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Original Research Article

ANTIBACTERIAL POTENTIAL OF MBKF16 ENDOPHYTIC FUNGI STRAIN ON CLINICAL PATHOGENS

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Abstract-

Endophytes reside in the tissue of live host plants and help them absorb nutrients, fix nitrogen, defend against diseases, and form symbiotic relationships without endangering the host plant. Secondary metabolites in diverse plants, animals, and microbes have been found to have therapeutic potential as a result of recent inquiry and investigation of these entities for their bioactive potential features. It has been studied how endophytic fungi function as bio factories for new bioactive compounds.

An endophytic fungus strain MBKF16 was isolated from edible shoots of *Bambusa tulda*. The submerged fermentation method was used to mass culture the isolated fungal culture in order to produce extracellular bioactive compounds. Chloroform, methanol, ethyl acetate, and water are the solvents used for the extraction method. The acquired extract was screened for phytochemical analysis. Agar well diffusion assay was performed for antagonistic efficacy against clinical pathogens *Salmonella typhi*, *Shigella sonnei*, *Escherichia coli*, and *Klebsiella pneumoniae*, which are acquired from KIMS Madikeri. MBKF16 Endophytic fungi are identified molecularly by the 18S rRNA partial gene sequencing method. ITS region obtained are compared with closely related species from the NCBI database. The sequences of *MBKF16* are submitted to NCBI with accession number ON287260. A phylogenetic tree is constructed using MEGHA 11 software. Results showed that *Rigidoporus vinctus* is a potential fungus for the production of numerous bioactive compounds. Ethyl acetate is a highly efficient solvent for extracting active antibacterial compounds, then water, methanol, and chloroform. Extracts confirm the presence of phenols, alkaloids and flavonoids. Ethyl acetate extract has significant antibacterial activity.

Keywords- Endophytic fungi , Antibacterial bioactive compound, Phytochemicals.

Introduction-

Endophytic fungi live in plant tissues for all or part of their life cycle without causing harm to the host plant or spreading illness. They do this by developing a mutually beneficial symbiotic relationship.[12] As a possible source of biological resources, endophytic fungal habitats have piqued the curiosity of environmentalists, microbiologists, and chemists. A host's health is preserved, abiotic and biotic stress tolerance, defence, eco-adaptation, and growth and expansion stimulus are just a few of the remarkable roles that endophytic fungus perform in the Host plant.[34] The use of endophytic fungi as biocontrol agents is acknowledged by researchers. Plant defence mechanisms against

endophytic fungal invasions are also beneficial to the plant's immune system. It is known that this endophytic interaction imposes additional defensive mechanisms on the management of the plant immune system. These mechanisms arise from the modulation of direct antimicrobial metabolites such as alkaloids to indirect phytohormones, jasmonic acid, or salicylic acid.[5] [6] Due to endophytic fungi's remarkable ability to produce chemical compounds, it is usually noticed that the biological activity of their compounds matches that of their host plant's chemicals.[7] There is evidence in the scientific literature that antibiotic resistance has grown significantly as a global issue in recent years.[8] In this regard, public healthcare systems worldwide face enormous challenges. Of course, a wide variety of microorganisms can infect both humans and animals.[9] Because of their capacity to generate unique bioactive substances with a range of biological characteristics, endophytic fungi have drawn a lot of interest and are used in pharmacological, medical, and agricultural settings. Endophytic fungus maintain the physiological and ecological characteristics of the host plant by living within the plant tissues without exhibiting any signs of illness. Pioneering lead compounds, derived from endophytic fungus, such penicillin and paclitaxel, have cleared the path for the investigation of new bioactive substances for industrial application. In spite of this, hardly much research has been done in this worthwhile and distinctive sector. These bioactive substances are classified into a number of structural classes, such as flavonoids, phenols, quinones, terpenoids, alkaloids, and steroids.[10] Antimicrobial resistance (AMR) has caused many of the medications now used to treat infectious infections to become less and less effective. Therefore, there is a continuing need to discover novel, potent chemicals that may be able to lessen some of this burden. Numerous researchers in this field have shown interest in endophytic fungus due to their remarkable capacity to generate novel bioactive chemicals, many of which have broad-spectrum antibacterial properties. Though very promising, this field of study is still very young. In a mutualistic symbiotic relationship, endophytes live in healthy plant tissues asymptomatically and create a wide range of bioactive substances that enhance the host plant's fitness. These substances exhibit a wide range of chemical variety.[2]

Materials and Method-

Collection and handling of samples – *Bambusa tulda* edible shoots are harvested in the Billigirirangana hills in Chamrajanagara, Karnataka. In the month of July The shoots are transported in a sterile, clean polythene bag to the laboratory within twenty-four hours. The upper culm sheath of the shoot was cut off. Then the edible part are rinsed with clean water to remove pathogens, surface soil particles, and adhering debris. After that, they are rinsed with distilled water for five times.

Endophytic fungal isolation – Shoot segments were immersed for three minutes in 70% ethanol, five minutes in a 2% sodium hypochlorite aqueous solution twice, and one minute in each instance. After removing the surface sterilising agents, the shoot samples should be rinsed six to eight times with sterile water in a laminar air flow hood and then dried in the sterilised paper in the same hood. This process should be repeated twice in sterile distilled water for five minutes[11]. After that, the segments are kept at room temperature in an SDA medium (Sabouraud Dextrose Agar media) for three to seven days, during which time the development of conidia and mycelia is monitored and a pure culture is produced.

Molecular identification of isolated fungi culture - The isolated fungi's genomic DNA was recovered using the CTAB method from their pure culture. ITS rDNA was amplified with forward primer ITS-1:5'-TCCGTAGGTGAACATGCGG-3' Reverse primer ITS-4: 5'-TCCTCCGCTTATTGATATGC-3' and the Sequences were compared with fungal ITS sequences in GenBank using BLAST searches[12].

Production of secondary metabolites by submerged fermentation -

The selected endophytic fungus isolates were cultured using submerged fermentation in order to produce secondary metabolites. Next, freshly sub cultured fungi were added to 500ml of autoclaved Sabouraud Dextrose Broth (SDB) in Erlenmeyer flasks. After that, the flasks were continuously shaken and allowed to incubate for 21 days at room temperature.

Solvent extraction was used to extract the fungal metabolites. The fermentation broth was filtered through a sterile muslin cloth on the twenty-first day following the collection of the fungal biomass that had fermented. The filtered broth was separated into four sections, each containing approximately 100 ml. Each flask was then filled with equal volumes of ethyl acetate, water, chloroform, and methanol solvent separately. The mixture was then moved to a separating funnel and swirled for 15 minutes. Using a rotating vacuum evaporator, the organic phase was collected and the solvent was extracted by evaporating it at 45°C under reduced pressure. For different biological characteristics, the dried crude extracts were weighed, labelled in different vials, and then kept at 4°C.

Confirmation of Phytochemicals -

Phytochemical analytical studies were conducted to determine the chemical composition of the active ingredients found in the endophytic fungal crude extracts. Alkaloids, flavonoids, saponins, tannins, phenols, and glycosides were among the components found. according to the method described by Banu and Cathrine [13 6]

Antibacterial assay-

Using a slightly modified version of Jayashankar's Agar well diffusion method, the dried crude secondary metabolites isolated from endophytic fungi were tested for their antibacterial efficacy against clinical pathogenic bacteria[14]. Escherichia coli, Shigella sonnei, Salmonella typhi, and Klebsiella pneumoniae, four clinical pathogens collected from KIMS Madikeri, Kodagu, were utilised in antibacterial assays. Dimethyl sulfoxide (DMSO) was used to dissolve endophytic extract in order to create stock solutions (1 mg/ml). In a test tube, 3 ml of nutrient broth was added to a loopful of test bacterial culture from the slant, and the tube was then incubated at 37°C for 24 hours. After preparing, autoclaving, and cooling to room temperature, nutrient agar media was created. Nutrient agar plates were coated with 100µl (~107 CFU/ml) of freshly created bacterial culture, which was equally distributed after being swabbed. Next, using a sterile metallic borer with a 6 mm diameter, wells were created in the media. Each of these samples of endophytic fungal extract (100µl) was applied to the corresponding well. A negative control of 100µl of pure DMSO and a positive control of 1 mg/ml of the reference antibiotic streptomycin were added to the other wells. Next, the plates were incubated throughout the entire night at 37°C. Outcomes were recorded.

Results and Discussion-

Endophytes are well-established bio-reservoirs of naturally occurring chemicals obtained from plants and represent an important symbiotic interaction in nature. Endophytes invade the internal tissues of plants without causing any outward changes or illness signs. The synthesis of novel metabolites with therapeutic significance has been facilitated by endophytes, which have garnered more interest in recent years [8]. The leaves of bamboo plants are rich in nutrients and have therapeutic properties. In traditional medicine, they are used to cure blood problems and inflammation. The hardened material found inside bamboo has been used to treat leprosy, asthma, and tuberculosis. The stalks are said to aid in digestion and stimulate appetite. The root has been used for ringworm. Flowers have been used to alleviate deafness and earaches. Some recent research have validated its anti-inflammatory and anti-tumor characteristics, as well as its effects on the uterus. [15]. Due to these characteristics, bamboo piques interest in exposing its endophytes, which have received less research. An attempt was undertaken to separate endophytic fungus from *Bambusa tulda* edible shoots in the Billigirirangana hills of Chamrajanagara, Karnataka.

The obtained shoot segments are surface sterilised to prevent contamination before being placed on SDA medium (Sabouraud Dextrose Agar medium) plates and cultured for three to seven days at room temperature. Plates are checked for the development of fungi from the sidewalls of shoot segments, and pure cultures are produced using the subculture process. Figure 1 shows a filamentous white circular growth pattern. A microscopic image of conidia and hyphae is shown below.

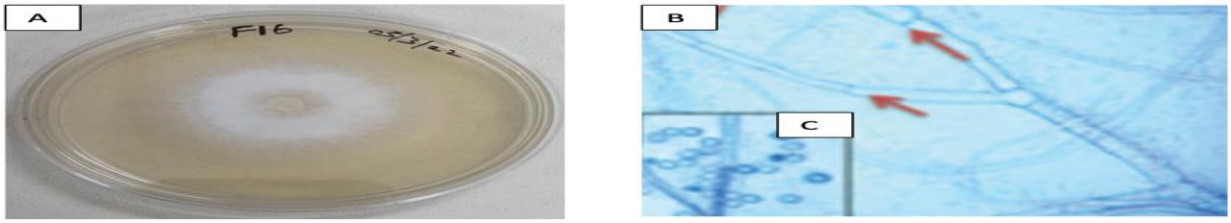

Figure 1. Photograph of A- Endophytic fungal growth on SDA media plate, B- Microscopic observation of Fungal hyphae, C- Enlarged microscopic view of Conidia.

The technique of 18S rRNA partial gene sequencing is used to identify isolated fungus. Bio Edit software is then used to modify and examine the obtained sequences. The obtained ITS region is compared to closely comparable species found in the NCBI database. These are the sequences that were obtained.

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTTATGGCGGAATTGTAGCTG
GCCCCAACCGGGCATGTGCACATTCTGTTCATTCCATTCTCATACACCTCTGTGCACTTT
ACATAGGTTTGGTATAGAAAAGGTCTTTATTGACTTTGGAAATACGAGTACTGACCTAT
GCTTTTATAAACGCTTCAGTTTTAGAATGTCATCCGCGTATAACGCAATAAATACAACT
TTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAAACGTATGCG
ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCATCTTGCGC
CCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGTGTCATGGTATTCTCAATACCCCAAA
TCTTTGCGGATAAGGGTGTGTTGGACTTGGAGGTTTTTGCAGGTAATGATTGTGTTACC
AGCTCCTCTTAAATGCATTAGCAGAGATAATACTGCTACTCTCCAGTGTGATAATTGTC
TACACTGTTAGTAGTGCGGTATAACAAAATGTCTATGCTTCTAATCGTCTTCGGACAAC
TTTTGACAATCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAA

These sequences exhibit 99.9% query coverage and 98.3% similarity to the *Rigidoporus vinctus* strain MFLUCC17-0007, accession no. MG545057.1. The degree of kinship is then ascertained by utilising MEGHA 11 software to evaluate the data. Using the bootstrap method and the Kimura 2 model, phylogenetic trees are produced, and neighbor-joining trees are produced with 1,500 replications. Afterwards, the acquired sequences are uploaded as *Rigidoporus vinctus* strain MBKF16 to the NCBI with accession number ON287260. The software MEGHA 11 is used to create a phylogenetic tree, as seen in Fig. 2.

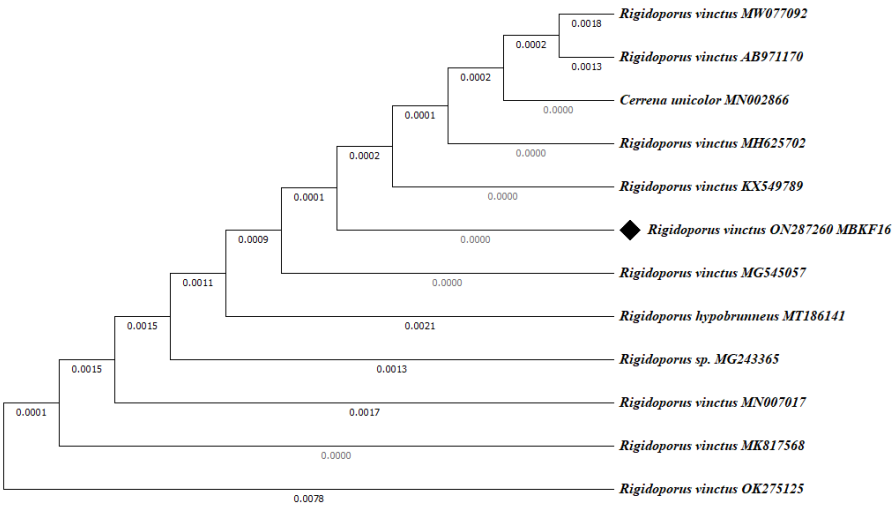

Figure 2. Phylogenetic tree of endophytic fungi strain MBKF16 isolate identified as *Rigidoporus vinctus*

Endophytes which are capable of producing metabolites and enzymes with characteristics similar to those of extracts from the host plant. [16]. It is reported that *Rigidoporus vinctus*, which is useful for the

manufacturing of agarwood, is said to have been isolated from the inner layers of infectious Aquilaria sinensis trees. [17]. The isolated fungal culture strain MBKF16 was subjected to mass culture by submerged fermentation method for the production of extracellular Bioactive compounds. Freshly subcultured fungi were cultured in Erlenmeyer flasks filled with 500ml of Sabouraud Dextrose Broth (SDB) that had been autoclaved. The flasks were then incubated for 21 days at room temperature with constant shaking. Obtained broth with bioactive compounds are filtered are taken for solvent extraction method by using 4 solvents Water, Ethyl acetate, Methanol, and Chloroform. Acquired extract were screened for phytochemicals- Alkaloids, Flavonoids, Saponins, Tannins, Phenols, and Glycosides. Results are tabulated in (Table 1) clearly shows Ethyl acetate is an effective solvent for the extraction of phenols, alkaloids and flavonoids followed by water, methanol and chloroform.

Table 1- Phytochemical constituents of Endophytic fungal extracts

Sl no	Solvent used	Alkaloids	Flavonoids	Saponins	Tannins	Phenols	Glycosides
1	Methanol	+	++	+	+	++	+
2	Ethyl acetate	++	++	+	-	+++	-
3	Chloroform	+	+	-	+	+	+
4	Water	++	+	++	-	++	+

The result of the phytochemical analysis of the fungal extracts showed the presence of various secondary metabolites including phenolic compounds and flavonoids. Phenols are found to be highly positive in the *Rigidoporus vinctus* extracts. By comparing this result the literature suggest they have the ability of supressing bacterial growth[18]. These phytochemicals may be acting as antioxidants and anti-microbial components[1920] hence they are validated by following assays Antibacterial activity of different solvent extracts was performed by agar well diffusion assay with three trials against clinical pathogens- *Escherichia coli, Shigella sonnei, Salmonella typhi* and *Klebsiella pneumoniae* obtained from KIMS Madikeri the details are tabulated in (Table 2). The ability of *Escherichia coli* to infect the gastrointestinal tract (IPEC) or areas outside of it (ExPEC) allows for differentiation. It has been proposed that ExPEC may carry resistance genes that can spread to pathogenic or opportunistic bacteria. Of the several pathogens, E. coli are becoming increasingly important[21]. Shigella sonnei is the second common infectious species that causes shigellosis, or bloody diarrhoea. Recent research has raised questions about its epidemiology, transmission, and pathogenic processes, making S. sonnei an emerging pathogen on a worldwide scale[22]. The opportunistic pathogen Klebsiella pneumoniae is commonly associated with nosocomial infections, primarily affecting those with compromised immune systems[23].

Table 2- List of Clinical pathogens and its characteristics

SL NO	PATHOGEN CODE	PATHOGEN	SPECIFICATION	OCCURRENCE	INFECTION
1	EC	*Escherichia coli*	Gram negative, Rod shape, motile, Non-Sporing, Flagellated,	bacteria found in the environment, foods, and intestines of people and animals	severe stomach cramps, diarrhea (often bloody), and vomiting. Some people may have a fever[24]
2	SS	*Shigella sonnei*	Gram-negative, nonmotile, facultatively anaerobic, non-spore-forming rods. Shigella are differentiated from the closely related Escherichia coli on the basis of pathogenicity, physiology (failure to	Shigella is found in the intestinal tract of infected people, and is spread by eating or drinking food or water contaminated with the bacteria. It can also be spread by direct contact with feces (even with microscopic amounts) from an infected person.	cause an infection called shigellosis. Most people with Shigella infection have diarrhoea (sometimes bloody), fever, and stomach cramps.[22]

			ferment lactose or decarboxylate lysine) and serology.[25]		
3	ST	*Salmonella typhi*	Gram-negative, facultatively anaerobic, rod-shaped bacteria generally 2–5 microns long by 0.5–1.5 microns wide and motile by peritrichous flagella. and can grow at 5–45°C.	lives only in humans their bloodstream and intestinal tract. where water and food may be unsafe and sanitation is poor.	Diarrhea, Stomach (abdominal)cramps, Nausea, Vomiting, Chills, Headache,Blood in the stool. Typhoid fever is a life-threatening illness caused by Salmonella Typhi bacteria.[26]
4	KP	*Klepsiella pnumoniae*	gram-negative, lactose-fermenting, non-motile, aerobic rod-shaped bacterium	Klebsiella bacteria can be spread through person-to-person contact (for example, from patient to patient via the contaminated hands of healthcare personnel, or other persons) or, less commonly, by contamination of the environment. The bacteria are not spread through the air. The bacterium typically colonizes human mucosal surfaces of the oropharynx and gastrointestinal (GI) tract.	Numerous illnesses, such as sepsis, pneumonia, urinary tract infections (UTIs), and bloodstream infections, are caused by Klebsiella species. These infections are especially problematic for the elderly, young people, and those with impaired immune systems.[27]

Agar well diffusion assay is used to detect the capability of a substance to inhibit the growth of microorganisms by measuring the diameter of the inhibition zone around the compound on an agar plate in milli meters. Results are tabulated in (Table 3)

Ethyl acetate extract shown highest antibacterial activity against *Escherichia coli* with high zone of inhibition with 16 mm and *Shigella sonnei* of 12mm which shows the extract has compounds which have antibacterial components which can be used for drug development. literature states that *Rigidoporus vinctus* have anti-bacterial and anti-fungal properties [28]. *Rigidoporus vinctus* can effectively decolorize dye-containing wastewater, thus reducing the hazardous effects of dyes on aquatic organisms and also acts as purifier of water by reducing toxicity[29]. Other than clinical uses it is known to produce aromatic compounds which exhibits the unique fragrance when it is infects agarwood[30 31].

Table 3- Antibacterial activity of Endophytic fungal solvent extracts.

Sl no	**The solvent used for extraction**	***Escherichia coli***	***Shigella sonnei***	***Salmonella typhi***	***Klebsiella pneumoniae***
1	Methanol	8 ± 0.10	00	7 ± 0.16	00
2	Ethyl acetate	16 ± 0.15	12 ± 0.24	11 ± 0.18	10 ± 0.30
3	Chloroform	9 ± 0.22	00	00	7 ± 0.20
4	Water	12 ± 0.15	7 ± 0.10	11 ± 0.22	9 ± 0.28
5	Negative control DMSO	00	00	00	00
6	Positive control Streptomycin (1mg/ml	18 ± 0.10	15 ± 0.10	15 ± 0.10	15 ± 0.10

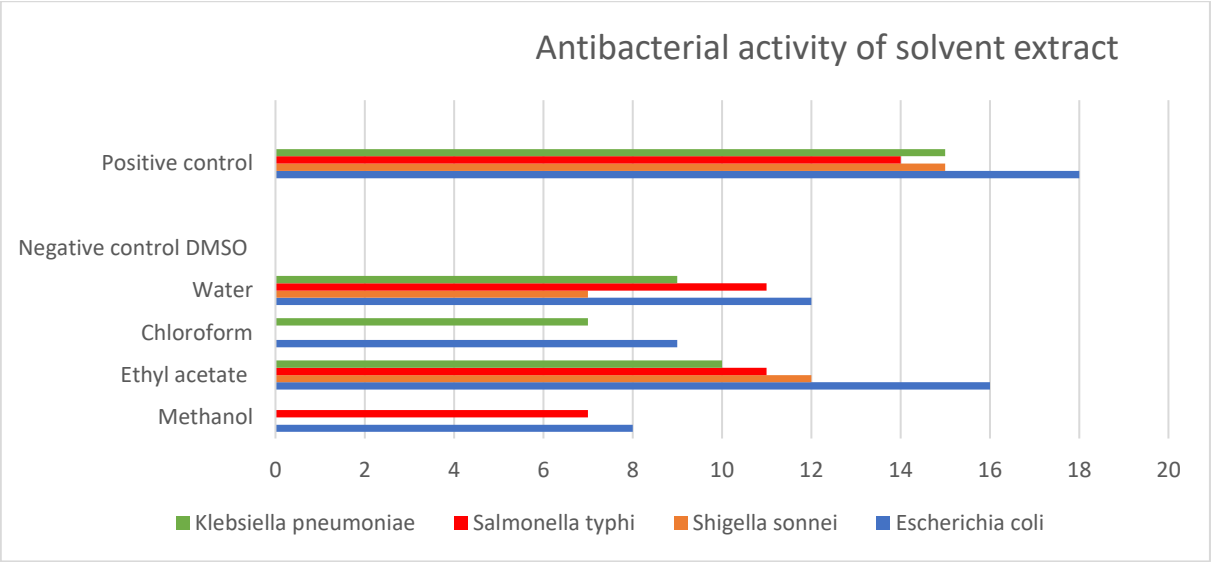

Figure 3. Graphical representation of antibacterial activity of different solvent extracts on four clinical pathogens *Escherichia coli, Shigella sonnei, Salmonella typhi* and *Klebsiella pneumoniae*

Conclusion-

There is increasing interest in studying the endophytes of various plants as a result of current discovery and investigation of these plants for bioactive potential. Endophytes are microorganisms that settle in the tissue of their host plants. It has been studied how endophytic fungi function as biofactories for new bioactive compounds. The endophytic fungus *Rigidoporus vinctus* was identified based on the edible shoots of *Bambusa tulda* that were isolated from the Billigirirangana hills in Chamrajanagara, Karnataka. One possible fungus that can produce a wide range of bioactive chemicals is *Rigidoporus vinctus*. When it comes to extracting active chemicals, ethyl acetate is far more effective than water, methanol, and chloroform. The presence of flavonoids, alkaloids, and phenols is confirmed by analysis. Significant antibacterial properties are present in ethyl acetate extract. Which proves ethyl acetate is an best solvent for extraction of bioactive compound from fungi. which declared *Rigidoporus vinctus* to be a worthy contender for pharmaceutical development.

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