



## **Protection of *Bombax Ceiba* by Revealing their Fungal Endophytic Diversity and Therapeutic Applications**

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**Abstract:** The objective of the present research investigation was to protect *Bombax ceiba* by revealing their endophytic fungi diversity for disease management. In the present research investigation, a total of 64 fungal endophytes were purified and, *Aspergillus tamari* was identified as a core fungal endophyte. The fungus showed the highest average linear growth rate of 6.2 mm/day and isolation frequency at 80% on tomato dextrose agar with white or dark brown or black or purple-brown to yellowish-green color variations with mycelial growth in between 65 mm to 90 mm on the media and 8 to 31 numbers of spores/ microscopic field. Further, the fungus revealed 2.86 g/100g of total wet biomass and 0.25 g/100g of the dried biomass of corn bran with 5.30 g/100ml of the aqueous crude extract in solid-state fermentation. Mycochemical screening of the crude extract showed alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols with  $29.0060 \pm 0.03223$  mg of total phenolic and  $12.2549 \pm 0.02345$  mg of flavonoids content. Thin layer chromatography was used to get purified potent spots of alkaloids [0.29], flavonoids [0.41] terpenoids [0.15] and saponins [0.91]. The fungus produced chitinase, amylase, protease, and cellulases enzyme. Further the aqueous crude extract worked as an agent of antioxidant in the FRAP at the rate of  $1.2407 \pm 0.00702$  while PM at  $0.7983 \pm 0.00351$ , antimicrobials against *E. coli* (100mg/ml at the rate of  $16.3 \pm 0.57$ ) and *Aspergillus niger* (100mg/ml at the rate of  $20.5 \pm 0.24$ ), and in anthelmintic activity, extract induced paralysis in earthworms within 3 minutes and death in 8 minutes. It is concluded that the *Bombax ceiba* has the potential to purify the potent fungal endophytes and their bioactive compounds that may be helpful to evaluate in vivo models against different diseases.

**Keywords:** *Bombax ceiba*, conservation, fungal endophytes, bioactive compounds, bioassays

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## I. INTRODUCTION

*Bombax ceiba* Linn is the bulky, deciduous, elegant tree which is found in Africa, Australia and temperate and tropical Asia. It belongs to the family Bombacaceae. It is applied for inflammations, diabetes, diarrhea, helminthic, leprosy, muscular injury, wounds, asthma, birth control, and sexual diseases<sup>1</sup>. Besides these, this same plant is used in agroforestry, providing food, fodder, fuel, and fiber<sup>2</sup>. In the past, *B. ceiba* was said to be the Yamadruma, the tree of Yama<sup>3</sup>. Also, tribes from Udaipur prohibit the use of *B. ceiba* explants because of unknown myths such as poison in the leaves, flowers, and stem, therefore, it led to natural protection of the same plant but due to this features tribes considered *B. ceiba* as a God tree and they use to burn them during Holi festival as a traditional Dahan of Holika<sup>3</sup>. Day by day, due to ethnobotany and commercial uses of *B. ceiba* led to an endangered zone, therefore, it was needed to plan an effective protection strategy. However, in the path of evolution, the fungi developed various types of associations with the plants. One of these associations is 'endophytes'<sup>4</sup>. This association was supported by the fossil records showing that plants are allied with endophytic and mycorrhizal fungi for roughly 400 million years<sup>5</sup>. During the isolation of fungal endophytes in the lab, they show different growth requirements; like nutrients, pH, osmotic conditions and temperature. These requirements may change from one fungus to another fungus<sup>6</sup>. Mycologists revealed natural media as the best choice of isolating media under any environmental condition and these are composed of natural substrates, such as herbaceous or woody stems, seeds, leaves, corn-meal, wheat germ, fruit pulps, and oatmeal<sup>7</sup>. The endophytic fungi extracts consists of different mycochemicals. These mycochemicals are biologically significant and play a vital role in cancer, tuberculosis, inflammation, kidney stone, diabetes, and many other blood-related diseases. Urolithiasis is a third prevalent urological disorder of humans formed usually within the kidney in the form of stones from ancient times<sup>8</sup>. There are quite a few types of kidney stones but the most common are calcium oxalate which represents 80% of analyzed stones. At present, kidney stone affects extra in industrialized countries owing to the change in lifestyle. Formation of the stone is a multifaceted process and involves several physicochemical actions which start with crystal nucleation, supersaturation, aggregation, and ending with retention inside the urinary tract<sup>9</sup>. Another focused area is microbial infections. Infections are caused by pathogens and these pathogens are bacteria, fungi, viruses, actinomycetes, and protists<sup>10</sup>. The greatest concern about these drugs is toxicity So it is the need of the day to look into alternative medicines for the treatment of the inflammatory responses<sup>11</sup>. Presently chemically synthesized drugs are in the market for curing diseases but these drugs may affect on human normal flora which may lead to the loss of natural immunity. To avoid such loss one can go for use of natural drugs including endophytic fungal oriented drugs. The objectives of the present investigations were to purify fungal endophytes from *B. ceiba* to produce, extract, analyze mycochemicals, and enzymes Bioassays.

## 2. MATERIALS AND METHODS

### 2.1 Study area, survey & sample collection

The ethno botany and commercial uses of *B. ceiba* were

obtained from the available literature<sup>1</sup>. To confirm and to get more information about *B. ceiba* myths and traditions, we carried out interview, a survey from the local tribal people, women, elder individuals, and worshipers during March to June 2018 in the western ghats of Karnataka. Further, the information was also collected from schools, colleges, and Ayurvedic teachers. The fresh and healthy samples of *B. ceiba* were collected from different areas of Western Ghats of Karnataka, India. The plant was identified and authenticated by Dr. Kotresha K., Department of Botany, Karnatak Science College, Dharwad, Karnataka, India and a voucher specimen (N0-01/2018) was deposited. All samples were immediately brought to the laboratory and processed for isolation of endophytic fungi.

### 2.2 Isolation, identification, & characterization of fungal endophytes

The explants were surface sterilized as per standard protocols<sup>12</sup>, then inoculated and incubated on Czapek Dox agar media plates<sup>13</sup> at 25°C ± 2°C for 15 days under dark condition. After incubation, purification and growth study was carried out according to standard methods<sup>13</sup>. The endophytic fungi were characterized by different media. Identification of fungal endophytes was performed by morphological methods<sup>14</sup> and the selected fungus was identified by ITS analysis<sup>16</sup>.

### 2.3 Fermentation, extraction, and purification of bioactive compounds

The production of bioactive compounds was carried out by solid-state fermentation in which corn bran was used as a substrate<sup>9</sup>. Three pieces of the grown pure culture of endophytic fungus were cut from the culture plate and inoculated in a 1000 ml Erlenmeyer flask containing 200g of corn bran and 30 ml of distilled water and incubated in the dark at 25°C ± 2°C for 15 days at static condition. At the end of the incubation period, the fermented media were processed for the extraction of bioactive compounds with the help of the aqueous solvent in the Soxhlet apparatus. 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 mycelia 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 aqueous solvent for the complete extraction of metabolites from fungal biomass for 18-20 hours at 40°C in Soxhlet apparatus. Then the concentration of the extract was performed on Rota evaporator and dried under oven at 40°C, weighed and stored at 15°C. In another hand, obtained mycelium was air-dried, weighed and recorded as mg/100mL.

### 2.4 Mycochemical analysis and thin layer chromatography of an aqueous crude extract of the fungus

Qualitative mycochemical analysis of the aqueous crude extract was performed for alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, and phenols, coumarins, quinones, and glycosides by following standard protocols. Quantitative analysis of flavonoids and phenol content was performed as per standard protocols<sup>19</sup>. 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 endophytic derived aqueous

crude extract by using solvent systems. Alkaloids [Methanol: conc. $\text{NH}_4\text{OH}$  (17:3)], flavonoids [Chloroform: methanol (18:2)], terpenoids [Benzene: Ethyl acetate (1: 1)], saponins [Chloroform: glacial acetic acid: methanol: water (6:2:1:1)] were used. TLC plates were spotted with aqueous crude extract 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 minutes. 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.

## 2.5 Primary screening of enzymes by endophytic fungus

Primary screening of chitinase<sup>9</sup>, amylase, cellulases<sup>12</sup>, and protease<sup>9</sup> was performed by an endophytic fungus on selective media and enzyme productions were confirmed by the observation of zone of hydrolysis.

## 2.6 Bioassay

### 2.6.1 Antioxidant Activity

Antioxidant activity of the aqueous crude extract was performed with the help of ferric ion reducing power assay and Phosphomolybdenum assay<sup>10-14</sup>. Antiulcer activity of aqueous crude extract was performed for the inhibition of oxalate crystals because of its satisfactory results simplicity and reproducibility in order to study the inhibitory capacity of crude extract as per standard methods<sup>13-16</sup>.

### 2.7 Antimicrobial activity

#### 2.7.1 Microorganisms

The crude extract was screened against different pathogens by using well diffusion method. The bacteria such as *E. coli*, *S. typhi*, *S. aureus*, *Bacillus spp.* and *Pseudomonas aeruginosa* and fungi such as *A. niger*, *A. fumigatus*, and *A. flavus* were used<sup>13-25</sup>.

#### 2.7.2 Well Diffusion Method

Muller-Hinton agar media (for Bacteria) and potato dextrose agar media (for fungi) plates were prepared and microbial cultures were spread to the concentration of  $1.0 \times 10^4$  CFU/ml attuned with saline. The Fluconazole and Telithromycin (10mg/mL) were used as optimistic control and DMSO was used as the control. Extracts were added in the prepared wells with controls and all plates were incubated 37°C for 24 hrs or 72 hrs and the inhibition zone was recorded with the assistance of zone reader<sup>17-19</sup>.

### 2.8 Anthelmintic activity

Healthy adult earthworms were collected from the department of agriculture, Agricultural University of Dharwad, Karnataka, India. All the earthworms were 3-5 cm in length and 0.1-0.2 cm in width. The same earthworms were used due to its anatomical and physiological resemblance with intestinal roundworm parasites of human beings. The earthworms were kept into an aqueous solution of fungus crude extract at 250  $\mu\text{g}/\text{ml}$  concentration and 100  $\mu\text{g}/\text{ml}$  of piperazine citrate standard drug. The time of paralysis and time of death were noted and the activity was recorded<sup>25</sup>.

## 2.9 Data analysis

Isolation results were recorded by Average Linear growth rate, Isolation frequency and colonization frequency. Date obtained from results (triplicates) was calculated for mean and standard deviation. Further, calculations were represented as Mean  $\pm$  Standard Deviation.

## 3. RESULTS AND DISCUSSION

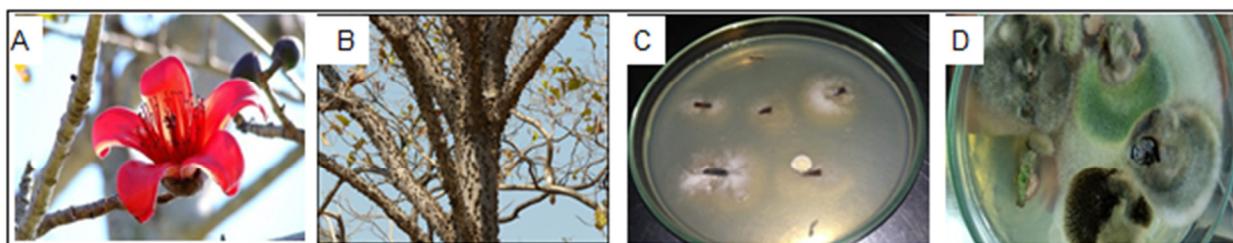
The Holi, the color festival comes in March and the *B. ceiba* is also blooming at the same time with red flowers and horny stem, therefore, the stem of the same plant is used to burn in Holi as a Holika-Dahan by Bhil, Garasia and Damor tribes in Western ghats of Karnataka, India. . *B. ceiba* stem is considered as virtuous Prahlad and planted almost a month before the festival day. Even the image of Holika and Prahlad is also prepared and attached over the prepared Holi<sup>1</sup>. In Bhils, before cutting a *B. ceiba* stem, a coconut is fixed on the branch, liqueur trickled and vermillion is applied and the tree is cut to have a head and two arms and usually the stem is removed from the ablaze pile. This conventional two armed Holi is still prepared and planted. But in some Bhil villages of Banswara district, they use bamboo with a red cloth tied on it, representing as Prahlad and there Bombax indicates the wicked aunt Holika, therefore it is allowable to be burnt and bamboo is detached presentation continued existence of Prahlad<sup>3</sup>. Due to the tradition, the mortal lay off always cascades on the *B. ceiba*. This unreasonable custom of wounding the plant for the purpose of Holika-Dahan is detrimental to the environment, troubling the eco-system and proving overwhelming for human health<sup>2</sup>. A survey in Western ghats of Karnataka and nearby forest areas has revealed illegally cutting of *B. ceiba* tree (Figure 1). According to local tribes and teachers, in the year 2017, around 2,300-3,000 trees or twigs or stem of *B. ceiba* were cut for Holika-Dahan. There was barely any anxiety concerning the forgo of such a big tree in the middle of Western ghats of Karnataka tribes. The seriousness of the circumstances can be further assessed as presently only 2,351 trees are surviving in Western ghats of Karnataka . A variety of ethnic conservation practices, in the shape of civilization, customs, myths, and folktales have made the continued existence of *B. ceiba* for so a lot of years. Now, there is requiring renewing these helpful societies for protection while discarding all other conventional practices that destroy the plant. The most significant part of the conservation strategy is to make people aware of its various helpful therapeutic applications. It is to be supposed a combined responsibility of nongovernmental and governmental organizations, forest officials, local environmentalists, village heads and teachers at the local school level. For conserving the ritual, only a little stick of *B. ceiba* can be used emblematically. In this regard, an

iron pole wrapped with dehydrated grassland and hay fabric in its place of the wood pole of *Bombax* can be used for on fire in Holi. Biotechnological applications can be used as a major tool to spread and preserve the species in a short time period. The plentiful species of fungal endophytes made an ecological place in the inner gap of plants. These ubiquitous fungi act together absolutely with their environment in a positive way and extremely superior for application in plant development and disease control<sup>21-25</sup>. The diversity of endophytic fungi was revealed from the different medicinal plants of rainfall area, desert area, and their effect was studied on a host plant with their biochemical occurrence<sup>23</sup>.

They revealed the diversity of leaves, flowers, stem, and roots for the isolation of fungal endophytes except they have isolated only total 24 fungal endophytes with 54% as an isolation frequency<sup>24</sup>. In the present study, we have isolated a total sixty-four fungal endophytes with isolation frequency of 74% from leaves and lowest from roots at the rate of 27%. All isolates were belonging to *Aspergillus tamari*, *Alternaria* Spp., *Phomopsis* Spp., *Nigrospora* Spp., and *Fusarium* Spp. with the colonization frequency of 75%, 30%, 22.5%, 10%, and 17.5% respectively. The results are shown in Table I and Figure 2.



**Fig 1. Cutting of the *B. ceiba* in Udaipur forests (A&B), Cut tree laying in the forests (C&D)**



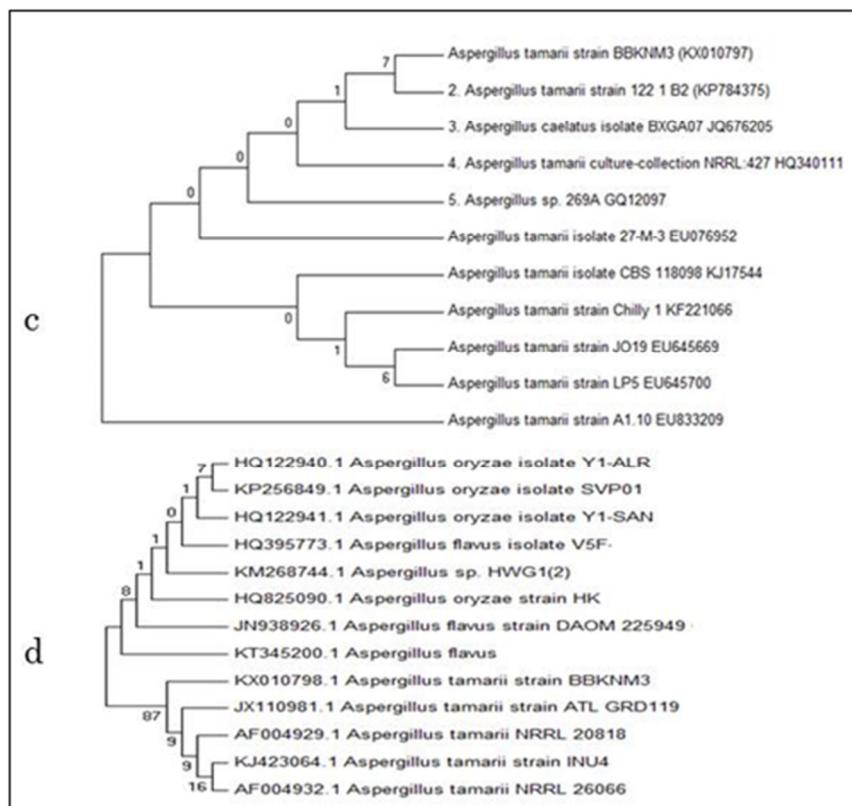
**Fig 2. Explants of *B. ceiba* were collected from Udaipur study area (A & B) and surface sterilized with the help of oxidants and chemicals at a definite concentration. Then all surface sterilized explants were implanted on Czapek Dox agar media and incubated at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 15 days, observed growth (C). After 26 days, contaminated fungal endophytes were observed with actinomycetes (D).**

Endophytic *Aspergillus tamari* showed core group in the isolation therefore, the same fungus was selected for further study. On the synthetic media endophytic *Aspergillus tamari* showed initially, dark green conidia, which next conquered colony look. They were commonly plain and flat at the boundaries but were raised in the center and wrinkled in an almost cerebriform pattern. They produced droplets of liquid, which were either uncolored or brown. The colonies were encircled by a white border, and the colony diameter ranged between 40-65 mm. The undersides of the colonies were slightly pale. While on natural media the same fungus

showed somewhat different colony characteristics. The colonies were initially white and had a spongy velvety surface. After 8 days of the incubation period, the colonies became raised and turned floccose at the center. The colonies produced yellowish-green and olive conidia, during sporulation. The conidia covered the complete exterior of the colonies apart from for the edges, where a white border was produced. The white border then moved out as the colonies became superior and shaped extra conidia. Sclerotia were produced and were originally white and turned a deep brown on the 13 days of the incubation period. No liquid

bubbles were produced. The reverse sides of the colonies were furrowed and slightly pale brown or yellowish. The colony diameter on the natural medium was ranged between 65-90 mm (Figure 3 and 4). On the natural media, the conidiophores were uncolored, thick-walled, and thickly roughened and were vesicles bearing. They ranged 600 and 1000  $\mu\text{m}$  in diameter. The vesicles were globose and were also variable in diameter, ranging between 1200 and 1600  $\mu\text{m}$ . They were blackish colored with either uniseriate or

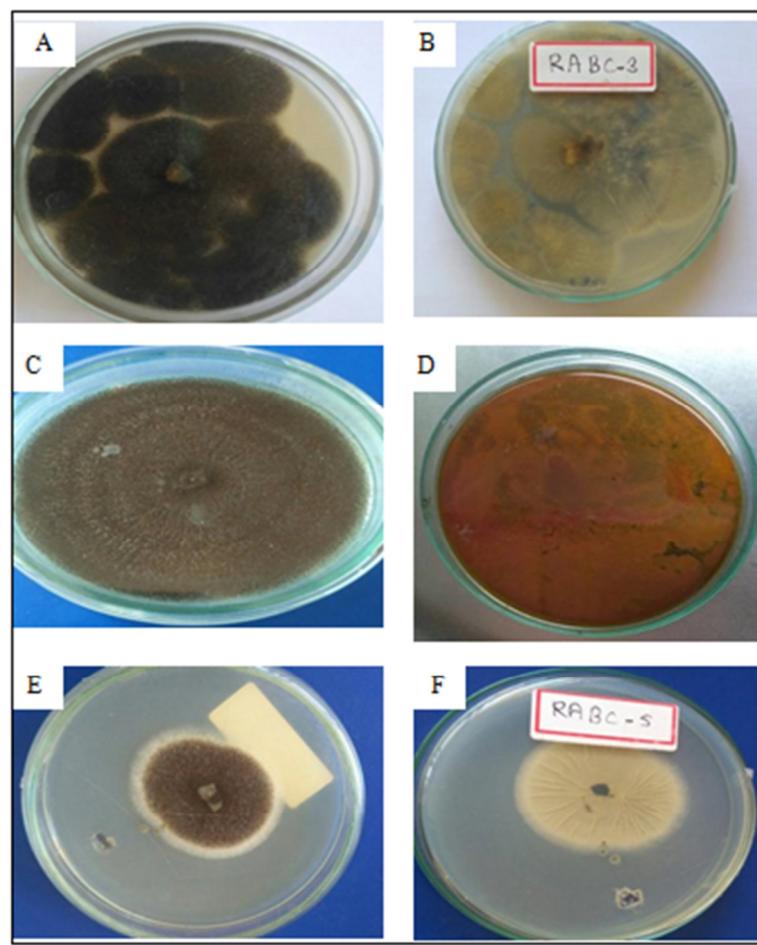
biseriate or both (Table 2). An endophytic *Aspergillus tamari* showed variations in sporulation on synthetic and natural media due to variations in nutrient compositions. They showed the highest sporulation on TDA in between 18-27 while lowest showed on SDA in between 4-9 per microscopic field. This result supports the findings that *Aspergillus tamari* showed the lowest sporulation on SDA in between the range 8-12 per microscopic field<sup>24</sup>.



**Fig 3. Identification of fungal endophytes C. The phylogenetic tree of LSU of *Aspergillus tamari* D. The phylogenetic tree of D1 and D2 regions of *Aspergillus tamari***

**Table 1 Fungal endophytes biodiversity within the explants of *B. ceiba***

Endophytic fungal species	Explants	Total analyzed explants	Isolates	Colonization frequency (%)
<i>Aspergillus tamari</i>	Leaves	40	11	27.5
	Flowers	40	6	15
	Stem	40	9	22.5
	Roots	40	4	10
<i>Alternaria</i> spp.	Leaves	40	2	5
	Flowers	40	3	7.5
	Stem	40	4	10
	Roots	40	3	7.5
<i>Phomopsis</i> spp.	Leaves	40	4	10
	Flowers	40	1	2.5
	Stem	40	3	7.5
	Roots	40	2	5
<i>Nigrospora</i> spp.	Leaves	40	1	2.5
	Flowers	40	3	7.5
	Stem	40	1	2.5
	Roots	40	-	-
<i>Fusarium</i> spp.	Leaves	40	3	7.5
	Flowers	40	1	2.5
	Stem	40	1	2.5
	Roots	40	2	5



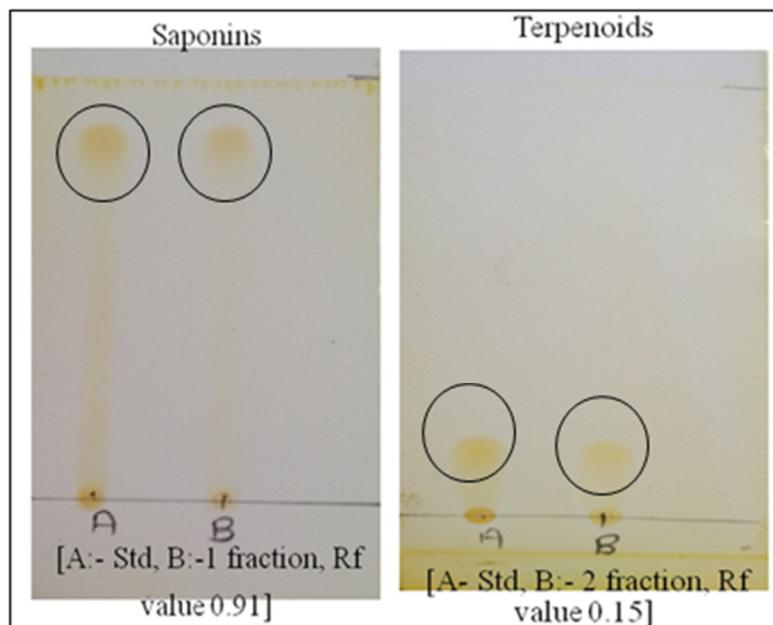
**Fig 4. Characterization of endophytic *Aspergillus tamari* on different media. Front (A) and Back (B) side of the potato dextrose agar media plate with moderate growth of endophytic *Aspergillus tamari*, Front (C) and Back (D) side of the tomato dextrose agar media plate with excellent growth of endophytic *Aspergillus tamari*, Front (E) and Back (F) side of the water agar with poor growth of endophytic *Aspergillus tamari***

The average linear growth rate of *Aspergillus tamari* on TDA was 6.2 mm/day while the lowest growth rate was 5.3 mm/day on WA; therefore, it indicates the good and rapid growth of the fungus on TDA as compared to other media. The isolation frequency of *Aspergillus tamari* was lowest at WA at the rate of 50% while the highest on TDA at the rate of 80%. Therefore natural media is effective and good for the growth of endophytic fungi as compared to synthetic media. Total wet biomass of endophytic *Aspergillus tamari* was recorded as 2.86 g/100g of corn bran and the dried biomass was 0.25 g/100g of corn bran. In another hand, obtained 80 ml of the fungal extract filtrate were concentrated into 5.30 g/100ml of the aqueous solvent by using Rota evaporator at 40°C after 6-8 hrs rotation at 90 rpm and used for further processes. Mycochemical screening of aqueous crude extract showed the presence of alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols and absence of coumarins, quinones, and glycosides. The total phenolic and flavonoids content of the aqueous crude extract of the fungus was estimated with Gallic acid as a reference standard, it showed  $29.0060 \pm 0.03223$  mg of phenolic and  $12.2549 \pm 0.02345$  mg of flavonoids content. The fungus crude extract was implanted on thin-layer chromatography for the detection of different bioactive compounds and it revealed the presence of four compounds having R<sub>f</sub> values of alkaloids [0.29] in Methanol: conc.NH<sub>4</sub>OH- 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 figure 5. These bioactive compounds are

considered as a natural source of antioxidant, antimicrobial and anti-inflammatory agents which have been exposed to decrease the peril and series of many diseases such as cancer and diabetes. Particularly phenol compounds act as a hydrogen donor to radical and a potent radical terminator also it inhibits lipid oxidation<sup>16</sup>. Saponins are used as dietary supplements and nutraceuticals and play a very important role to produce an inhibitory effect on inflammation. Tannins are measured to have antiviral, antibacterial, antiphastic, anticancer, antiulcer and antioxidant agents. Even though Phenols and Flavonoids are well thought-out to be a multitasking and broad group of natural components which possess a broad spectrum of biological activities including terminating free radicals, reducing the oxygen concentration, transforming primary goods of oxidation into non-oxidant molecules and acts as metal chelators<sup>19</sup>. The endophytic *Aspergillus tamari* showed the ability for the production of chitinase, amylase, protease, and cellulases by primary screening. This result supports the findings that *Penicillium* and *Aspergillus* spp showed the production of different enzymes<sup>19</sup>. The results are shown in Figure 6. In ferric ion reducing power assay, the fungus aqueous crude extract showed effective antioxidant activity at the rate of  $1.2407 \pm 0.00702$  while in the PM assay showed  $0.7983 \pm 0.00351$  antioxidant capacity, compared to the standard ascorbic acid. The aqueous crude extract of the fungus was utilized to evaluate their antimicrobial activity against food pathogens including *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus* species, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus*

*niger* using disc diffusion method. The results revealed that fungus crude extract was potentially efficient in suppressing food poisoning bacteria with variable potency. The fungus crude extract was most effective against *Escherichia coli* to retard their growth at the concentration of 100mg/ml with the rate of  $16.3 \pm 0.57$  while in the case of fungal pathogens;

the extract was effective against *Aspergillus niger* at the concentration of 100mg/ml at the rate of  $20.5 \pm 0.24$ . The Anthelmintic activity of the fungus aqueous crude extract is given in table 3. It is very clear that 100  $\mu\text{g}/\text{ml}$  of the extract was effective on earthworms with shortest time of paralysis ( $3.84 \pm 0.17$ ) and death ( $8.92 \pm 0.23$ ).



**Fig 5. Thin layer chromatography**

**Table 3 Anthelmintic activity of endophytic *Aspergillus tamari* aqueous crude extract**

Concentration of Extracts	Paralysis Time (mm)	Time
Piperazine citrate extract (100 $\mu\text{g}/\text{ml}$ )	$31.33 \pm 1.67$	$38.01 \pm 1.34$
Aqueous fungus crude extract	50 $\mu\text{g}/\text{ml}$	$38.33 \pm 0.67$
	100 $\mu\text{g}/\text{ml}$	$3.84 \pm 0.17$
	200 $\mu\text{g}/\text{ml}$	$12.33 \pm 1.04$
	300 $\mu\text{g}/\text{ml}$	$63.07 \pm 0.21$
	400 $\mu\text{g}/\text{ml}$	$11.33 \pm 0.04$

#### 4. CONCLUSION

The present study concluded that the isolation of pharmaceutically potent fungal endophytes from *B. ceiba* could be promising, safe, and cheap approach for the protection of the same plant. The aqueous *Aspergillus tamari* crude extract showed potency against food pathogens, earthworms, and different oxidant radicals, therefore present research investigation would be helpful for the drug discovery and development in the pharmaceutical industry.

#### 5. ACKNOWLEDGEMENTS

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#### 6. AUTHORS CONTRIBUTION STATEMENT

Mr. Rohit Shankar Mane perceived the idea, carried out the research study, evaluated the results and drafted the manuscript. Prof. A. B. Vedamurthy guided to Mr. Mane in conducting this research study and also reviewed the manuscript.

#### 7. CONFLICT OF INTEREST

Conflict of interest declared none.

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