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STRUCTURE ELUCIDATION AND THERAPEUTIC APPLICATIONS OF ENDOPHYTIC FUNGI DERIVED BIOACTIVE COMPOUNDS OBTAINED FROM *XIMENIA AMERICANA* WESTERN GHATS OF KARNATAKA INDIA

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Keywords:

An endophytic *Thielaviopsis basicola*, Antagonistic activity, Antioxidant activity, Phytochemicals, Solid state fermentation, *Ximenia americana*

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ABSTRACT: The objective of the present investigation was the bioprospection of pharmaceutically vital bioactive compounds from endophytic *Thielaviopsis basicola* with antagonistic and antioxidant activity isolated from *Ximenia americana*, Western Ghats of Karnataka, India. The fresh and healthy leaves and roots of *Ximenia americana* were collected from the forests of Western Ghats of Karnataka, India and submerged for the enumeration and Purification of fungal endophytes by using different media. Isolated fungal endophytes were processed for the bioprospection of pharmaceutically vital bioactive compounds by referring to standard protocols. At a glance, endophyte derived bioactive compounds were used i*n-vitro* antagonistic and antioxidant activity. A total of 32 pure isolates were obtained, 8 morphologically distinct fungal endophytes were identified from 200 explants of *X. americana*. Endophytic *Thielaviopsis basicola* was a core candidate identified and cultivated in submerged fermentation in potato dextrose broth under the controlled condition at 25 °C ± 2°C for 15 days in incubator shaker. From this fermented medium, 3.30 g of aqueous crude extract per 100 ml of potato dextrose broth was obtained by the solvent-solvent extraction method. The qualitative phytochemical analysis showed a broad spectrum of bioactive compounds such as alkaloids, terpenoids, flavonoids, phenols, tannins, and glycosides. It was found that the aqueous crude extract contains 23.0090 ± 0.04129 mg of phenol and 53.47 ± 0.88059 mg of flavonoids content. From these crude extract TBXA1 and TBXA2, two fractions were purified by column chromatography and spotted potent bioactive compounds on thin-layer chromatography. The FTIR data of TBXA1 and TBXA2 showed different functional groups such as alcohol, phenols, alkanes, alkenes, aromatic, alkyl halides, amines, ether, and nitrile. The TBXA1 exhibited prominent antagonistic activity and MIC against *Staphylococcus aureus* (24 mm with 100 µg/mL) and *Candida albicans* (22 mm with 75 µg/mL) while TBXA2 exhibited against *Salmonella typhi* (22 mm with 75 µg/mL) and *Aspergillus fumigatus* (18 mm with 75 µg/mL). The TBXA1 showed potent antioxidant activity (86.24 ± 0.35) with 5.26 µg/mL of IC_{50} value as compared with TBXA2. This is the first innovative report of endophytic *Thielaviopsis basicola* for their phytochemicals detection and prominent *in-vitro* activities.

INTRODUCTION: Nowadays, there is a need to search for novel bioactive compounds from natural sources because of the increased number of emerging diseases in the globe with their rapid development and spread of multidrug-resistant pathogens.

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Mostly novel antioxidant and anticancer bioactive compounds have to search from the natural entity [1, 2]. Somehow due to incomplete knowledge and unknown uses, many medicinal plants are lagging behind in the list of worldwide pharmaceutical research.

The Indian research institutes such as the National Chemical Laboratory, Department of Science and Technology and Department of Biotechnology has given high priority to the search for novel bioactive compounds to treat HIV, Tuberculosis, Kidney Stone, Cancer, and other microbial diseases [3]. Fungi are widely identified as a potent candidate

for the production of novel bioactive compounds from the First World War [4-6].

During the last decades, researchers revealed different bioactive compounds such as phenols, flavonoids, tannins, saponins, glycosides, terpenoids, *etc*. from the different fungal genera that possess a wide variety of biological activities including antimycobacterial, antibacterial, antifungal, antioxidant and anticancer [2, 7]. Optionally, these all fungi are isolated and purified from different medicinal plants as endophytic fungi due to their ethnobotany and pharmaceutical applications [7-12]. In 1866, De Barry introduced the term "endophyte" for those microbes that reside inside the healthy tissues of plants without causing any apparent diseases [13-16]. After 100 years, Carroll (1986) used the term 'endophyte' for those organisms that cause asymptomatic infections within the plants [17-19]. Petrini elucidated Carroll's definition as commensalism in plants. Wilson further expanded endophytes and included both fungi and bacteria as commensals. Nowadays, this term is expanded as endophytic microorganisms which include fungi and bacteria with actinomycetes, resides intracellularly for their whole or at least part of life cycle [20-22].

In the present research investigation, *Ximenia americana* was selected as an effective plant candidate because of its unknown endophytic fungal diversity and bioactive compounds composition with antagonistic and antioxidant activity. The objectives of the present research study were

1. Enumeration and Purification of fungal endophytes from *Ximenia Americana.*
2. Bioprospection of pharmaceutically vital bioactive compounds.
3. *In-vitro* antagonistic and antioxidant activity.

MATERIALS AND METHODS:

Reagents and Chemicals: All chemicals used in the present study were purchased from Sigma-Aldrich, USA, and Hi-media, India. The chemicals like 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Sulphorodamine B were purchased from Sigma-Aldrich, USA. 75% ethanol, 4% Sodium hypochlorite, Sodium azide, 250 g Potato dextrose agar medium, 250 g Potato dextrose broth, 250 g of Muller-Hinton agar medium Aluminum chloride, Potassium acetate, Folin-Ciocalteu reagent, 60-120 mesh silica gel, silica gel 60 F-254, Potassium bromide, TPVG were obtained from Hi-media, India. Corn bran and other chemicals were obtained from the local market.

Isolation and Characterization of Endophytic Fungi: The fresh and healthy leaves and roots of *Ximenia americana* were collected from the forests of Karwar, Uttar Kannada district, Western Ghats of Karnataka, India in the month of July 2018. The selection of plant *Ximenia americana* was based on ethnobotanical history and abundance. The plant was identified and authenticated by Dr. Kotresha K., at the Department of Botany, Karnatak Science College, Dharwad, Karnataka, India and a voucher specimen kept under the number N0-01/2018. All explants were immediately brought to the laboratory and processed for further work. The explants were washed under running tap water to remove the dust fond of to the surface and rinsed three times with distilled water. To eradicate the epiphytic microorganisms, the explants were surface sterilized according to the standard protocol [2]. Surface sterilized explants were dried on a sterile tissue paper and were cut with a sterile blade into 0.5 to 0.3 cm pieces; each piece was placed onto a potato dextrose agar (PDA) plates. To avoid possible contamination, the final-rinse distilled water was placed on a fresh PDA plate as a negative control. All plates were incubated in dark at 25 °C ± 2 °C for 15 days. When mycelia appeared round the edge of the pieces on the PDA plates, the hyphal tips were transferred onto a freshly prepared PDA plate to obtain a pure culture. Isolated endophytic fungi were subjected for 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 by referring standard protocols [6]. The cultures were submitted to Mycology and Plant Pathology Group, Agharkar Research Institute, Pune, India for the identification and confirmation of endophytic fungi.

Analysis of Data:

Isolation Frequency: The isolation frequency of emerging hyphae was determined for each of the explants.

IF = Number of explant pieces showing growth × 100 / Total number of explant pieces

Mean: The mean of isolated endophytic fungi was carried out by using the following formula.

$$\overline{X} = \Sigma X/N$$

Where,
$\overline{X}$ = Mean,
ΣX = the sum of total numbers in a list
N = the number of items in that list.

All experiments were performed in triplicates (n=3), and the data are presented as the mean ± standard deviation.

Production, Extraction, and Phytochemical Analysis of Bioactive Compounds: The production of bioactive compounds was carried out by submerged fermentation in which potato dextrose broth was used. Three pieces of the grown pure culture of the fungus were cut from the culture plate and inoculated in a 1000 ml Erlenmeyer flask containing 200 ml of potato dextrose broth and incubated under the controlled conditions at 25 °C ± 2 °C for 15 days in incubator shaker. At the end of the incubation period, the fermented media were processed for the extraction of bioactive compounds. Fermented media was submerged for the extraction of bioactive compounds in the Soxhlet apparatus by using an aqueous solvent. In brief, 100 ml of aqueous solvent was added in fermented media and kept on the rotary shaker for 24 h. After 24 h the mycelium and culture media were separated from each other by vacuum filtration.

On the first hand, the filtrate was extracted three times with an equal volume of aqueous solvent for the complete extraction of metabolites from fungal biomass for 18-20 h at 40 °C in the Soxhlet apparatus. Then the concentration of the extract was performed on the Rota evaporator and dried under oven at 40 °C, weighed and stored at 15 °C. On another hand, obtained mycelia were air-dried, weighed and recorded as mg/100mL. Qualitative phytochemical analysis of an aqueous crude extract of *Thielaviopsis basicola* was employed for the detection of alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, and phenols, coumarins, quinones and glycosides by preferring standard protocols [19, 23-27]. Further, the flavonoids content and phenol content were estimated by using the Folin-Ciocalteu colorimetric method as per standard protocols [19]. Quantification was done on the basis of the standard curve of gallic acid and results were expressed as gallic acid equivalent.

Purification of Endophyte Derived Bioactive Compounds: Endophyte derived crude extract was run on 60-120 mesh silica gel (Spectrochem India) in column chromatography (CC). A clean, dried glass column was filled with the silica gel slurry and petroleum ether filled up to 20 cm. The column was repeatedly washed and flushed with petroleum ether to set the bed of the silica gel in the column. 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 and further used for structure elucidation. Thin Layer Chromatography (TLC) was performed by using precoated TLC plates with silica gel 60 F-254 for the investigation of bioactive compounds from the purified fractions using different solvent systems.

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 saponins chloroform: glacial acetic acid: methanol: water (6:2:1:1)

TLC plates were spotted with purified fractions with standard solutions of alkaloids, flavonoids, terpenoids, and saponins, and then developed in their respective eluent solvent systems. The chromatogram was developed in the closed TLC chamber in the selected solvent system for 5 min. 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 (R_f) 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.

FTIR was used for the detection of the different functional groups present in the purified fractions. 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.

Bioassays:

***In-vitro* Antagonistic Activity:**

Test Microorganisms: The two fractions of the fungus were used for *in-vitro* antagonistic activity against bacteria include *Staphylococcus aureus*, *Pseudomonas aeruginosa, Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi,* and fungi include *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger,* and *Aspergillus fumigatus* by well diffusion method [2, 6, 12, 28].

Well Diffusion Assay Method: The bacterium and fungal inoculum were prepared to the concentration of 1.0×10^4 CFU/ml adjusted with saline. The culture suspension was prepared and used as a stock culture for the experiment purpose. The culture suspension was spread on nutrient agar media for verification of other microbial contamination. Fluconazole and Telithromycin (10mg/mL) were used as positive control and solvent DMSO was used as the negative control. The verified microbial culture suspensions were spread on Muller-Hinton agar media plates and purified extract samples were added in the wells with standard antibiotics. Plates were incubated at 37°C for 24 h or 72 h and the zone of inhibition was recorded with the help of zone reader. All experiments were performed in triplicates.

Determination of MIC: For the determination of MIC of purified fractions, the broth microdilution technique was used in 96- well microtitre plate [32-34]. The microbial suspensions was adjusted at the concentration of 1.0×10^{-4} CFU/ml [OD= 0.34]. The different concentrations of purified fractions [25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, and 125 µg/mL] was prepared and added in 96- well microtitre plate containing microbial suspensions. Telithromycin (10 µg/mL) was used as positive control and solvent DMSO was used as the negative control. The plates were incubated at 37 °C for 48 h and absorbance was recorded at 630 nm in a UV-visible spectrophotometer. IC_{50} was expressed as the concentration of purified fractions [mg/ml] essential to produce a 50% reduction of microbial culture growth.

***In-vitro* Antioxidant Activity:**

Ferric Ion Reducing Power Assay (FRAP): Ferric ions reducing power was measured according to the standard methods [19, 24] with minimal modifications. The purified fractions of fungus were pipette in different concentrations ranging from 100 µl to 500 µl were mixed with 2.5 ml of 20 mM phosphate buffer and 2.5 ml (1% w/v) potassium ferricyanide, and then the mixture was incubated at 40 °C for 20 min.

To this mixture, 2.5 ml of (10% w/v) trichloroacetic acid and 0.5 ml of (0.1% w/v) ferric chloride were added to the mixture and kept for 10 min at room temperature to produce green colored complex. Ascorbic acid was used as a positive reference standard. The absorbance of the color developed was measured at 700 nm using a UV-Vis spectrophotometer.

DPPH Radical Scavenging Assay: The DPPH (2, 2 Diphenyl- 2- Picryl Hydrazyl) radical scavenging assay was carried out with purified fractions of fungus according to the method proposed by[24]. About 100 µl of a DPPH solution is prepared in ethanol (60 µmol/l) was mixed with 100 µl of the test samples in various concentrations. The blend was incubated for half an hour in dark at room temperature and the resultant absorbance was measured at 517 nm. Ascorbic acid was used as a standard. The DPPH scavenging activity of each sample was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = A_c\text{-}A_t / A_c \times 100$$

Where,

Ac = the absorbance of the control reaction (100 µl of ethanol with 100 µl of the DPPH solution).

At = the absorbance of the test sample.

RESULTS:

Isolation of Fungal Endophytes: In the Present investigation, *Ximenia americana* was collected from the forests area of Karwar, Western Ghats of

Karnataka, India. A total 32 fungal endophytes were isolated and enumerated from 100 pieces of 200 explants such as leaves, and roots of *Ximenia americana*. Most of the endophytic fungi were belonging to Ascomycetes. The endophytic *Thielaviopsis basicola* found to be a core group with the colonization frequency of 63.20%. The endophytic *Aspergillus niger* found to be second largest core group with colonization frequency of 41.60% followed by *Fusarium oxysporum* (38.33%), *Sterile mycelia* (33.20%), *Pestalotiopsis inflexa* (27.20%), *Nigrospora* species (20%), *Nigrospora* species (20%) and *Alternaria alternata* (15.30%). The colonization frequency of leaves explant pieces was varied between 0 to 46.6% and roots explant pieces were varied from 0% to 20%. The leaf pieces showed the highest colonization frequency by *Thielaviopsis basicola* (46.6%) and lowest colonization frequency by *Phomopsis* species (0%) while the root pieces showed the highest colonization frequency by *Phomopsis* species (20%) and lowest colonization frequency by *Nigrospora* species (0%). Results are shown in **Plate 1**, and **Table 1**.

Characterization of Fungal Endophytes:

***Fusarium oxysporum*:** *Fusarium oxysporum* showed moderate radial growth with 46 mm diameter on Potato dextrose agar media after 15 days of incubation period under the dark condition at 25 ℃ ± 2 ℃. They showed 0.9 cm/day diameter growth with white to pink or violet colored mycelium. They produce two types of conidia such as microconidia and macroconidia. Microconidia are hyaline, septate, and small with 0-3 µm size while macroconidia are hyaline, circular, look-alike heel, septate and small with 0-2 µm size. They showed 20-30 spores per microscopic field. Conidiogenous cells hyaline, enteroblastic in nature.

***Alternaria alternate*:** *Alternaria alternata* showed good radial growth with 79 mm diameter on Potato dextrose agar media after 15 days of incubation period under the dark condition at 25 ℃ ± 2 ℃. They showed 1.2 cm/day diameter growth with white color at start somehow changed into light brown with white mycelium after 10 days of incubation period. They showed pale brown colored conidiophores with 28.45 µm. these conidia are oval in shape. They showed 10-16 spores per microscopic field.

Aspergillus niger: *Aspergillus niger* showed good radial growth with 76 mm diameter on Potato dextrose agar media after 15 days of incubation period under the dark condition at 25 ℃ ± 2 ℃. They showed 1.6 cm/day diameter growth with dark brown to black or purple colored mycelium. They produce two types of conidia such as microconidia and macroconidia. These conidiophores were raised from the substratum varying from 200 µm to several millimeters long and 10-20 µm. These were smooth, vesicle globose, phialides borne directly on the vesicles. They showed 30-42 spores per microscopic field.

TABLE 1: ISOLATION OF FUNGAL ENDOPHYTES FROM *XIMENIA AMERICANA*

Endophytic fungi	Explants	Total Number of explant pieces	Isolates	IF (%)	Total	Mean
Fusarium	Leaves	10	3	30.0	38.33	
oxysporum	Roots	12	1	8.33		
Sterile mycelia	Leaves	12	2	16.6	33.20	
	Roots	12	2	16.6		
Alternaria alternata	Leaves	13	-	0	15.30	
	Roots	13	2	15.3		
Aspergillus niger	Leaves	12	3	25.0	41.60	32.35
	Roots	12	2	16.6		
Phomopsis species	Leaves	15	-	0	20.00	
	Roots	10	2	20.0		
Nigrospora species	Leaves	15	3	20.0	20.00	
	Roots	14	-	0		
Thielaviopsis	Leaves	15	7	46.6	63.20	
basicola	Roots	12	2	16.6		
Pestalotiopsis	Leaves	11	3	27.2	27.20	
inflexa	Roots	12	-	0		
Total	-	200	32	16		

***Phomopsis* Species:** *Phomopsis* species showed a slow growth because of sporulation nature. The colony of *Phomopsis* species looked dark in the form with mycelia being immersed, branched, septate and brown in color. The pycnidia are formed at the top of the mycelial mat, were globose in nature. After 10 days of incubation, the media become black because of secreted black pigment, therefore, the reverse side of the colonies was appeared black in color. They showed 8-12 spores per microscopic field.

***Nigrospora* Species:** *Nigrospora* species showed slow radial growth with 56 mm diameter. The colony was white at first then later it was observed as a brown to black with more sporulation. They produced black and brown pigments on media due to which media were blackish in color. Conidiophores were branched, flexuous, colorless to brown, smooth in nature. They were black, shining, smooth unseptate 10-14 μm diameter. They showed 12-16 spores per microscopic field.

***Thielaviopsis basicola*:** *Thielaviopsis basicola* showed good radial growth with 86 mm diameter. The colony of *Phomopsis* species looked dark all over the plate due to the production of black pigments. They produced two types of conidia namely endoconidia and aleuriospores. Endoconidia were single-celled, hyaline, spores with rounded ends. They were arranged in the chain formate. They showed 3 to 5 μm in diameter. Aleuriospores were darkly pigmented in nature.

They showed cylindrical spores that contain 2 to 6 cells and measures 20-25 μm in length. Both showed nucleated septa. They showed 27-32 spores per microscopic field.

***Pestalotiopsis inflexa*:** *Pestalotiopsis inflexa* showed moderate radial growth with 61 mm diameter on Potato dextrose agar media after 15 days of incubation period under the dark condition at 25 °C ± 2 °C. The colony of *Phomopsis* species looked light brown all over the plate. They showed conidia which were clavate to fusiform, rarely curved, smooth-walled, straight 5-6 celled, 23-29 X 80-95 μm. Apical and basal cell hyaline was long and broad cylindrical in nature. Septa were observed with the hardly constricted site. They showed 17-22 spores per microscopic field.

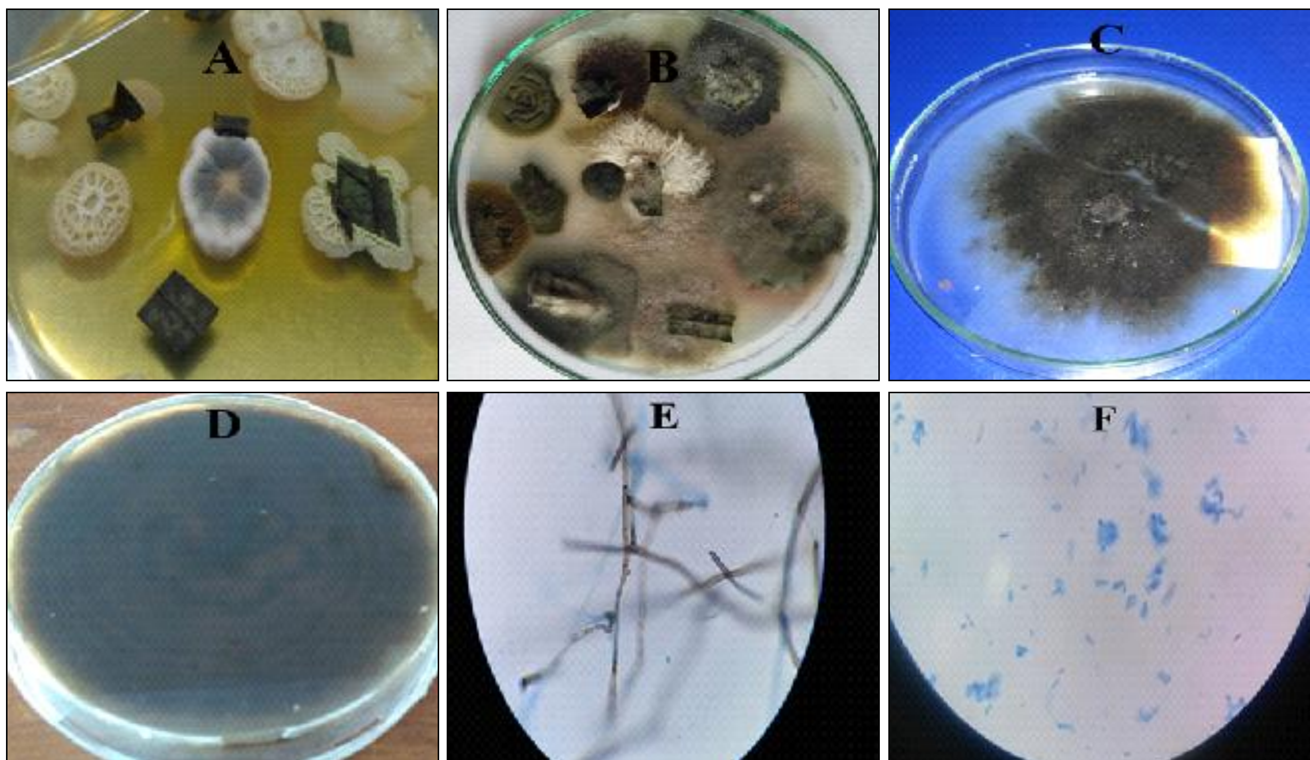


PLATE 1: ISOLATION AND CHARACTERIZATION OF FUNGAL ENDOPHYTES FROM LEAVES AND ROOTS OF *XIMENIA AMERICANA* (A) & (B) ISOLATION OF FUNGAL ENDOPHYTES ON PDA MEDIUM PLATES IN DARK AT 25 °C ± 2 °C FOR 15 DAYS (C) & (D) CHARACTERIZATION AND PURIFICATION OF *THIELAVIOPSIS BASICOLA* ON PDA MEDIUM PLATES IN THE DARK AT 25 °C ± 2 °C FOR 15 DAYS, PLATES SHOWING FRONT AND BACK SIDE (E) & (F) MICROSCOPIC CHARACTERIZATION OF MYCELIUM AND SPORES

Production, Extraction, and Phytochemical Analysis of Bioactive Compounds: Submerged fermentation was employed for the production of bioactive compounds under static conditions by using 100 ml of potato dextrose broth in the presence of *Thielaviopsis basicola* culture. After 15 days of the incubation period, 100 ml of aqueous solvent was added in the fermented media and then submerged for vacuum filtration. The filtrate and biomass were separated. The wet biomass of *Thielaviopsis basicola* was recorded as 2.86 g/100ml of PDB and the dried biomass recorded as 0.25 g/100ml of PDB. In the hand, obtained 80 ml of the filtrate were concentrated into 3.30 g/100ml of the aqueous solvent by using the Rota evaporator at 40 °C after 6-8 h rotation at 90 rpm and used for further processes. The qualitative analysis of the aqueous crude extract of endophytic *Thielaviopsis basicola* revealed a broad spectrum of secondary metabolites such as Phenols, Tannins, Flavonoids, Saponins, Alkaloids, and Glycosides. The quantitative analysis of the total phenolic content of the aqueous extract was estimated with Gallic acid as a reference standard. The aqueous extract showed high phenolic content (23.0090 ± 0.04129 mg). Total flavonoids content was performed using the $AlCl_3$ method using quercetin as a standard. The aqueous extract showed high flavonoids content (53.47 ± 0.88059 mg). The results were expressed as mg/g GAE per gram of endophytic extract. The results are shown in **Table 2**.

Purification and Evaluation of Endophyte Derived Bioactive Compounds: The column and thin-layer chromatography were used for the purification of crude aqueous extract of endophytic *Thielaviopsis basicola*. Total two fractions were purified from the column chromatography namely TBXA1 and TBXA2. Further, both fractions were implanted in thin layer chromatography for the detection of different bioactive compounds. TLC of TBXA1 and TBXA2 revealed the presence of four compounds having R*f* values of alkaloids [0.29] in Methanol: conc.NH_4OH- 17:3, flavonoids [0.41] in Chloroform: methanol- 18:2, terpenoids [0.15] in Benzene: Ethyl acetate -1: 1, and saponins [0.91] in Chloroform: glacial acetic acid: methanol: water- 6:2:1:1 solvent systems. The results are shown in **Plate 2**. The Fourier Transform Infrared Spectrophotometer (FTIR) Analysis of TBXA1 and TBXA2 revealed different functional groups.

Graph 1 and **Table 3** represent different functional groups of TBXA1. The very strong absorption bands observed at 3392.28 cm^{-1} which represents O-H strong stretching vibrations. It indicates alcohol and Phenols as a functional group. The very less absorption bands were observed at 605.83 cm^{-1} which represents C-Br strong stretching vibrations. It indicates Alkyl halide as a functional group. **Graph 2** and **Table 4** represent different functional groups of TBXA2. The very strong absorption bands were at 3745.33 cm^{-1} which represents N-H strong stretching vibrations. It represents Amide groups. The very less absorption bands were at 458.24 cm^{-1} which represents C-I strong stretching vibrations. It indicates an Alkyl halide functional group.

TABLE 2: PHYTOCHEMICAL ANALYSIS OF AQUEOUS CRUDE EXTRACTS OF ENDOPHYTIC *THIELAVIOPSIS BASICOLA* (+ INDICATES PRESENCE AND – INDICATES ABSENCE)

S. no.	Tests	Observation
1	Alkaloids	+
2	Terpenoids	+
3	Steroids	-
4	Tannins	+
5	Saponins	+
6	Flavonoids	+
7	Phenols	+
8	Coumarins	-
9	Quinones	-
10	Glycosides	+

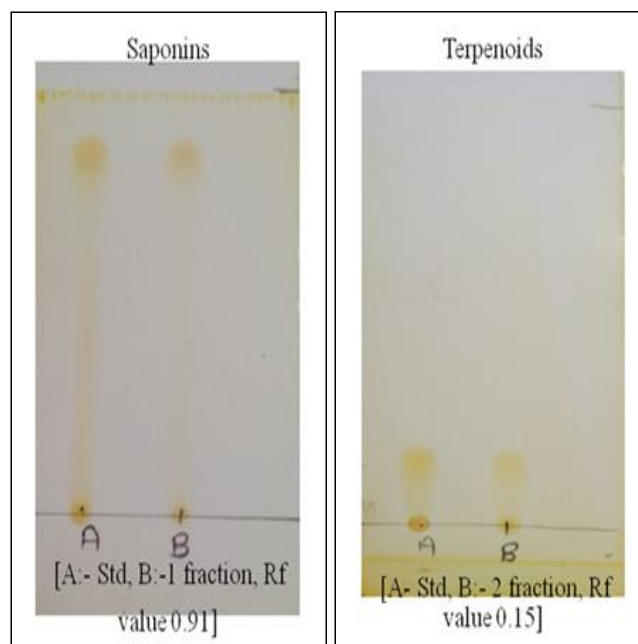
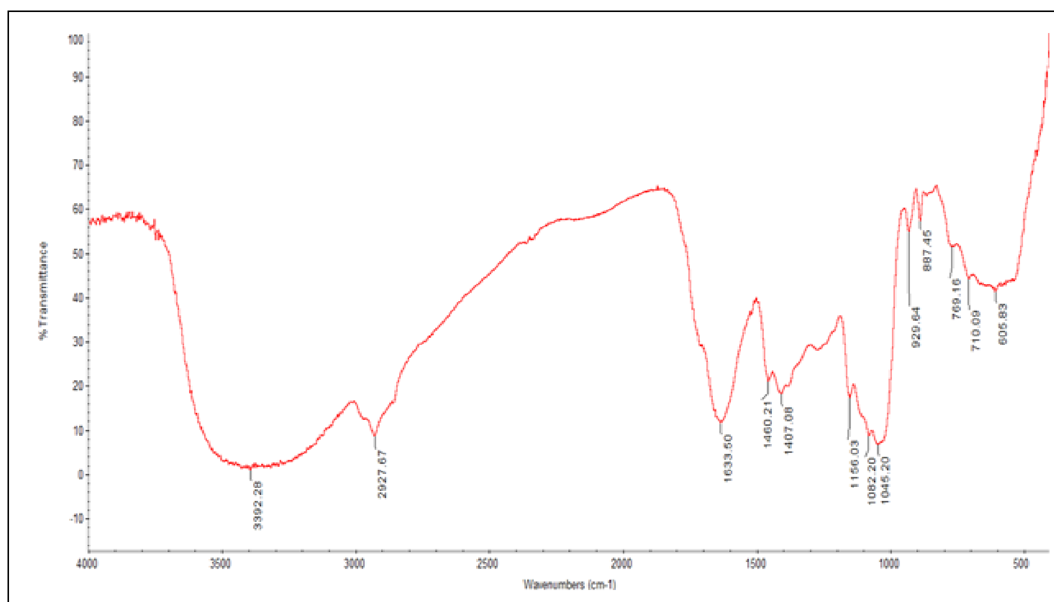


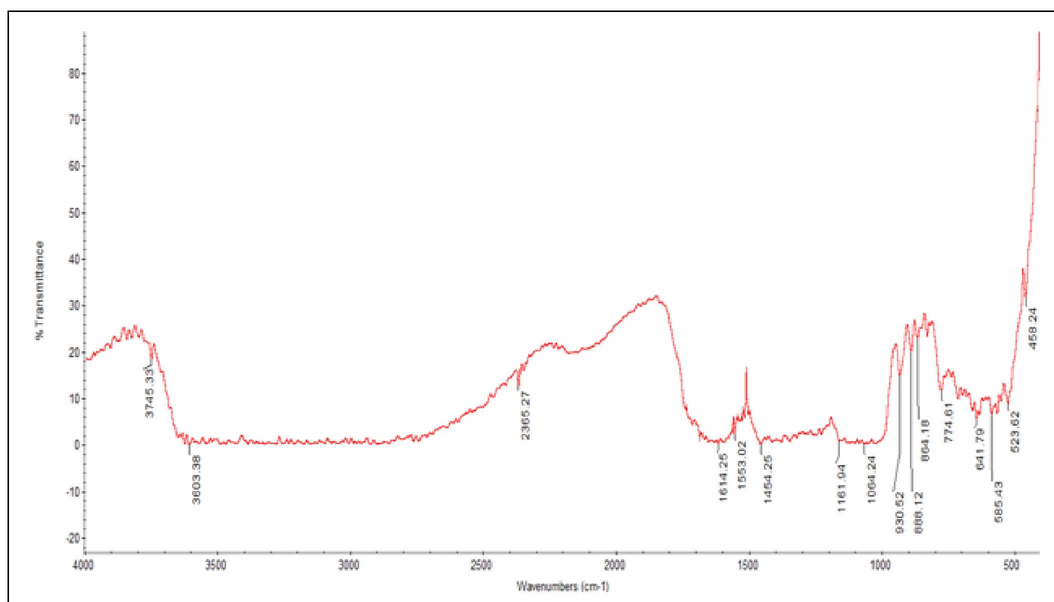
PLATE 2: THIN LAYER CHROMATOGRAPHY SPOTS UNDER NORMAL LIGHT FOR THE DETECTION OF BIOACTIVE COMPOUNDS COMPARED WITH STANDARDS



GRAPH 1: FTIR GRAPH OF TBXA1 OBTAINED FROM CC OF THE AQUEOUS CRUDE EXTRACT OF *THIELAVIOPSIS BASICOLA*

TABLE 3: FTIR FREQUENCIES OF DIFFERENT FUNCTIONAL GROUPS WITH THEIR INTENSITIES OF TBXA1 OBTAINED FROM CRUDE AQUEOUS EXTRACT OF *THIELAVIOPSIS BASICOLA*

Functional Groups	Type of Vibration	Frequency	Intensity
O-H Alcohol and Phenols	Stretch	3392.28	Strong
-C-H Alkanes	Stretch	2927.67	Weak
C=C Alkenes	Stretch	1633.50	Weak
-C-H Alkanes	Bending	1460.21	Medium
C=C Aromatic groups	Stretch	1407.08	Weak
C-F Alkyl halide	Stretch	1156.03	Strong
C-N Amine	Stretch	1082.20	Strong
C-O Ether	Stretch	1045.20	Strong
=C-H Alkene	bending	929.64	Strong
=C-H Alkene	Bending	887.45	Strong
C-Cl Alkyl halide	Stretch	769.16	Strong
C-Cl Alkyl halide	Stretch	710.09	Strong
C-Br Alkyl halide	Stretch	605.83	Strong



GRAPH 2: FTIR GRAPH OF TBXA2 OBTAINED FROM CC OF THE AQUEOUS CRUDE EXTRACT OF *THIELAVIOPSIS BASICOLA*

TABLE 4: FTIR FREQUENCIES OF DIFFERENT FUNCTIONAL GROUPS WITH THEIR INTENSITIES OF TBXA2 OBTAINED FROM CRUDE AQUEOUS EXTRACT OF *THIELAVIOPSIS BASICOLA*

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

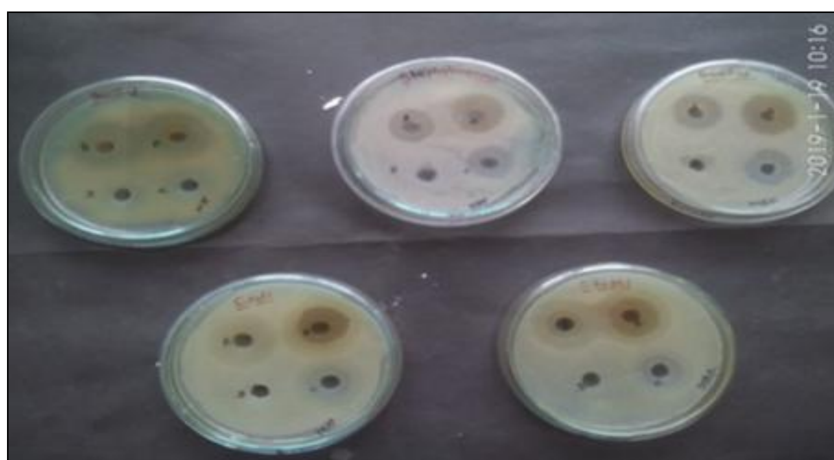


PLATE 3: ANTIBACTERIAL ACTIVITY OF TBXA1 AND TBXA2 OF *THIELAVIOPSIS BASICOLA*, THE PLATES OF *BACILLUS SUBTILIS* (1), *STAPHYLOCOCCUS AUREUS* (2), *PSEUDOMONAS AERUGINOSA* (3), *ESCHERICHIA COLI* (4), *SALMONELLA TYPHI* (5). THE WELL A CONTAINS TBXA1 SUSPENSION, B CONTAINS TBXA2 SUSPENSION, C CONTAINS DMSO AND D CONTAINS TELITHROMYCIN. THE ALL OBTAINED PURE CULTURES WERE SPREAD ON MULLER HINTON AGAR, AND WELLS WERE PREPARED. ADDITION OF ALL PREPARED SUSPENSIONS, POSITIVE CONTROL, AND NEGATIVE CONTROL WAS DONE AND INCUBATED AT 37 °C FOR 24 h AND AFTER 24 h ZONE OF INHIBITION WAS RECORDED WITH THE HELP OF ZONE READER

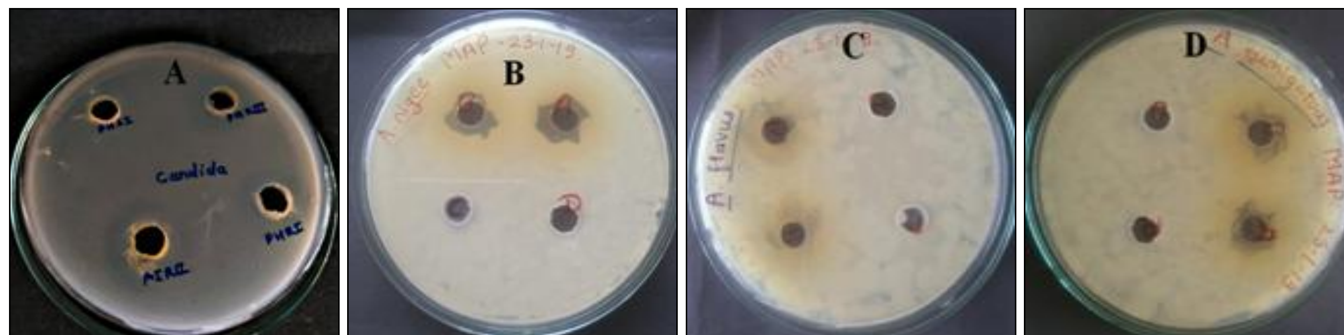


PLATE 4: ANTIFUNGAL ACTIVITIES OF TbCC1 AND TbCC2 OF *THIELAVIOPSIS BASICOLA*. THE PLATES OF *CANDIDA ALBICANS* (A), *ASPERGILLUS NIGER* (B), ASPERGILLUS *FLAVUS* (C), AND *ASPERGILLUS FUMIGATUS* (D). THE WELLS A CONTAIN TBCC1 SUSPENSION, B CONTAINS TbCC2 SUSPENSION, C CONTAINS DMSO AND D CONTAINS TELITHROMYCIN. THE ALL OBTAINED PURE CULTURES WERE SPREAD ON MULLER HINTON AGAR, AND WELLS WERE PREPARED. ADDITION OF ALL PREPARED SUSPENSIONS, POSITIVE CONTROL, AND NEGATIVE CONTROL WAS DONE AND INCUBATED AT 37 °C FOR 72 h AND AFTER 72 h ZONE OF INHIBITION WAS RECORDED WITH THE HELP OF ZONE READER

TABLE 5: *IN-VITRO* ANTAGONISTIC ACTIVITY OF TBXA1 AND TBXA2 OF *THIELAVIOPSIS BASICOLA*

Well no.	CC Fractions and Standard antibiotics	Zone of inhibition (mm)								
		S. aureus	*P. aeruginosa*	*B. subtilis*	*E. coli*	*S. typhi*	*C. albicans*	*A. flavus*	*A. niger*	*A. fumigatus*
A	TbCC1	24	21	20	23	23	22	16	21	17
B	TbCC2	15	19	16	21	22	12	13	17	18
C	Telithromycin [Bacterial]	28	24	22	25	23	NA	NA	NA	NA
C	Fluconazole [Fungi]	NA	NA	NA	NA	NA	18	21	22	24
D	DMSO	-	-	-	-	-	-	-	-	-

[NA] = Not Applicable, [-] = absence of zone of inhibition

Bioassays: In the present study, *Thielaviopsis basicola* endophytic fungi were subjected to Solid-state fermentation for the production of secondary metabolites followed by subsequent extraction by solvent-solvent extraction method and Soxhlet extraction method to obtain the crude extract. This crude extract was purified by column chromatography and thin-layer chromatography. TBXA1 and TBXA2 fractions were obtained from CC and both these fractions assessed for their bioactive potential *viz*. antagonistic and antioxidant activity.

***In-vitro* Antagonistic Activity:** *In-vitro* antimicrobial activity of TBXA1 and TBXA2 of *Thielaviopsis basicola* against *Staphylococcus aureus*, *Pseudomonas aeruginosa, Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, and fungi include *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger,* and *Aspergillus fumigatus* by agar well diffusion method. The results of antimicrobial activity of TBXA1 and TBXA2 of *Thielaviopsis basicola* were summarized in **Plate 3, 4** and **Table 5**. The results proved that the minimum inhibitory concentration value of TBXA1 and TBXA2 against selected microorganisms was varied and this inconsistency depends upon the microbial strains. The MIC values of TBXA1 against bacteria were 100 µg/ml (*Staphylococcus aureus*), 75 µg/ml (*Pseudomonas aeruginosa*), 100 µg/ml (*Bacillus subtilis*), 75 µg/ml (*Escherichia coli*), and 50 µg/ml (*Salmonella typhi*) while against fungi were 75 µg/ml (*Candida albicans*), 100 µg/ml (*Aspergillus flavus*), 100 µg/ml (*Aspergillus niger*), and 100 µg/ml (*Aspergillus fumigatus*). The results are shown in figure 4. The MIC values of TBXA2 against bacteria were 75 µg/ml (*Staphylococcus aureus*), 100 µg/ml (*Pseudomonas aeruginosa*), 75 µg/ml (*Bacillus subtilis*), 100 µg/ml (*Escherichia coli*), and 75 µg/ml (*Salmonella typhi*) while against fungi were 125 µg/ml (*Candida albicans*), 75 µg/ml (*Aspergillus flavus*), 100 µg/ml (*Aspergillus niger*), and 75 µg/ml (*Aspergillus fumigatus*).

***In-vitro* Antioxidant Activity:** Ferric ion reducing power assay was performed with TBXA1 and TBXA2 by using ascorbic acid as standard. In this assay, the highest antioxidant activity at the rate of 1.2407 ± 0.00702 showed by TBXA1 as compared to TBXA2. DPPH (2, 2 Diphenyl, 2- Picryl Hydrazyl) assay was performed with TBXA1 and TBXA2 and ascorbic acid standard. The results of this assay showed potent antioxidant activity with TBXA1 being the highly potent (86.24 ± 0.35) as compared with TBXA2 (82.5333 ± 0.23714). The IC_{50} of TBXA1 found to be 5.26 µg/mL and TBXA2 found to be 7.24 µg/mL.

DISCUSSION: According to the world health organization, roughly 65-80% of developing countries including India, depend on herbal medicines for their fitness care due to difficulties in accessing modern medicines. Natural products or derivatives have been established to have noteworthy antimicrobial, antioxidant and anticancer activity due to their aptitude to slow growth, in case of cancer angiogenesis and metastasis without any side effects [6-12].

Karwar lies in the hilly region of Western Ghats of Karnataka, India, and situated on the rows of the Kali River. It is a coastal city on the west coast of the Indian isthmus, located at 14.820 °N and 74.135 °E. It has a warm summer from March to May, where the temperature may reach 37 °C. Winter from December to February is actual slight (24 °C and 32 °C). The wind period from June to September has a usual rainfall of over 400 centimeters (160 in) and wind speed ranges from

1.2 to 2.5 m/sec. This area is horny with different plants such as shrubs, herbs, and trees [17, 18, 19]. This all plants are growing in unusual habitats with distinctive ecological environmental niche and holding novel strategies for survival, ethnobotanical antiquity and used for traditional medicines. It has been found that all plants seize fungal endophytes in their tissues of leaves, petioles, stems, twigs, bark, xylem, roots, fruits, flowers and seeds [2-14]. Merely fungal endophytes are studied from the Karnataka region even though great biodiversity is implemented by nature in the wide range. Mainly few researchers selected Mercara region, Karnataka for the study of fungal endophytes [19] and some studied endophytic fungi from the tropical region of Karnataka, India [28] therefore in accordance with an above reference study and facts about fungal endophytes, we selected Karwar, Western Ghats of Karnataka, India as a study area. During the plant selection from the selected area non-apparent hypothesis was used; it deals with only herbs and harvests low molecular weight organic compounds with low toxicity and high bioactivity.

In the present study *Ximenia americana* Linn. The plant was selected as an endophytic inhabiting candidate. It belongs to Olacaceae family [31]. It is a small tree or shrub, resident to the tropical area of Africa and also seen dispersed in different parts of the world. This species is widely famous in rural communities in Africa and Asia. They are using in the treatment of a wide variety of ailments such as wild olive or sour plum or yellow plum and widely used as herbal medicine in action of malaria, leproutic ulcer, and skin infections. The leaves are reported to have antibacterial activity and also used in the management of fever, tuberculosis, tooth decay and wounds [31].

Morphological studies revealed that the isolates have belonged to four endophytic fungal classes Deuteromycetes, Ascomycetes, Zygomycetes and Basidiomycetes with different isolation frequencies. The highest colonization frequency showed by leaves explants while the lowest showed by root explants. Most dominant fungal endophytes enumerated were *Thielaviopsis basicola* at the rate of 46.6% belonged to Ascomycetes while the lowest was *Alternaria alternata* at the rate of 15.30%. A total 14 fungal endophytes were isolated from the different medicinal plants such as *Azadirachta indica*, *Terminalia arjuna*, and *Catharanthus roseus* [15]. Further, they showed the highest isolation frequencies of fungal endophytes from the leaf samples and lowest by stem samples [15] while in other studies a total 77 fungal endophytes were reported from the inner bark of *Acalypha indica* belonging to 15 genera [16]. The recorded Ascomycetes as core fungal endophytic class from medicinal plants [18, 30]. Deuteromycetes (55-72%) with high isolation frequencies and Ascomycetes (10-35%) with low isolation frequencies from different medicinal plants [29]. Mainly studied endophytes from different medicinal plants and they were foliar endophytes [25]. The colonization rate of fungal endophytes is significantly higher in the stems than in the leaves, but we found the opposite; nevertheless, the results of several previous studies were similar to those obtained in the current study [33]. The probable reasons for these discrepancies are dissimilar organ arrangement, chemical composition, and even the period length of organs hang about residual in the tree, possibly leading to change in plant intracellular substance composition. Even though a number of differences in endophytic fungi isolates were experiential between organs, similarities between the distributions of endophytic fungi in a range of organs were also noted [34].

In contrast with previous study samples [33, 34, 16], our samples were collected from the unusual area of India, and therefore the diversity of the obtained isolates was high; nevertheless, we did not collect stem or flowers of *Ximenia americana* explants, therefore a few fungal endophytes may have been under-represented. Previous reports of fungal endophytes from India may account for the high CF in the roots of medicinal plants except for our results. There may be two reasons for the different enumeration count. One likely reason is the relationship between soil fungi and rhizospheric fungi. They have prevalent and diversified an endophytic relationship with the roots. The additional reason is that roots as significant sources of the easily nearby substrate may give a comparatively steady environment favoring much fungal continued existence [34].

Fungal endophytes are the great sources of the novel bioactive compounds. In current years, there

has been confirmed that the production of bioactive compounds by an endophyte is not accidental but seems to be correlated with his environmental niche. The metabolic communications of endophytes with its host may errand the synthesis of bioactive compounds [34]. Fungal endophytes were isolated from grasses and further, they showed the *in-vitro* and *in-vivo* production of different ergot alkaloids, loline alkaloids, lolitrems, growth hormone and paramine alkaloids [18]. In our study, we used submerged fermentation for the production of bioactive compounds under the influence of PDB under the controlled conditions at 25 °C ± 2 °C for 15 days. After fermentation, extraction was carried out by vacuum filtration followed by solvent-solvent extraction method and rotary evaporation to obtain the crude extract by using an aqueous solvent.

The wet and dried biomass of *Thielaviopsis basicola* was recorded as 2.86 g/100ml and 0.25 g/100ml of the PDB respectively with 3.30 g/100ml of concentrated aqueous crude extract. The endophytic fungi of grass grown in synthetic medium produced precursors of auxin *in-vitro* [19] while others showed production of Cryptocin by *Cryptosporiopsis quercina* with their potent activity against *Pyricularia oryzae cavara* and a number other plant pathogenic fungi [14, 18]. Merely *Streptomyces* was reported as a novel bioactive compounds producer from marine water [5]. The bioactive compounds production may vary from organism to organism because of the substrate provided, metabolic processes, the formation of products and byproducts, environmental conditions. A specific bioactive compound requires a specific substrate to make the chemical body with respect to provided semi-natural conditions [18].

There are two substrate-based methods used for the construction of bioactive compounds namely solid-state fermentation and submerged state fermentation. Previously production of bioactive compounds was reported from fungal endophytes by submerged state fermentation only while merely studied by solid-state fermentation [18]. Rice bran were used as a substrate in solid-state fermentation for the production of bioactive compounds at room temperature and revealed few bioactive compounds with their biological activities in rice blast disease [7] while Liquid Wickerham medium and solid rice bran medium was used for the production of bioactive compounds by submerged and solid-state fermentation respectively and revealed different bioactive compounds with their bioactivity [9]. The phytochemical analysis of the aqueous crude extract revealed that the presence of phenols, tannins, terpenoids, flavonoids, saponins, alkaloids and glycosides with phenolic content (23.0090 ± 0.04129 mg) and flavonoids content (53.47 ± 0.88059 mg). The TBXA1 and TBXA2 fractions of crude extract of *Thielaviopsis basicola* obtained from column chromatography revealed the presence of alkaloids, flavonoids, terpenoids, and saponins in the solvents MA, CM, BE, CGMW respectively in thin layer chromatography. The R_f values matched with standards and confirmed the purification and efficiency of bioactive compounds.

Further, FTIR showed different functional groups that are mainly found in natural drugs. The graph 1 of TBXA1 revealed the different stretching and bending of the alcohol and phenols, alkanes, alkenes, aromatic groups, alkyl halides, amines, and ether groups while the graph 2 of TBXA2 revealed the alcohol and phenols, alkanes, alkenes, aromatic groups, alkyl halides, amines, ether, and nitrile.

The TBXA1 showed highest antagonistic activity against *Staphylococcus aureus* with 24 mm zone of inhibition and lowest against *Bacillus subtilis* with 20 mm zone of inhibition in case of bacteria while in case of fungi, it showed highest antagonistic activity against *Candida albicans* with 22 mm zone of inhibition and lowest against *Aspergillus niger* with 16 mm zone of inhibition. The TBXA2, showed highest antagonistic activity against *Salmonella typhi* with 22 mm zone of inhibition and lowest against *Staphylococcus aureus* with 15 mm zone of inhibition in case of bacteria while in case of fungi, it showed highest antagonistic activity against *Aspergillus fumigatus* with 18 mm zone of inhibition and lowest against *Candida albicans* with 12 mm zone of inhibition.

The TBXA1 showed inhibitory MIC at the rate 100µg/ml in the case of bacteria while 75 µg/ml in the case of fungi. The TBXA2 showed inhibitory, effective MIC at the rate of 75 µg/ml in case of bacteria while 100µg/ml in the case of fungi. The MIC value of extract <100 µg/ml were classified as

first-class antibacterial activity, while extracts with MIC price ranging from 100 µg/ml to 500 µg/ml classified as modest activity [7, 18]. The antioxidant activity of bioactive compounds mainly depends on the efficiency of bioactive compounds towards that toxin or protein [19]. The IC_{50} of alkaloids obtained from *Aspergillus niger* fermented medium showed 3.22 µg/mL which was effective against DPPH and these activities revealed effective antioxidant activity [18, 20].

The antioxidant activity of TBXA1 and TBXA2 was performed by DPPH assay and FRAP assay. DPPH radicals receive electrons from TBXA1 and TBXA2 and reduced into DPPH. The violet color of DPPH radical gives yellow-colored reduced ferric ions. In the FRAP assay, the highest antioxidant activity showed by TBXA1 at the rate of 1.2407 ± 0.00702 as compared to TBXA2. The highest DPPH activity was showed by TBXA1 at the rate of 86.24 ± 0.35 as compared with TBXA2 (82.5333 ± 0.23714). The IC_{50} of TBXA1 found to be 5.26 µg/mL, which was effective against DPPH and these activities revealed effective antioxidant activity.

CONCLUSION: The results of the study showed, that the leaves and roots of *Ximenia americana* have the potential to reveal the diversity of fungal endophytes. *Thielaviopsis basicola* were the dominant fungal taxa isolated and selected for further study. The *Thielaviopsis basicola* has latent to produce bioactive compounds by using potato dextrose broth under submerged fermentation and these compounds have significant *in-vitro* antagonistic and antioxidant activity.

The results highlighted the potential of the leaves of the selected plant and isolated fungal endophytes as a source of natural remedy. Therefore, we concluded that the present research work is the first report available from the *X. americana* for an endophytic *Thielaviopsis basicola* with biological activities from Western Ghats of Karnataka, India. This report may lead to exploring valuable source for drug discovery and development.

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