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Production, purification and evaluation of different functional groups from endophytic *Penicillium* species derived bioactive compounds isolated from *Aloe vera*

Asmita B Gangurde[1], Priyanka R Jagtap[2], Manoj A Vyawahare[3], Girish P Kukreja[4], Rohit S Mane[5*]
[1-4] Department of Microbiology, New arts, commerce and science college, Ahmednagar, Maharashtra, India
[5] Department of Biotechnology and Microbiology, Karnatak University, Dharwad, Karnataka, India

Abstract
Distorted environment and human lifestyle require novel drugs in the different diseases; therefore, the present study was undertaken with fungal endophytes from *Aloe vera*. Total of sixteen fungal endophytes was isolated from the fifty root samples of *Aloe vera* collected from the different locations of Shrirampur, Ahmednagar district, India. All sixteen fungal endophytes were belonging to *Fusarium oxysporum* (20%), *Nigrospora* species (33%), *Alternaria alternata* (8%), *Aspergillus* species (50%), and *Penicillium* species (60%). The endophytic *Penicillium* species found to be a core group; therefore, the same fungus was grown in potato dextrose broth for the production of bioactive compounds and extracted with methanol solvent by solvent extraction method. The wet biomass of *Penicillium* species was recorded as 1.06 g/200ml and dried biomass recorded as 0.4 g/200ml of potato dextrose broth and 2.30 g/200ml of the crude extract were obtained. Further, six fractions were purified by the column chromatography and different bioactive compounds with Rf values such as alkaloids [0.32], flavonoids [0.43], flavonoids [0.43], terpenoids [0.16], and polar basic compounds [0.78] were purified from these six fractions under the influence of different solvent systems. FTIR analysis revealed different functional groups such as amide, alkanes, alkenes, alcohol and phenols, alkyl halide, ether, amines, and nitrile.

Keywords: fungal endophytes, bioactive compounds, extraction, purification

1. Introduction

Endophytic microorganisms are those that occur intracellularly within the roots of Aloe vera. Almost all classes of herbs and other plants examine to date found to host endophytic microorganisms [1]. The characteristic feature of these fungi is the symbiotic association with other microbes in plant microflora and this association helps to produce different secondary metabolites. These secondary metabolites are mainly recognized for their use as remedies in different infectious diseases [2]. The fungal extracts have been used in many biological activities including antimicrobial activity, antioxidant activity, anti-urolithiasis activity, anticancer activity, and different microbial diseases. Studies have been reported on natural products including the substance of alkaloids, terpenoids, flavonoids, steroids etc. from isolated entophytes [3]. In the present study, *Aloe vera* was selected as an effective candidate for the study of fungal endophytes. *Aloe vera* is the succulent evergreen plant, mainly found in tropical climates around the world. It is the medicinal, short-stems herb [4]. *Aloe vera* roots contain phytochemicals which are mostly active against different pathogens. The plant tissues, especially roots are excellent reservoirs for endophytic microorganism isolated from up till uncharted areas and from the extreme environment is the choice for development of potential novel metabolite [5].

The objectives of the present study were (1) Isolation and purification of fungal endophytes from the root samples of *Aloe vera* (2) Enumeration of fungal endophytes (3) Production and extraction of bioactive compounds from *Penicillium* species (4) Purification and identification of functional groups from endophyte derived crude extracts.

2. Material and methods

2.1 Study site and plant materials

Total often locations was selected from the Shrirampur area, Ahmadnagar district Maharashtra, India. The locations were named as Loc 1 to Loc 10. The locations were selected on the basis of water availability and type of soil. The *Aloe vera* plant was selected for endophytic fungi study however only roots were selected and collected from the above-said region in the month of November 2018. The Aloe vera were identified and authenticated by Prof. Kukreja G., Department of Microbiology, NACSC, Ahmadnagar and a voucher specimen (N0-01/2018) was deposited. All samples were immediately brought to the laboratory and processed for isolation of endophytic microorganisms.

2.2 Isolation and purification of fungal endophytes

The root samples were washed under running tap water to remove the dust fond of to the surface and rinsed five times with distilled water. To eradicate the epiphytic microorganisms, the explants were surface sterilized according to the protocol of Mane and Vedamurthy [1]. Surface sterilized root samples were dried on a sterile tissue paper and were crushed into 1M phosphate buffer solution then 01.mL of this solution was inoculated on the sterile PDA plates containing ampicillin (50 mg/L). The plates were incubated at in the dark at 25°C ± 2°C for 10 days. After 10 days when a mycelium appeared around the edge

of the pieces on the PDA plates, the hyphal tips were transferred onto freshly prepared PDA plates to obtain pure culture for the preservation of fungal endophytes. The macroscopic and microscopic characterization such as the color and nature of the growth of the colony, the morphology of fruiting structures and spore morphology according to Mane and Vedamurthy [1]. The cultures were submitted to Mycology and Plant Pathology Group, Agharkar Research Institute, Pune, India for the identification and confirmation of endophytic fungi.

2.3 Production and Extraction of bioactive compounds

Selected endophytic fungus were subjected to liquid surface fermentation for the production of bioactive compounds. Flasks containing 100 mL potato dextrose broth were inoculated with eight-day old spore and mycelial suspensions of the endophytic fungus. All flasks were incubated for two weeks in the dark at 25°C ± 2°C for 10 days at static condition. After incubation, bioactive compounds from the broth culture were extracted using methanol solvent extraction. Methanol solvent (150 mL) was added to the broth culture and the formed mycelial mat was manually macerated. Then, the broth was mixed for four hours at 100 rpm and then left to soak overnight. After 24 hours, the methanol extract was separated from the broth using a filtration. The bioactive compounds layer was then concentrated *in vacuo* and the resulting mixture was dried overnight. The dried extracts were weighed and then used as the crude bioactive compounds for the purification and bioassays.

2.4 Purification and identification of functional groups from endophyte derived bioactive compounds

Endophyte derived crude extract was purified by column chromatography (CC) and thin layer chromatography (TLC). A clean, dried glass column was filled with the silica gel slurry and petroleum ether filled up to of 20cm. The extract was dissolved in DMSO to obtain a free-flowing powder. Then the powder was loaded in the silica gel column through funnel and elution was started with petroleum ether. Each fraction was collected, named as CECC. Further, collected fractions were spotted on precoated TLC plates with silica gel 60 F-254. The chromatogram was developed in the closed TLC chamber in the selected solvent system for 5 minutes. The solvents were selected as

1. for alkaloids Methanol: conc.NH_4OH (17:3)
2. for flavonoids Chloroform: methanol (18:2)
3. for terpenoids Benzene: Ethyl acetate (1: 1)
4. for polar basic compounds Chloroform: glacial acetic acid: methanol: water (6:2:1:1)

After 5 minutes, plates air dried and observed under sunlight and UV light (254 and 366 nm) for the observation of compound bands. Retention factor (Rf) value was calculated by using the following formula.

$$Rf = A/B$$

A = distance between sample spot and central point of the observed spot.

B = distance between the sample spot and the mobile phase front.

2.5 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR was used for the detection of the different functional groups present in the methanol crude extract. The FTIR was handled by the diffuse reflectance technique in which the dried fraction sample was assorted with potassium bromide to form a very well powder and then compressed into a thin pellet. The pellet was used for the analysis of different functional groups. The samples were irradiated by a broad spectrum of infrared light and the stage of absorbance at a meticulous incidence was plotted after. The absorbance was measured between 400-600 nm for the identification and quantification of organic groups.

3. Results and discussion

3.1 Isolation and purification of fungal endophytes

Total of sixteen fungal endophytes was isolated from the fifty root samples of *Aloe vera* collected from the different locations of Shrirampur, Ahmednagar district, India.

These sixteen fungal endophytes were identified on the basis of microscopic observation, colony morphology, and growth pattern. These all fungal endophytes were belonging to *Fusarium oxysporum, Nigrospora* species, *Alternaria alternata*, *Aspergillus* species, and *Penicillium* species. They were mainly belonging to the Ascomycetes group. The endophytic *Penicillium* species found to be a core group with the isolation frequency 60%. The endophytic *Aspergillus* species found to be second largest core group with colonization frequency of 50% followed by *Nigrospora* species (33%), *Fusarium oxysporum* (20%), and *Alternaria alternata* (8%). The results are shown in table 1, figure 1 & Plate 1. Tolulope *et al.* [7], reported seven fungal endophytic species as *Macrophomina* species, *Trichoderma* species, *Aspergillus* species, and *Penicillium* species from *A. boonei* while Mane *et al.* [3], purified total 8 fungal endophytes from *Aloe vera* which were belonging to mainly *Aspergillus niger*, and *Trichoderma* species.

Table 1: Isolation of fungal endophytes from the roots of *Aloe vera*

Endophytic fungi	Explants	Location	Total samples	Isolates	IF (%)
Fusarium oxysporum	Roots	All	10	2	20
Alternaria alternata	Roots	All	12	1	8
Aspergillus species	Roots	All	6	3	50
Nigrospora species	Roots	All	12	4	33
Penicillium species	Roots	All	10	6	60
Total		All	50	16	32

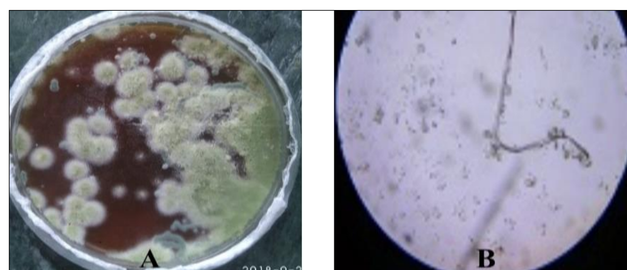

Fig 1: Macroscopic and microscopic characterization of endophytic *Penicillium* species

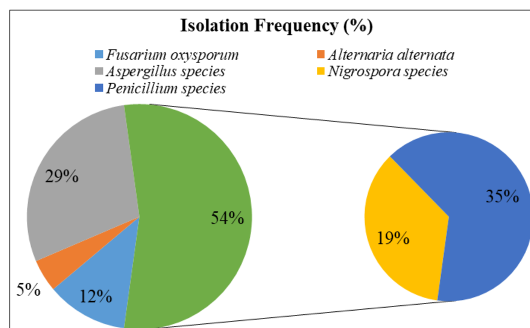

Fig 2: Isolation frequencies of fungal endophytes from the roots of *Aloe vera*

3.2 Production and extraction of bioactive compounds from Penicillium species

Submerged fermentation was employed for the production of bioactive compounds under static conditions by using 100 ml of potato dextrose broth and *Penicillium* species culture. After 10 days of incubation period, 100 ml of methanol was added as a solvent in the fermented media and then submerged for vacuum filtration. The filtrate and biomass were separated. The wet biomass of *Penicillium* species was recorded as 1.06 g/200ml of potato dextrose broth and the dried biomass recorded as 0.4 g/200ml of potato dextrose broth. In another hand, obtained 60 ml of filtrate were concentrated into 2.30 g/200ml of the methanol solvent by using Rota evaporator at 40°C after 6-8 hrs rotation at 90 rpm and used for further processes. Zhao *et al.* [8], produced different bioactive compounds from endophytic actinomycetes from different medicinal plants. They reported endophytic biomass of more than 2 g/200 ml of fermented broth. Mane *et al.* [4], have reported a crude extract of more than 2 g/100 ml of broth. As compared to Zhao *et al.* [8], and Mane *et al.* [4], our present results are better.

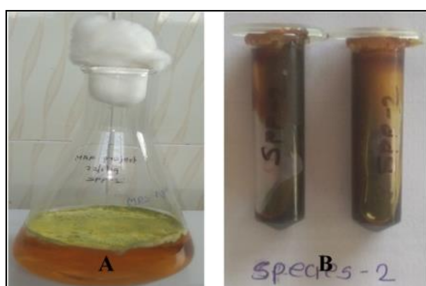

Fig 3: Production and extraction of bioactive compounds by *Penicillium* species. A shows feremented media and B shows dried crude extract.

In Ayurveda, many studies have shown that alkaloids, glycosides are known to have antimicrobial properties. Saponins are used as dietary supplements and nutraceuticals and play a very important role to produce inhibitory effect on inflammation. Tannins are measured to be having antiviral, antibacterial, antiphrastic, anticancer, antiulcer and antioxidant agents. Our results support to Ayurveda medicinal studies [4]. Microbial endophytes from medicinal plant are major reservoir of secondary metabolites. Tropical and temperate endophytes found to be different with respect to metabolite production ability. The discovery of novel antimicrobial metabolites from medicinal plants is an important alternative to overcome the increasing levels of drug resistance by human pathogen [6].

3.3 Purification and identification of functional groups from endophyte derived crude extracts

The column and thin layer chromatography were used for the purification of crude methanol extract of endophytic *Penicillium* species. Total six fractions were purified from the column chromatography namely CECC1, CECC2, CECC3, CECC4, CECC5 and CECC6. Further, all fractions were implanted in thin layer chromatography for the detection of different bioactive compounds. The results are shown in table 2. TLC of six fractions samples revealed the presence of four compounds having R*f* values of alkaloids [0.32] in Methanol: conc.NH_4OH- 17:3, flavonoids [0.43] in Chloroform: methanol- 18:2, terpenoids [0.16] in Benzene: Ethyl acetate -1: 1, and polar basic compounds [0.78] in Chloroform: glacial acetic acid: methanol: water- 6:2:1:1 solvent systems.

Table 2: Purification of different bioactive compounds by CC and TLC

CC Fractions	TLC spots			
	Alkaloids	Flavonoids	Terpenoids	polar basic compounds
CECC1	-	+	+	+
CECC2	+	+	+	+
CECC3	+	+	-	+
CECC4	+	+	-	+
CECC5	+	+	+	+
CECC6	+	+	+	+

FTIR analysis of methanol crude extract of *Penicillium* species revealed different functional groups. It includes amide, alkanes, alkenes, alcohol and phenols, alkyl halide, ether, amines and nitrile groups.

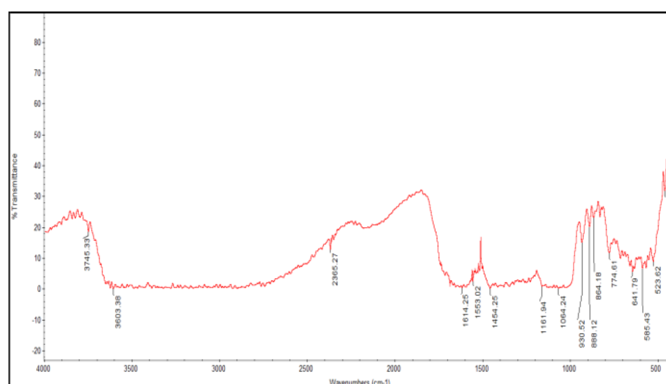

Fig 1: FTIR graph of methanol crude extract obtained by *Penicillium* species

Table 3: FTIR analysis of *Penicillium* crude extract of methanol solvent

Functional Groups	Type of Vibration	Frequency	Intensity
N-H Amide	Stretch	3745.33	Strong
OH Alcohol and Phenols	Stretch	3603.38	Strong
C≡N Nitrile	Stretch	2365.27	Medium
N-H_2 Amines	Stretch	1614.25	Strong
C=C Aromatic groups	Stretch	1553.02	Weak
C=C Aromatic groups	Stretch	1454.25	Strong
C-O Ether	Stretch	1161.94	Strong
C-O Ether	Stretch	1064.24	Strong
=C-H Alkene	Bending	930.52	Strong
=C-H Alkene	Bending	888.12	Strong
=C-H Alkene	Bending	864.18	Strong
=C-H Alkene	Bending	774.61	Strong
C-Cl Alkyl halide	Stretch	641.79	Strong
C-Br Alkyl halide	Stretch	585.43	Strong
C-Br Alkyl halide	Stretch	523.62	Strong
C-I Alkyl halide	Stretch	458.24	Strong

Graph 1 revealed different stretching's and bendings with high and low intensities therefore different functional groups had observed. These all functional groups state many future novel bioactive compounds.

4. Conclusion

The roots of *Aloe vera* hold endophytic fungi and they are able to produce bioactive compounds by using potato dextrose broth under static condition and room temperature. These bioactive compounds revealed different functional groups by FTIR study. In future GC-MS and NMR study of same extract may reveal novel bioactive compound against fungal and bacterial pathogens. The present study suggests that the present fungal endophytes and their extract may use in drug discovery and development.

5. Author contributions

Asmita B Gangurde, Priyanka R Jagtap, Manoj A Vyawahare, Girish P Kukreja wrote the manuscript. Rohit S Mane designed the concept, edited the manuscript. All authors read and approved the manuscript.

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