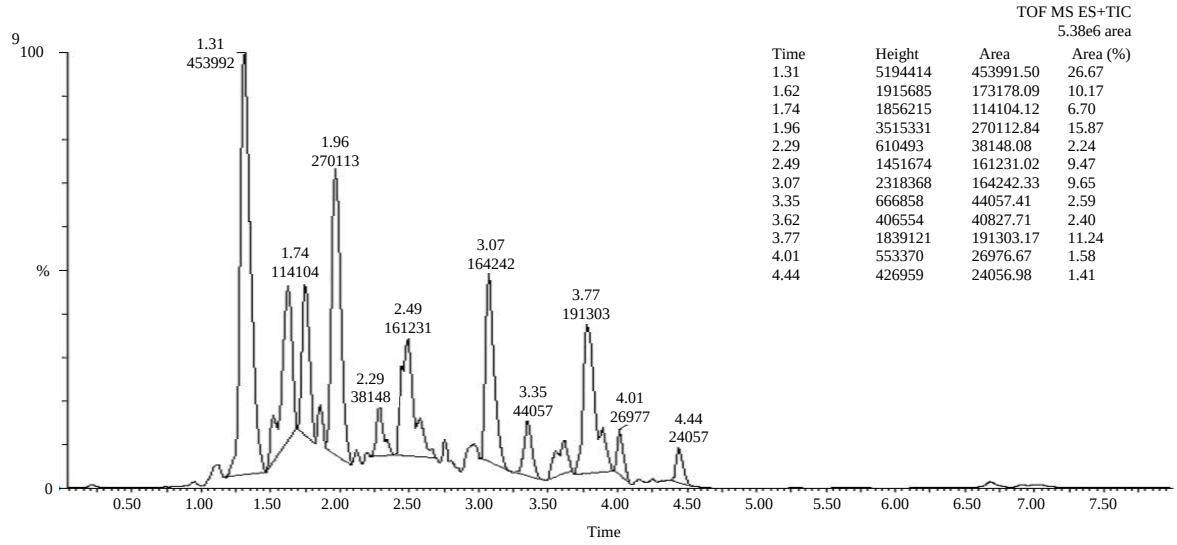

Fig. 6: LC-MS analysis of ethyl acetate extract of *P. citrinum*

as 1-(3-bromophenyl)-N-[4-(2,4-dimethoxyphenyl)-1,3-thiazol-2-yl]-1,2,3-triazole-4-carboxamide, 3-{[5-(4-bromophenyl)-1, 3, 4-oxadiazol-2-yl]methyl}-4-oxophthalazine-1-carboxylic acid, N-[(9Z)-14-bromo-1,10-diazatricyclo[9.4.0.0^{3},] pentadeca-3,5, 7,10,12,14-hexaen-9-ylidene]-5-nitrofuran-2-carboxamide, 3-chloro-N-{[4-(5-chloro-2methoxyphenyl)oxan-4-yl]methyl}-4-fluorobenzene-1-sulfonamide,4-(3-bromopropanesulfonyl) oxane, 4,4-dichlorobenzophenone and 4,4-dichlorobenzophenone along with other minor phenolic compounds (Fig. 6).

DISCUSSION

Marine microbes, particularly fungi, have recently been utilized as a new source of novel bioactive secondary metabolites[21]. The present study was carried out to evaluate the therapeutic potential of endophytic fungi, *P. citrinum* isolated from the sea weed, *S. wightii.* The preliminary phytochemical tests on ethyl acetate extract of *P. citrinum* showed the presence of diverse secondary metabolites such as phenolic compounds, tannins, glycosides and flavonoids. Even the LC-MS analysis of the endophytic extract confirmed the presence of the different secondary metabolites. These phytochemicals are the major contributors to the medicinal property of the endophytic extract. Phenolic compounds are reported to be responsible for reducing the lipid peroxidation and other types of oxidative process that leads to the generation of free radicals[22,23]. The antioxidant activity of extract was determined by DPPH scavenging activity. The fungal extract showed significant radical scavenging activity. The DPPH scavenging assay is one of the easiest and basic methods to assess the antioxidant potential of synthetic and natural products[24]. *Penicillium citrinum* extract showed significant Fe^{3+} reducing power. Electron donation capacity and free radical quenching capability are the hall marks of reducing power assay[21]. This study also showed a relationship between phenolic and flavonoid content, free radical scavenging activity by DPPH method and reducing power of the extract. This study is in agreement with the studies reported earlier about the positive correlation between the phenolic content and the antioxidant potential[23-26]. The ethyl acetate extract showed the presence of significant amount of flavonoids. Flavonoid is a class of phenolic compounds found in fruits, vegetables and flowers. There are different groups of flavonoids and each group reported to be containing diverse medicinal properties[27]. These compounds may also involve in the antioxidant potential of this extract.

Angiogenesis is essential for the tumor growth and metastasis as the process provides necessary oxygen and nutrition for the growing tumor. Antiangiogenic therapy and neovascularization inhibition is a promising approach to the treatment of cancer. The CAM assay was carried out to verify the antiangiogenic activity of the endophytic extract *in vivo*. Angiogenesis on the chick chorioallontoic membrane of fertilized eggs is the basis for CAM assay. The CAM assay is frequently used to find out the angiogenic as well as the angiosuppressive potential of various molecules[28].

In the present study, ethyl acetate extract of *P. citrinum* was subjected for its angiosuprresive activity in CAM assay. There was an increase in the vessel numbers and branches in control group. However, a reduced length and number of vessel branches were observed in the *P. citrium* extract

treated group. Even avascular regions were observed around the insert in the treated group showing the suppression of angiogenesis on CAM. Haemetoxylin and eosin staining of the control and treated group of CAM also confirmed the antiangiogenic capabilities of endophytic extract. Even though marine endophytes are studied for antioxidant, cytotoxic and other pharmacological potential; no report was available on the antiangiogenic activity. Hence, we could not compare angiogenic potential of *P. citrinum* with other marine endophytes. However, the antiangiogenic potential of *P. citrinum* extract was comparable to the activity of terrestrial endophytes and sea weeds reported earlier[29-31].

Administration of *P. citrinum* extract (1 mg mL^{-1}) and positive control (Thalidomide, 1 µg mL^{-1}) showed a delayed process of wound closure compared with the untreated MCF-7 cells. In this study also we did not find any report regarding the scratch assay using sea weed endophyte extracts. The wound healing potential of *P. citrinum* extract was much lower than thalidomide and other purified compound from the terrestrial endophytes. However, the purification of the lead molecule may show significant wound healing potential as that of standard drug (as crude extract was used for this study).

The LC-MS analysis of the sea weed endophytic extract showed the presence of different natural compounds as mentioned in the result section. Different biological activities to varying extent may be attributed to the vast number of active small functional molecules present in the *P. citrinum.* This study is in agreement with the finding of Palem[32] who have studied relationship between the metabolic compounds in the endophytic fungi to its biological activity. The presence of an array of secondary metabolites in *P. citrinum* makes it an interesting species to study the relation between the secondary metabolites and therapeutic potential.

CONCLUSION

We have reported for the 1st time about the inhibition of angiogenesis by ethyl extract of *P. citrinum*. This fungal extract may provide a new source of antiangiogenic agents which can be exploited as a potential candidate in the treatment of angiogenesis related diseases such as cancer, psoriasis, rheumatoid arthritis and diabetic retinopathy.

SIGNIFICANT STATEMENTS

- Regulation of angiogenesis will aid in controlling many diseases such as aging, psoriasis, cancer, etc.
- More than 300 antiangiogenic drugs reported till date and most of them have side effects and toxic to normal cell. These side effects prompted us to look for an alternative source
- Natural products are used since ages to treat many diseases and the marine endophytes have the added advantage of producing novel chemicals due to their special ecological niche
- This study is the first report on the antiangiogenic activity of endophytic fungi isolated from seaweeds
- This study will help in developing a cost effective, less toxic drug for the prevention of angiogenesis related diseases such as cancer

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