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THERAPEUTIC APPLICATIONS OF BIOACTIVE COMPOUNDS OBTAINED BY ENDOPHYTIC *Thielaviopsis basicola* ISOLATED FROM X*imenia americana*, WESTERN GHATS OF INDIA

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ABSTRACT

The objective of the present study was the bioprospection of bioactive compounds from endophytic *Thielaviopsis basicola* with therapeutic applications, 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. The collected explants were submerged for the enumeration and purification of fungal endophytes by using selective, and different media. Isolated fungal endophytes were processed for the production, extraction, phytochemical analysis, purification and evaluation of bioactive compounds by referring standard protocols. At a glance, endophyte derived bioactive compounds were used in *in-vitro* antagonistic, antioxidant antiurolithisis, and anticancer 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 solid-state fermentation on corn bran under the dark condition at 25℃ ± 2℃ for 15 days. Obtained 5.30g of aqueous crude extract per 100g of cornmeal were processed for qualitative phytochemical analysis, in alkaloids, terpenoids, flavonoids, phenols, tannins, and glycosides were found. The aqueous crude extract showed 0.761g of phenol and 0.276g of flavonoids content. From these crude extract TbCC1 and TbCC2, two fractions were purified by column chromatography and spotted potent bioactive compounds on thin layer chromatography. The FTIR data of TbCC1 and TbCC2 showed different functional groups such as alcohol, phenols, alkanes, alkenes, aromatic, alkyl halides, amines, ether, and nitrile. The TbCC1 exhibited prominent antagonistic activity and MIC against *Staphylococcus aureus* (24mm with 100µg/mL) and *Candida albicans* (22mm with 75µg/mL) while TbCC2 exhibited against *Salmonella typhi* (22mm with 75µg/mL) and *Aspergillus fumigatus* (18mm with 75µg/mL). The TbCC1 showed potent antioxidant activity (86.24±0.35) with 5.26µg/mL of IC50 value as compared with TbCC2. The maximum antiurolithiasis activity showed with 100% concentration at the rate of 67.5±1.49 of aqueous crude extract. The Sulphorodamine B assay showed MCF7 human breast cancer cell line viability 98.39±0.0250 at 100µg concentration and 40.81±0.0470 at 500µg with an IC50 value of 459.60µg.This is the first innovative report of endophytic *Thielaviopsis basicola* for their phytochemicals detection and prominent *in-vitro* activities from the selected plants.

Keywords: Endophytic *Thielaviopsis basicola*; microbial pathogens; kidney stone; MCF7 human breast cancer cell line; phytochemicals; *Ximenia americana*

INTRODUCTION

Nowadays there is a need to search 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 (**Ananda *et al.*, 2010; Mane *et al.*, 2017**). Mostly novel antioxidant and anticancer bioactive compounds have to search from the natural entity. Somehow due to incomplete knowledge and unknown uses, many medicinal plants are lagging behind in the list of worldwide pharmaceutical research (**Suryanarayanan *et al.*, 2002; Aditi *et al.*, 2017**). The Indian research institutes such as 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 (**Padmanabhan *et al.*, 1977; Julia *et al.*, 2009; Dharmaraj *et al.*, 2010**). During the last decades, researchers revealed different bioactive compounds such as phenols (**Fisher *et al.*, 1991; Julia *et al.*, 2009**), flavonoids, tannins (**Zeng *et al.*, 1987**), saponins, glycosides, terpenoids (**Zhou *et al.*, 2012; Zhang *et al.*, 2013**) etc. from the different fungal genera that possess a wide variety of biological activities including antifungal (**Padmanabhan *et al.*, 1977**), antioxidant (**Zhou *et al.*, 2012**), antimycobacterial (**Zhang *et al.*, 2013**), antibacterial, and anticancer (**Julia *et al.*, 2009**). Optionally, these all fungi are isolated and purified from different plant sources as endophytic fungi due to their ethnobotany and pharmaceutical applications (**Sun et al., 2011; Sunayana *et al.*, 2014**). In 1866, De Barry introduced the term "endophyte" for those microbes that reside inside the healthy tissues of plants without causing any apparent diseases (**Romas *et al.*, 2011**). After 100 years, Carroll (1986) used the term 'endophyte' for those organisms that cause asymptomatic infections within the plants (**Rodriguez *et al.*, 2008; Li *et al.*, 2008**). 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 (**Padmanabhan *et al.*, 1977; Mane *et al.*, 2017, 2018**). In the present research study, *Ximenia americana* was selected as an effective plant candidate because of their unknown endophytic fungal diversity and bioactive compounds composition with *in-vitro* bioassay. The objectives of the present research study was 1. Enumeration and Purification of fungal endophytes from *Ximenia americana* 2. Production, extraction, phytochemical analysis, purification and evaluation of endophyte derived bioactive compounds 3. *In-vitro* bioassay.

MATERIAL 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 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 were 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[19]. Surface sterilized explants were dried on a sterile tissue paper and were cut with a sterile blade into 0.5 to 0.3cm 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℃ ± 2℃ for 15 days. When a mycelium appeared round the edge of the pieces on the PDA plates, the hyphal tips were transferred onto a freshly prepared PDA plates to obtain pure culture. Isolated endophytic fungi was subjected for the macroscopic and microscopic characterization such as the colour and nature of the growth of colony, 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.

$$IF = \frac{\text{Number of explant pieces showing growth}}{\text{Total number of explant pieces}} \times 100$$

Mean

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

$$X = \Sigma X / N$$

Where,

X = Mean,

ΣX = the sum of total numbers in a list

N = the number of items in that list.

Production, extraction, and phytochemical analysis

The production of bioactive compounds was carried out by solid state fermentation in which corn bran was used as a substrate. Three pieces of the grown pure culture of *Thielaviopsis basicola* were cut from the culture plate and inoculated in a 1000 ml Erlenmeyer flask containing 200 g of corn bran and 50 ml of distilled water and incubated in the dark at 25℃ ± 2℃ for 15 days at static condition. At the end of the incubation period, the fermented media were processed for the extraction of bioactive compounds. *Thielaviopsis basicola* feremented media was submerged for the extraction of bioactive compounds in Soxhlet apparatus by using distilled water as a solvent. In brief, 100 ml of distilled water was added in fermented media and kept on the rotary shaker for 24 hrs. After 24 hrs the mycelium and culture media were separated from each other by vacuum filtration. In the first hand, the filtrate was extracted three times with equal volume of distilled water as solvents for the complete extraction of metabolites from fungal biomass for 18-20 hours at 40℃ in Soxhlet apparatus. Then the concentration of extract was performed on Rota evaporator and dried under oven at 40℃, weighed and stored at 15℃. In another hand, obtained mycelium was air dried, weighed and recorded as mg/100mL. Qualitative and quantitative phytochemical analysis of 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. Quantification was done on the basis of the standard curve of gallic acid and results were expressed as gallic acid equivalent.

Column chromatography, thin layer chromatography, and Fourier transform infrared spectroscopy

Endophyte derived crude extract was run on 60-120 mesh silica gel (Spectrochem India) in column chromatography. A clean, dried glass column was filled with the silica gel slurry and petroleum ether filled up to of 20cm. 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 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 TbCC 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 TbCC1 and TbCC2 using different solvent systems.

For alkaloids Methanol: conc.NH_4OH (17:3)

For flavonoids Chloroform: methanol (18:2)

For terpenoids Benzene: Ethyl acetate (1: 1)

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

TLC plates were spotted with TbCC1 and TbCC2 obtained samples with standard solutions of alkaloids, flavonoids, terpenoids and saponins, and then developed in their respective eluent solvent systems. Chromatogram was developed in the closed TLC chamber in selected solvent system for 5 minutes. After 5 minutes, plates air dried and observed under sun light and UV light (254 and 366 nm) for the observation of compound bands. Retention factor (Rf) value was calculated by using the following formula,

$$Rf = A/B$$

Where,

A = distance between sample spot and central point of 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 TbCC1 and TbCC2. 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 infra red 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 functional groups.

***In-vitro* Bioassays**

Antagonistic activity

The two fractions TbCC1 and TbCC2 of *Thielaviopsis basicola* were used for *in-vitro* antagonistic activity against bacteria includes *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 assay. In the well diffusion assay, the bacterium and fungal inoculum was 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 spreaded on nutrient agar medium for verification of other microbial contamination. Fluconazole and Telithromycin (10mg/mL) were used as positive control and solvent DMSO was used as negative control. The verified microbial culture suspensions were spreaded on Muller-Hinton agar medium plates and purified extract samples were added in the wells with standard antibiotics. Plates were incubated at 37℃ for 24 hrs or 72 hrs and zone of inhibition was recorded with the help of zone reader. All experiments were performed in triplicates. For the determination of MIC of TbCC1 and TbCC2 fractions broth micro dilution technique was used in 96- well microtitre plate. The microbial suspensions was adjusted at the concentration of 1.0×10^{-4} CFU/ml [OD= 0.34]. The different concentrations of TbCC1 and TbCC2 [25µg/mL, 50µg/mL, 75µg/mL, 100µg/mL, and 125µg/mL] was prepared and added in 96- well microtitre plate containing microbial suspensions. Telithromycin (10 µg/mL) were used as positive control and solvent DMSO was used as negative control. The plates were incubated at 37℃ for 48 hrs and absorbance was recorded at 630 nm in UV-visible spectrophotometer. IC50 was expressed as the concentration of TbCC1 and TbCC2 [mg/ml] essential to produce 50% reduction of microbial culture growth.

Antioxidant activity

Antioxidant was performed by ferric ion reducing power assay and DPPH radical scavenging Assay. The TbCC1 and TbCC2 fractions of *Thielaviopsis basicola* were pipette in different concentrations ranging from 100µl to 500µl 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 positive reference standard. The absorbance of the color developed was measured at 700 nm using UV-Vis spectrophotometer. The DPPH (2, 2 Diphenyl- 2- Picryl Hydrazyl) radical scavenging assay was carried out with TbCC1 and TbCC2 fractions of *Thielaviopsis basicola* according to the method proposed by[24]. About 100 µl of a DPPH solution in prepared in ethanol (60µmol/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:

DPPH scavenging activity (%) = A_c-A_t/A_c x 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.

Antiurolithiasis activity

Antiurolithisis activity was performed for the inhibition of oxalate crystals because of its satisfactory results simplicity and reproducibility in order to study inhibitory capacity of fractions. The solutions of $CaCl_2.2H_2O$ (9mM) and $Na_2C_2O_4$ (3mM) were prepared using a buffer containing 0.15 M NaCl and 0.05 M Tris HCl at pH 6.5. The inhibitory solution was prepared by adding 0.25 ml of fractions in 100 mL of 0.15 M NaCl solution. 1 mL of $CaCl_2$ solution was assorted with 1 mL of inhibitory solution of fractions with different concentration (25%, 50%, 75%, 100%). Blank reading were reported and sodium oxalate (1 mL) was added. The absorbance was measured at 620nm with the help of UV spectrophotometer at different time intervals.

Anticancer activity

The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown-up in 25 cm^2 culture flasks and every experiment were performed in 96 microtitre plates. For Cytotoxic activity, all weighed test drugs were disjointedly dissolved in DMSO and volume was completed up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were organized from this for carrying out cytotoxic studies. Anticancer activity of TbCC1 and TbCC2 of *Thielaviopsis basicola* was performed by using Sulphorodamine B (SRB) assay on MCF7 human breast cancer cell line at Rakesh Clinical Laboratory, Jath Maharashtra, India.

RESULTS

Isolation of fungal endophytes

Ximenia americana was collected from the forests area of Karwar, Western Ghats of Karnataka, India. Total 32 fungal endophytes were isolated and enumerated from 100 pieces of 200 explants such as leaves, and roots of *X. 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% and further were confirmed by 18S rRNA sequencing (Mane *et al*., Unpublished data). 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%), and *Alternaria alternata* (15.30%). The colonization frequency of leaves explant piece was varied between 0 to 46.6% and roots explant piece were varied from 0% to 20%. The leaves piece showed highest colonization frequency by *Thielaviopsis basicola* (46.6%) and lowest colonization frequency by *Phomopsis* species (0%) while the root piece showed highest colonization frequency by *Phomopsis* species (20%) and lowest colonization frequency by *Nigrospora* species (0%). Results are shown in Plate 1, Figure 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.

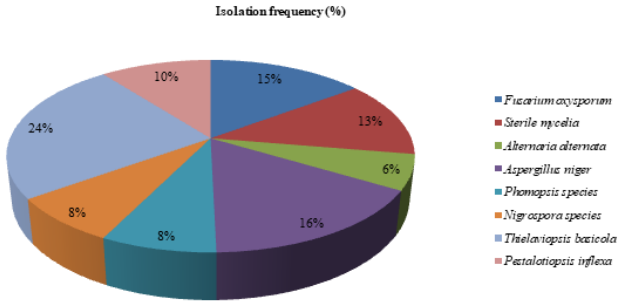


Figure 1 Isolation frequencies of fungal endophytes from *Ximenia americana*

Table 1 Isolation of fungal endophytes from *Ximenia americana*

Endophytic fungi	Explants	Total Number of explant piece	Isolates	IF (%)	Total	Mean
Fusarium oxysporum	Leaves	10	3	30.0	38.33	32.35
	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	
	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 basicola	Leaves	15	7	46.6	63.20	
	Roots	12	2	16.6		
Pestalotiopsis inflexa	Leaves	11	3	27.2	27.20	
	Roots	12	-	0		
Total	-	200	32	16		

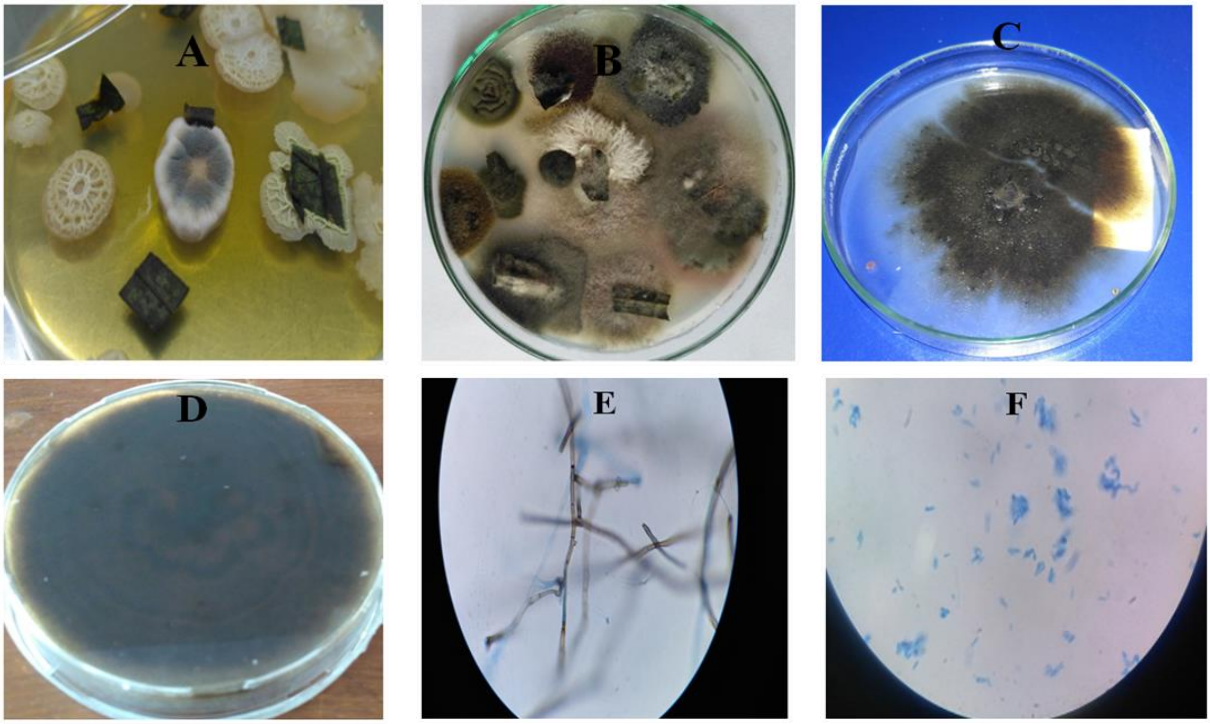


Plate 1 Isolation and characterization of fungal endophytes from leaves and roots of *Ximenia americana* (A) & (B) Isolation of fungal endophytes on PDA medium plates in dark at 25℃ ± 2℃ for 15 days (C) & (D) Characterization and purification of *Thielaviopsis basicola* on PDA medium plates in the dark at 25℃ ± 2℃ for 15 days, plates showing front and back side (E) & (F) Microscopic characterization of mycelium and spores.

Alternaria alternata

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.

***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 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℃ ± 2℃. 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.

Production, extraction and phytochemical analysis of bioactive compounds

Solid state fermentation was employed for the production of bioactive compounds under static conditions by using 200g of corn bran and 100 ml of distilled water in the presence of *Thielaviopsis basicola* culture. After 15 days of 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 fungus was recorded as 2.86 g/100g of corn bran and the dried biomass recorded as 0.25 g/100g of corn bran. In another hand, obtained 80 ml of filtrate were concentrated into 5.30 g/100ml of the aqueous solvent by using Rota evaporator at 40℃ after 6-8 hrs rotation at 90rpm and used for further processes. The results are shown in figure 2. The qualitative analysis of the aqueous crude extract of fungus revealed 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 extract showed high phenolic content (23.0090±0.04129 mg). Total flavonoids content was performed using $AlCl_3$ method using quercetin as a standard. The 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.

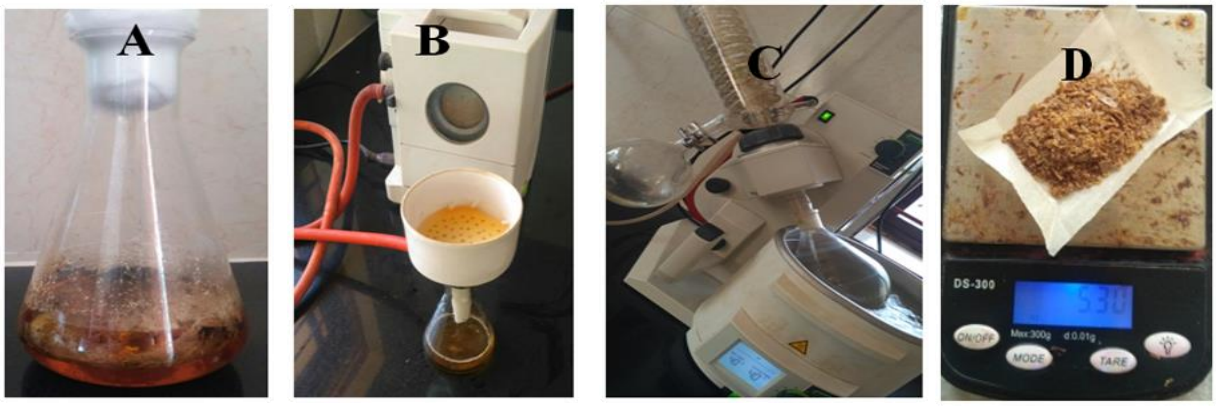


Figure 2 Fermentation and Extraction of bioactive compounds of aqueous crude extract obtained by the solid state fermentation from endophytic *Thielaviopsis basicola* (A) Fermented media (B) vacuum filtration to separate out fungal mycelium and extract (C) Rotary Evaporator for the concentration of aqueous crude extract (D) weighing.

Column chromatography, thin layer chromatography, and Fourier transform infrared spectroscopy

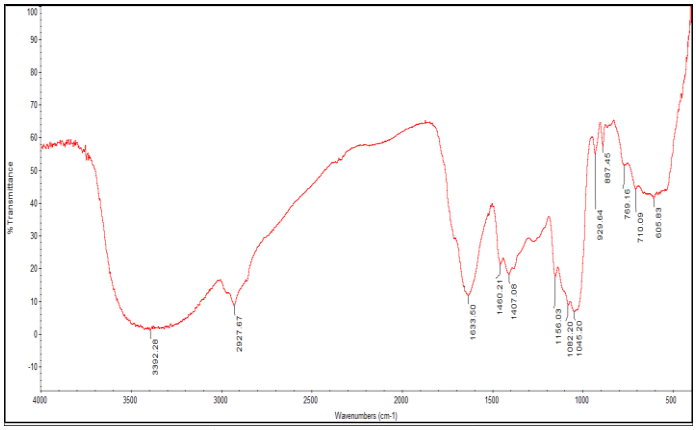
The column and thin layer chromatography were used for the purification of aqueous crude extract of the fungus. Total two fractions were purified from the column chromatography namely TbCC1 and TbCC2. Further, both fractions were implanted in thin layer chromatography for the detection of different bioactive compounds. TLC of TbCC1 and TbCC2 samples revealed the presence of four compounds with different R_f values such as 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 analysis of TbCC1 and TbCC2 revealed different functional groups. The graph 1 and table 3 represents different functional groups of TbCC1. The very strong absorption bands observed at 3392.28cm^{-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. The graph 2 and table 4 represents different functional groups of TbCC2. The very strong absorption bands were at 3745.33cm^{-1} which represents N-H strong stretching vibrations. It represents Amide groups. The very less absorption bands was at 458.24cm^{-1} which represents C-I strong stretching vibrations. It indicates Alkyl halide functional group.

Table 2 Phytochemical analysis of the fungus aqueous crude extract (+ indicates presence and – indicates absence).

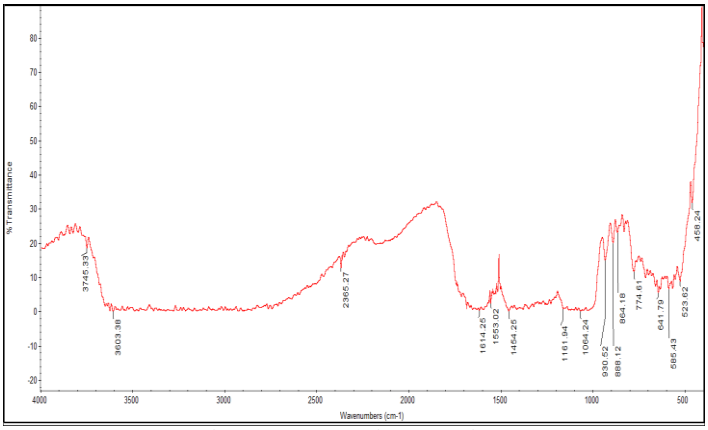
Sr. No.	Tests	Observation
1	Alkaloids	+
2	Terpenoids	+
3	Steroids	-
4	Tannins	+
5	Saponins	+
6	Flavonoids	+
7	Phenols	+
8	Coumarins	-
9	Quinones	-
10	Glycosides	+

Table 3 FTIR frequencies of different functional groups with their intensities of TbCC1 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 1 FTIR graph of TbCC1 obtained from CC of the aqueous crude extract of *Thielaviopsis basicola*



Graph 2 FTIR graph of TbCC2 obtained from CC of the aqueous crude extract of *Thielaviopsis basicola*.

Table 4 FTIR frequencies of different functional groups with their intensities of TbCC2 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

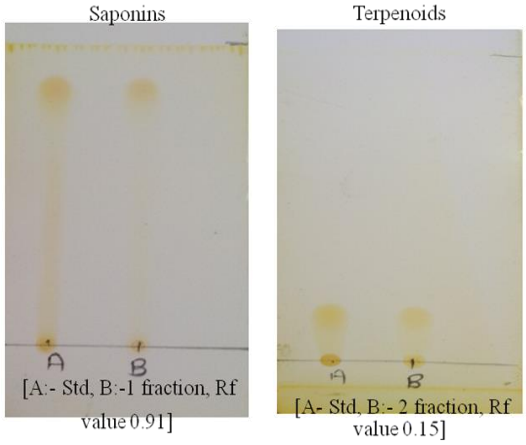


Plate 2 Thin layer chromatography spots under normal light for the detection of bioactive compounds compared with standards.

Bioassay

Antagonistic activity

The antimicrobial activity of TbCC1 and TbCC2 were performed 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 TbCC1 and TbCC2 of *Thielaviopsis basicola* were summarized in Plate 3 and Table 7. The results proved that the minimum inhibitory concentration value of TbCC1 and TbCC2 against selected microorganisms was varied and this inconsistency depends upon the microbial strains. The MIC values of TbCC1 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 TbCC2 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*).

Antioxidant activity

Ferric ion reducing power assay was performed with TbCC1 and TbCC2 by using ascorbic acid as standard. In this assay, the highest antioxidant activity at the rate of 1.2407±0.00702 showed by TbCC1 as compared to TbCC2. DPPH (2, 2 Diphenyl, 2- Picryl Hydrazyl) assay was performed with TbCC1 and TbCC2 and ascorbic acid standard. Results of this assay showed potent antioxidant activity with TbCC1 being the highly potent (86.24±0.35) as compared with TbCC2 (82.5333±0.23714). The IC50 of TbCC1 found to be 5.26µg/mL and TbCC2 found to be 7.24 µg/mL.

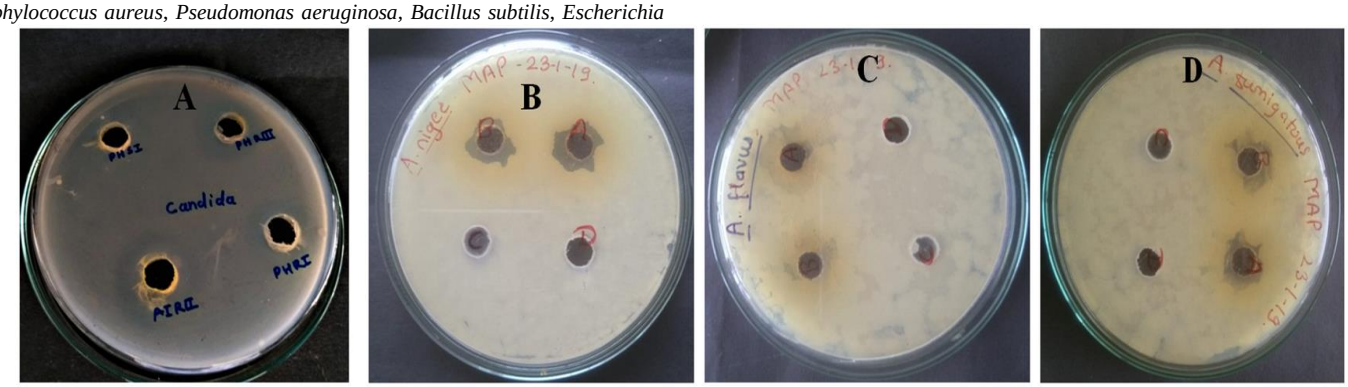


Plate 3 Antifungal activities of TbCC1 and TbCC2 of *Thielaviopsis basicola*. The plates of *Candida albicans* (A), *Aspergillus niger* (B), Aspergillus *flavus* (C), and *Aspergillus fumigatus* (D). The wells A contain TbCC1 suspension, B contains TbCC2 suspension, C contains DMSO and D contains Telithromycin. 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℃ for 72 hrs and after 72 hrs zone of inhibition was recorded with the help of zone reader.

Table 5 *In vitro* antagonistic activity of TbCC1 and TbCC2 of *Thielaviopsis basicola*. [NA] = Not Applicable, [-] = absence of zone of inhibition

Well No.	CC Fractions and Standard antibiotics	Zone of inhibition (mm)								
		Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Escherichia coli*	*Salmonella typhi*	*Candida albicans*	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus 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 [Bacteria]	28	24	22	25	23	NA	NA	NA	NA
C	Fluconazole [Fungi]	NA	NA	NA	NA	NA	18	21	22	24
D	DMSO	-	-	-	-	-	-	-	-	-

Antiurolithisis activity

Antiurolithiasis activity of the fungus extract was effective against calcium oxalate crystals. The most effective concentration was at 100% with 67.5±1.49 (Figure 3).

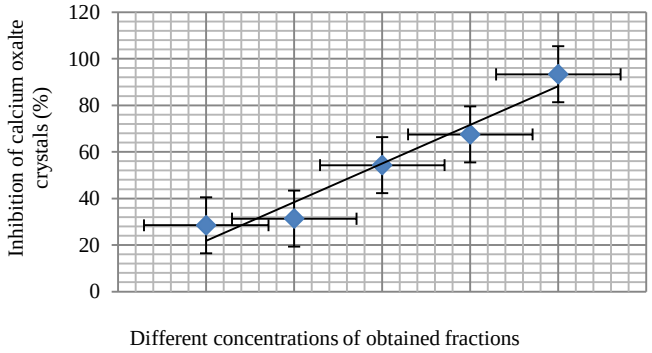


Figure 3 antiurolithiasis activity of the fungus

Anticancer activity

The TbCC1 and TbCC2 fractions were tested for their anticancer activity against MCF7 human breast cancer cell line by SRB assay. After completion of the protocol, the absorbance was recorded on an Elisa plate reader at a wavelength of 540 nm. In detail, untreated MCF7 breast cancer cell lines taken as the control group, MCF7 breast cancer cell lines treated with standard drug *Cisplatin* considered as positive control group whereas TbCC1 and TbCC2 treated with MCF7 breast cancer cell lines taken as a treated group. The different concentration of the standard drug and TbCC1, TbCC2 (200µg, 300µg, 400µg, and 500µg) were selected to learn morphological changes and cell growth inhibition of MCF7 breast cancer cell lines. Morphological studies revealed that significant increase in detached cells in the culture medium as compared with the control group treated group and positive control group. The normal cells showed regular and normal shape while treated MCF7 breast cancer cell lines were turgid and shrunken in shape. Morphological changes in nucleus representing apoptosis and also chromatin concentration, elongation of cells and reduction in cell count and density were observed which the features of apoptosis. Further, microscopic assessment exposed that morphological changes and decline of cells leading to cell apoptosis induced by the TbCC1. At the 100 µg concentration of TbCC1 cell viability was 98.39±0.0250 and at the 500 µg concentration cell viability was 40.81±0.0470, therefore, this clearly indicates that cells were alive at a minimum concentration only while they decreased their viability as the concentration of TbCC1 increased.

Table 6 Comparison of Effect of TbCC1 and Standard Drug *Cisplatin* on MCF7 breast cancer cell lines viability.

Sr. No	Sample	Concentration (µg)	Cell Viability (%)	IC_{50} (µg)
1	TbCC1	100	98.39±0.0250	459.60
2		200	84.75±0.0235	
3		300	77.98±0.0020	
4		400	59.80±0.0305	
5		500	40.81±0.0470	
6	*Cisplatin* Std Drug	30	43.39±0.0065	20.0

DISCUSSION

Karwar was selected as a study area because of great diversity of medicinal plants and unusual habitats with distinctive ecological environmental niche, novel strategies for survival, ethnobotanical antiquity. It 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^{O}N and 74.135^{O}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 (Suryanarayanan *et al*., 2000; Mane *et al*., 2018). The plant selection from Karwar area was tough job therefore we used non-apparent hypothesis which deals with only herbs and harvests low molecular weight organic compounds with low toxicity and high bioactivity (Zhou *et al*., 2012; Zhang *et al*., 2013). 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 (Ananda *et al*., 2002; Dharmaraj *et al*., 2010; Aletaha *et al*., 2018). The plant tissues, especially leaves and roots are excellent reservoirs for endophytic fungi, isolated from up till uncharted areas and from extreme environment is the choice for development of potential novel metabolite. Due to their wide ethnobotany and pharmacology *Ximenia americana* was selected as an endophytic inhabiting candidate. Total of 32 fungal endophytes were isolated and most of them belonging to the classes Deuteromycetes, Ascomycetes, Zygomycetes and Basidiomycetes. Most dominant endophytic fungi was *Thielaviopsis basicola* at the rate of 46.6% and further were confirmed by 18S rRNA sequencing (Mane *et al*., Unpublished data). This result supports the findings of Khan *et al*., 2012; Mane *et al*., 2018 the fungal endophytes from *Azadirachta indica*, *Terminalia arjuna*, and *Catharanthus roseus*, belonging to *Thielaviopsis* Species and *Aspergillus niger*. Rodriguez *et al*., 2008 studied endophytes from different medicinal plants and they were foliar endophytes. 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 ones obtained in the current study (Wilson *et al*., 1994). 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 (Zeng *et al*., 1987). Even though a number of differences in endophytic fungi isolates were experiential between organs. In contrast with previous study samples, 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*, therefore a few fungal endophytes may have been underrepresented. Previous reports of fungal endophytes from India may account for the high frequency from 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 (Zeng *et al*., 1987). 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. 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 (Rice *et al*., 1997). The endophytic fungi of grass grown in synthetic medium produced precursors of auxin *in vitro* while others showed production of Cryptocin by *Cryptosporiopsis quercina* with their potent activity against *Pyricularia oryzae cavara* and a number other plant pathogenic fungi. Merely *Streptomyces* was reported as a novel bioactive compounds producer from marine water. The bioactive compounds production may vary from organism to organism because of the substrate provided, metabolic processes, formation of products and byproducts, environmental conditions. Specific bioactive compound requires specific substrate to make chemical body with respect to provided semi-natural conditions (Lodge *et al*., 1996). Solid state fermentation revealed total of the wet and dried biomass of fungus at the rate of 2.86 g/100g and 0.25 g/100g of the corn bran respectively with 5.30 g/100ml of concentrated aqueous crude extract.The aqueous crude extract showed phytochemical analysis variation with 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). Two fractions namely TbCC1 and TbCC2 of crude extract obtained from the fungus by column chromatography revealed which further revealed the presence of alkaloids, flavonoids, terpenoids, and saponins in the solvents MA, CM, BE, CGMW respectively in thin layer chromatography. The Rf values matched with standards and confirmed the purification and efficiency of bioactive compounds. Further FTIR showed different functional groups those are mainly found in natural drugs. The results of TbCC1 revealed the different stretching and bending of the alcohol and phenols, alkanes, alkenes, aromatic groups, alkyl halides, amines, and ether groups while the TbCC2 revealed the alcohol and phenols, alkanes, alkenes, aromatic groups, alkyl halides, amines, ether, and nitrile. Phenolic and flavonoids compounds mainly observed as an antibacterial agents while terpenoids and alkaloid; cardiac glycosides have been reported to possess antifungal and antiurolithiasis activity (Murthy *et al*., 2010; Torres *et al*., 2015) Till date no reports were available on bioactive compounds from endophytic *Thielaviopsis basicola* in urolithiasis hence this study revealed new approach for urolithiasis. Partial purified fractions showed effective antagonistic, antioxidant, antiurolthisis and anticancer activities under controlled conditions. An endophytic *Thielaviopsis basicola* has ability to produce various bioactive compounds which will be used for various human diseases so it would be reported as an organism of pharmaceutical importance. However, further studies will need to undertaken to reveal the chemical structure of bioactive compounds and their toxic profile.

CONCLUSION

The present study aimed at evaluating different therapeutic applications of purified aqeous extract from an endophytic *Thielaviopsis basicola* in order to withdraw biologically active compounds. In this view, we purified different bioactive compounds as far as antagonistic, antioxidant, antiurolthisis and anticancer activities are concerned by using ecofriendly aqeous solvent by solid state fermentation. The obtained data may provide innovative information about the present topic which may helpful in the drug designing and development in future.

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