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# ANTIMICROBIAL ACTIVITY OF *FUSARIUM OXYSPORUM*, ENDOPHYTIC FUNGUS, ISOLATED FROM *PSIDIUM GUAJAVA* L. (WHITE FRUIT)

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Medicinal plant, Secondary metabolite, Endophytic fungus, Inhibitory activity, ITS rRNA gene sequence

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**ABSTRACT:** The endophytic fungi isolated from the medicinal plant are an essential part of the current drug discovery from natural products. The endophytic fungus isolated from the healthy leaves of *Psidium guajava* was identified based on microscopic and cultural characteristics. The phylogenetic analysis inferred based on the internal transcribed spacer rRNA region sequence showed close similarity to *Fusarium oxysporum*. The secondary metabolite produced in solid-state, and submerged fermentation was extracted using ethyl acetate and acetone. The antimicrobial activity of the crude extract was screened by the agar well diffusion method. The inhibitory effect of the extract against bacterial pathogens was evaluated by minimum bactericidal concentration MBC and microdilution MIC methods. The antibacterial activity of the crude extract is significant at $p<0.05$ level. The extract presented a broad spectrum of inhibition against all the tested bacteria with inhibition zone diameter from 6.67 ± 0.58 mm to 22.00 ± 1.00 mm. The extract showed strong antimicrobial activity with MIC value from 0.156 mg/ml to 5.0 mg/ml and the MBC from 0.625 mg/ml to 10.0 mg/ml. From the tested fungi, the crude extract showed inhibitory activity against *Saccharomyces cerevisiae*, but no inhibition to *Candida albicans* and *Trichophyton interdigitale*. Therefore, the fungus can be an ideal resource for the biological prospecting of antimicrobial agents.

**INTRODUCTION:** Higher plants harbor a large array of diverse microorganisms with varying types of associations. The plant-microbe interactions include symbionts, mycorrhizal and rhizospheric microorganisms [1]. The symbiotic microbes play a role in protecting their host photobiont against the attacks of herbivory and pathogens whereas mycorrhizal and rhizospheric microorganisms buffer the effects of soil toxic compounds and soil-borne pathogens [1].

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Endophytic microorganisms provide plant protection and enhance plant responses. Endophytes are symbiotic micro-organisms residing asymptomatically in the different tissues of all living plant species [2, 3]. Most commonly, they are microfungi and bacteria [3], but few studies reported the endophytic existence of microscopic algae [4].

The endophytic fungi produce interesting bioactive secondary metabolites with pharmaceutical, Industrial, and agricultural importance [5, 6]. They are a reservoir of various natural products that possess bioactivities against various diseases [7]. Endophytes, on the contrary to synthesized chemical compounds, are a rich source of healthy, safe, and environmentally friendly bioactive natural products useful in pharmaceutical, environmental and agricultural industries [8].

Endo-phytic fungi are the major contributor to most of the natural products produced by endophytic microorganisms [9]. The natural products produced by endophytic fungi have potential applications in the development of antimicrobial, antiviral [10], anti-leishmanial [11], anti-oxidant, anticancer, antiparasitic, and bio-control agents [12].

*Psidium guava* is used as both fruit crop [13] and an ethnomedicinal plant with plenty of medical values [14]. There are several reports on the medicinal properties of the plant extract, for example, antidiabetic, anti inflammatory, antimicrobial [15], anticancer, and antioxidant activities.

More interestingly, medicinal plants and crop plants harbor 64% of endophytic fungi screened for antimicrobial activities [16]. Therefore, we selected *Psidium guajava* for the investigation of endophytic fungi for natural bioactive product discovery. Being both traditional medicinal and food crops, *P. guajava* leaves have been used in the therapy of various diseases [17]. The health benefits of the plant may be due to the bioactive compounds produced by the plant and/or by their endophytic fungi. However, the research report on endophytic fungi from *P. guajava* is very scanty in comparison to its multipurpose ethnobiological uses. Therefore, it is imperative to exploit the endophyte to prospect the industrial lead bioactive compounds.

The endophytic fungi are a reservoir of natural antibacterial agents. They are a potential source of bioactive compounds [18]. Currently, the bacterial infection is increased due to the development of drug resistance by infectious agents. This poses a strong need for potent antibacterial to combat this increasing infection. In India, the endophytic species from medicinal plants have exhibited antimicrobial, antifungal, antioxidant and anti-malarial activity [19]. Endophytic fungi can be a dependable source for the discovery of bio-therapeutics against microbial infections parti-cularly in poverty-driven tropical countries [20].

Recently, there are increasing trends in the discovery of current drug candidates in drug development from natural origin [21]. The endophytic fungi from plants of different ecology are the major contributor to the discovery of natural products with diverse bioactivity. In this study, the endophytic fungus *Fusarium oxysporum* is reported from *Psidium guajava* leaves. This investigation aimed to isolate, characterize and identify endophytic fungus from *P. guajava* and to evaluate the *in-vitro* antimicrobial activity of the endophytic fungus *F. oxysporum*.

## MATERIALS AND METHODS:

**Study Area Description**: This research was carried out in the Department of Applied Botany, at Mangalore University of the Dakshina Kannada District. Dakshina Kannada is one of the three coastal districts of Karnataka State, India. The district is situated between $12.57^0$ and $13\ 50^0$ North Latitude and $74^0$ and $75\ 50^0$ East Longitude [22, 23].

**Sample Collection:** The leaf samples from *P. guajava* were randomly collected from Mangalore University staff quarters. The healthy fresh leaves sample was collected in a sterile plastic bag and brought to the laboratory. The sample was processed for endophytic fungi isolation within two hours after collection.

**Isolation of Endophytic Fungi from *Psidium guajava* Leaves:** The samples were washed thoroughly under running tap water until all the debris was removed from the leaf surfaces [24]. The endophytic fungi from inside the tissue of fresh, healthy leaves were isolated after a series of surface sterilization processes. The leaf sample surface was sterilized by dipping the leaf into 95% EtOH (for 2 min), rinsed with sterile distilled water twice, dipped into 5% Sodium hypochlorite (NaOCl) for 4 min, dipped into 95% EtOH (for 1 min) and rinsed with sterile distilled water twice to remove the surface sterilizing chemicals and allowed to air dry in aseptic conditions. The surface sterilization effectiveness was confirmed by inoculating 0.3 ml distilled water from the last rinse into the PDA culture medium.

The sterilized leaves samples were cut into small pieces by sterile stainless scissors and plated on the Petri dishes containing PDA supplemented with 250 $\mu$g/ml streptomycin sulfate to inhibit bacterial contaminants. The inoculated plates were sealed with parafilm and incubated at ambient temperature (28 ºC) for 21 days. The emergence of the hyphae from the leaf was visually inspected every day. The hyphae that emerged before 3 days were considered as surface mycobiota and discarded.

The endophytic fungal isolates were purified by sub-culturing into freshly prepared PDA medium supplemented with streptomycin sulfate. The purified cultures were inoculated into PDA slant and incubated for six days at ambient temperature (28 $^{o}$C). The grown pure cultures of the endophytic fungal isolates were kept in the refrigerator for future microbiological studies. Extra two slants of the isolates were kept in 20% glycerol at -80 ℃ for long-time preservation.

**Identification of the Endophytic Isolate:** The endophyte isolate was identified based on cultural characteristics: macroscopic and microscopic characteristics.

**Macroscopic Characteristics of the Isolate:** The endophyte isolate was cultured on PDA medium for 7 days. The culture growth condition was observed every day, and the morphological features such as mycelia growth pattern, color on a plate, and growth rate were recorded every day.

**Microscopic Characteristics of the Isolate:** The microscopic feature of the isolate was characterized by slide culture preparation on a sterile microscope glass slide. Five days grew culture was inoculated into PDA cube cut and aseptically placed on the slide, covered by sterile coverslip, moistened with sterilized distilled water, and incubated at ambient temperature for 7 days.

At the end of the seventh day, the coverslip was removed from the glass slide culture, stained by lactophenol cotton blue, and observed under the Nikon phase-contrast microscope (Nikon Y-TV55 ECLIPSE). Based on the cultural characteristics, the isolate was identified by using standard mycological manual [25, 26].

**Sequencing of the Endophyte Fungus Isolate ITS rRNA:** The PCR and DNA sequencing was done by the National Collection of Industrial Microorganisms, CSIR-National Chemical Laboratory (NCL), Pune, India.

Chromosomal DNA was extracted by using a spin column kit (HiMedia, India, or similar manufacturers). Fungal ITS rRNA gene (600 bp) [27] was amplified using polymerase chain reaction (PCR) in a thermal cycler and were purified using Exonuclease I-Shrimp Alkaline Phosphatase (Exo-SAP) [28]. The PCR condition for sequencing the ITS rRNA region was run using the ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) primer sequences. PCR conditions for amplification of IT'S THE region were: No of cycles (35), initial denaturation temp (95℃) for 5 min, denaturation temp (95℃) for 30 sec, annealing temp (55℃) for 30 sec, extension temp (72 ℃) for 1 min, and final extension temp (72℃) for 7 min.

The purified amplicons were sequenced by the Sanger method in ABI 3500 × l genetic analyzer (Life Technologies, USA). Sequencing files (.ab1) were edited using Chromaslite (version 1.5) and further analyzed by the Basic Local Alignment Search Tool (BLAST) with the closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences [29]. The nucleotide sequences were compared to sequence databases and calculated the statistical significance of matches [30].

The BLAST algorithms were used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. (i) The Blastn program was searched to find the potentially closely related type strain sequences [29] (ii) The sequence similarity values between the query sequence and the sequences identified in step (i) were calculated using pairwise alignment [31]. Therefore, the isolate was reported with the first five-ten hits observed in the said database [32, 33].

**Phylogenetic Relationship:** The phylogenetic relationship of the endophytic isolate was inferred by using MEGA-X. Multiple sequences were aligned from the Gene database involving *Fusarium* and related species from type material and outgroup taxa. The Maximum likelihood (ML) phylogram was obtained from the its rRNA gene sequences of *Fusarium* species and related genera [34].

**Fermentation and Extraction of Secondary Metabolites:**

**Selection of the Best Candidate Endophyte:** The endophytic fungal isolate with the best antimicrobial activity was selected among the isolates. The 5 days old cultures of endophytes cut

from the PDA medium and inoculated to the nutrient agar media seeded with 18 h old pathogenic test bacteria. The inoculated cultures were incubated at 37 ºC for 48 h. The endophytic cube produces a clear zone against the tested bacteria that were considered as potential bioactive isolates and selected for further study. The endophyte produced relatively wide clear zone was selected for this study.

**Solid-State Fermentation (SSF):** Endophytic fungus exhibiting biological activity was subjected to secondary metabolite production. The endophytic fungal isolate was grown on a fresh PDA medium at 28 ºC for 5 days. To 500 ml Erlenmeyer flasks, 100 gm of rice was soaked with 100 ml distilled water overnight, autoclaved twice at 121 ºC for 30 min and the fresh fungus culture cut by "flame sterilized cork borer" (6 mm) was inoculated and incubated for 30 days at 30 ºC. The fermented culture flask was filled with 200ml of ethyl acetate, stand for overnight, shaken thoroughly and filtered by four fold Whatman 1 filter paper. The filtration process was repeated, five times in our cases, until most of the metabolite was extracted [34].

**Submerged Fermentation (SmF):** Three pieces of the pure endophytic fungal culture (5 days old) from PDA media was cut by sterile cork borer (6 mm diameter) and transferred into a 500 ml Erlenmeyer flask containing 200 ml of PDB media in ten flasks (for quantification). The endophytic fungal cultures were then incubated at 28 ºC for one month.

The fermented media was separated from the fungal mat by filtration using cheesecloth. The metabolite, extracellular and intracellular, was extracted by adding 200 ml of ethyl acetate solvent into culture filtrate flasks and mixed intermittently and kept overnight to ensure the death of fungal cells.

The treated mixture was then transferred into a separatory funnel and kept to stand for 10 minutes until the organic solvent layer and the media components formed two distinct layers. Thereafter, the upper organic solvent layer containing the extracts was separated and the solvent was evaporated in a water bath at 50 ºC.

The separated mycelia were dried at 55 ºC in a hot air oven and the dried mycelial mat was ground into powder. To the dried mycelia powder, 40 ml acetone was poured, kept for 24 h and the acetone extract was separated on filtration by using whatman 1 filter paper (four folded). Finally, the ethyl acetate and acetone extracts from both SSF and SmF were pooled together; the crude extract collected after evaporating the extraction solvent was dried in a hot air oven at 55 ºC and the dried extract was stored in the refrigerator for future biological activity evaluation [24].

**Screening Antimicrobial Activity of the Extract:** The antimicrobial activities of the endophytic fungal isolate, *Fusarium oxysporum*, was evaluated against seven bacteria and three fungi by a method of agar well diffusion and microdilution minimum inhibitory concentration (MIC) assay, and minimum bactericidal concentration (MBC) assays. The antibacterial test pathogenic bacteria were *E. faecalis, K. pneumoniae, P. aeruginosa, B. subtilis, E. coli, P. vulgaris, and S. aureus.* The antifungal test fungi were *C. albicans*, *S. cerevisiae*, and *Trichophyton interdigitale.*

**Test Microorganisms:** The pathogenic test bacteria were obtained from the Microbiology Department, Yenepoya Medical College, Derlakatte, Mangalore, India, and Biochemistry Department, Mangalore University, India.

**Inoculum Preparation and Standardization of the Test Bacteria:** The test bacteria were cultured in Mueller Hinton Broth (MHB) medium at 37 ºC for 24 h according to the procedure of Clinical and Laboratory Standards Institute (CLSI) document for minimal inhibitory concentration (MIC) bacterial assays [35, 36]. The grown test bacteria cultures were re-inoculated into MHA medium and incubated for 24 h at 37 $^{o}$C. Five colonies of 24 hours old test bacteria were inoculated using a flame sterilized inoculating wire loop into freshly prepared MHB and incubated overnight [37]. The overnight cultures bacteria were re-inoculated into 0.85 % normal saline solution incubated at 37 ºC until the growth reaches 0.5 Mac Farland units through periodic check by taking a portion from the inoculum and measured by Systronics µc Colorimeter 115.

**Preparation and Standardization of the Test Fungi:** The test fungi were sub-cultured in Saborauds Dextrose Broth (SDB) medium for 48 hours at 37 ºC in the BOD incubator. The cultures were transferred to Saborauds Dextrose Agar (SDA) medium and incubated. Five pure colonies of the cultures were picked by a wire loop, inoculated into SDB medium, and incubated for 48 hours. The incubated test fungi load was adjusted to 0.5 Mac Farland units through periodic check by taking a portion from the inoculum and measured by Systronics µc Colorimeter 115. The adjusted test fungal cultures were used for antifungal bioactivity assay.

**Extract Preparation:** The dried and preserved crude extract of the endophyte was dissolved in DMSO, and filtered with 0.45 µm filter paper (Millipore).

**Antimicrobial Evaluation of Endophyte Extract by Agar Well Diffusion:** The evaluation of antimicrobial potency of the fungal endophyte crude extract was carried out by agar well diffusion assay. The extract was prepared in four varying concentrations of 80, 60, 40, and 20 mg/ml in 2.5% DMSO. The test bacteria and fungi cultures (200µl) were uniformly cotton swab inoculated into a sterile MHA and SDA media, respectively. From each concentration of extract preparations, 30 µL was pipetted to each of the 5 wells (6mm) punched by a gel puncher (4 holes for extract, 1 well for DMSO). Chloramphenicol disc (30 µg/disc) and Fluconazole 25 mcg/disc (FLU [25] Himedia) were used as a standard positive control for bacteria and fungi, respectively. DMSO (2.5%) was used as a negative control reference. The inoculated culture preparations were refrigerated for 1hr to permit diffusion of extract to medium and incubated in the BOD incubator. The test was done in a triplicate and the inhibition zone was measured [38].

**MIC Determination of the Extract:** The minimum inhibitory concentration (MIC) of the crude extract that inhibited the bacterial culture growth was determined by microdilution. Due to weak antifungal activity, the MIC of the extract was not determined for fungi.

**Microdilution Assay:** The crude extract initially screened at a concentration of 80-20 mg/ml was subjected to MIC evaluation. The endophytic extract MIC was evaluated against the test bacteria at concentration ranges from 10 to 0.039 mg/ml in 9 series of two-fold serial dilution.

The MIC assay experiment encompasses a sterility check, negative control, and positive control (32 µg/ml Chloramphenicol). The Meuller Hinton Broth medium was prepared and bacterial inoculum was standardized according to NCCLS [37].

The 96-well microplates were prepared by adding 90 µl of sterile distilled $H_2O$ into each well, 10 µl of extract dissolved in DMSO, 80µl of MHB medium and 20 µl of the inoculum and incubated for 22 h [39]. Finally, the content of the final volume in each microtiter plate well was 200 µl [39]. The microplate tray with its content was sealed in a plastic bag [36] and incubated at 37 ºC for 22 h in the BOD incubator.

For bacterial growth visualization, 0.5 % of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) was prepared in distilled sterile water at the time of recording the MIC result. After incubation, 20 µl TTC was dispensed to the microplate well and incubated for 2 h. The microplate was checked for color changes (to the pink color indicated bacterial growth and retained original culture color showed growth inhibition). In addition to visual inspection, the microplate was evaluated by reading the absorbance at 600 nm on the Thermo Fisher Scientific microplate reader (Multiskan G0 Spectrophotometer, Molecular Devices).

**Minimum Bactericidal Concentration (MBC) Determination:** After reading the plates incubated for MIC value, MBC was determined by re-inoculating 100 µl culture mixture from 2 dilution series above and below the MIC value into the MHA medium. The inoculum was spread by using sterile bent glass rods to dryness and incubated at 37 ºC for 24 h. The absence of any growth of the bacterial pathogens showed the MBC of the crude extract [40].

**Statistical Data Analysis:** All the experiments were performed in triplicate and the data were statistically analyzed by SPSS statistical software, version 25.0 (SPSS Inc, Chicago, Illinois, USA) and the result was presented in Mean ± standard deviation (SD). Dunnett t test was used to determine the significance of antimicrobial activity

at p<0.05. The MIC data was graphically presented by using GraphPhad Prism software.

## RESULTS AND DISCUSSION:

**Isolation of Endophytic Fungi from *Psidium gua java* Leaves:** The endophytic fungal isolates have emerged from the surface-sterilized healthy leaves **Fig. 1** after three days of inoculation on to the PDA medium.

**Morphological Characteristics of the Endophytic Isolate:** The isolated endophyte fungus produced thin cottony mycelia with a tinge of pink mycelium color and displayed a moderate growth rate in PDA culture medium **Fig. 2** and **3** [25]. Conidiophores are inconsistent, slender, and simple, born in single or grouped. Two types of phialosporous conidia produced: macroconidia boat-shaped, with slightly tapering apical cells and hooked basal cells, 4-celled; and microconidia ellipsoidal, 1 to 2-celled **Fig. 4** and **5**. Chlamydospores are globose and solitary **Fig. 6** [25].

**FIG. 1: ENDOPHYTIC FUNGAL ISOLATE EMERGING FROM THE SURFACE-STERILIZED LEAVES OF *P. GUAJAVA***

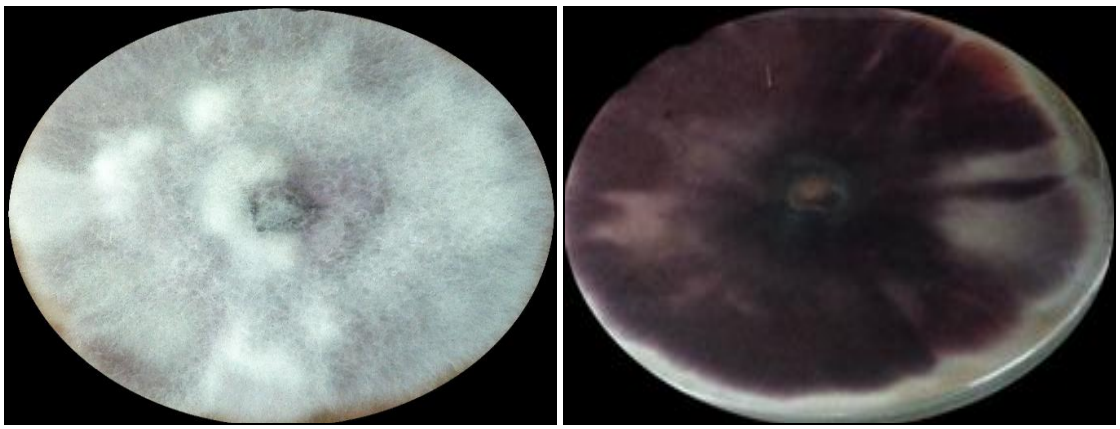

**FIG. 2 AND 3: *FUSARIUM OXYSPORUM* ENDOPHYTE CULTURE IN PDA MEDIA** (2: front color; 3: reverse colony color)

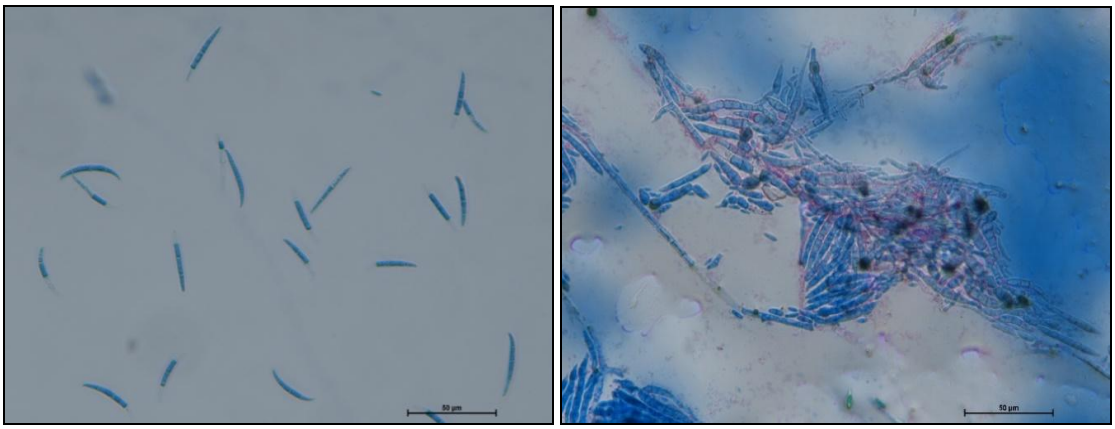

**FIG. 4, 5 AND 6: *FUSARIUM OXYSPORUM* SLIDE CULTURE STAINED BY LACTOPHENOL COTTON BLUE** (4: conidia on conidiophores; 5: conidia; 6: conidia and chlamydospores). bars 50 µm.

**Phylogenetic Relationship of the Endophyte Fungus Isolate:** Based on the analysis of the ITS rRNA gene sequence, the endophyte fungal culture isolate showed 99.63% identity to *Fusarium oxysporum* (accession number: MT420611). The phylogenetic tree was constructed using MEGA-X and the evolutionary relationship inferred by the Maximum Likelihood (ML) method **Fig. 7**. Phylogenetically, the endophytic isolate is closely related to the *F. oxysporum*.

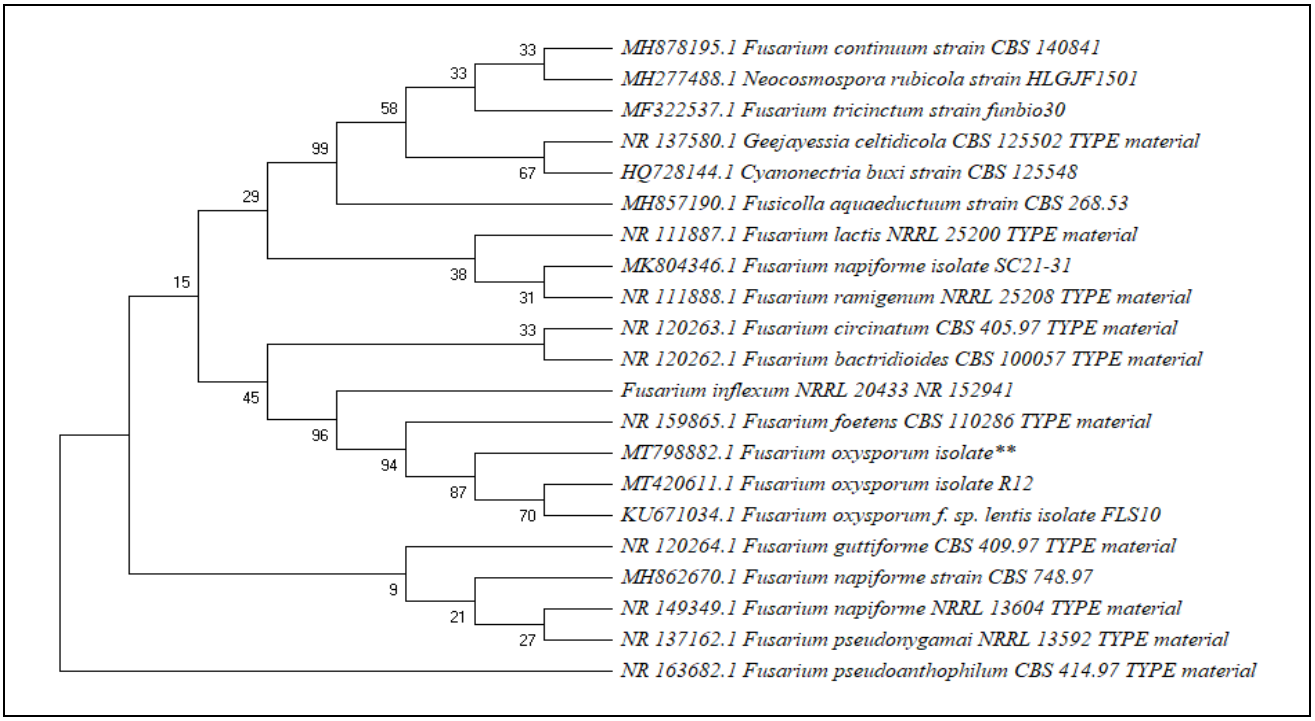


**FIG. 7: PHYLOGENETIC TREE OF THE ENDOPHYTIC ISOLATE CONSTRUCTED BY THE MAXIMUM LIKELIHOOD (ML) METHOD BASED ON ITS RNA SEQUENCES.** **Endophyte reported in this study (accession number: MT798882). The evolutionary analysis involved 21 nucleotide sequences

**Cultivation of *Fusarium oxysporum* and Anti-microbial Screening of the Extract:** The cultivated fungus grew, and the medium was completely covered with white-pinkish dense mycelium. The endophyte fungus has produced secondary metabolites by SSF **Fig. 8** and SmF **Fig. 9** in rice medium and PDB medium, respectively.

The antimicrobial activity screening of endophytic fungal extract was assayed against the human pathogenic bacteria *Bacillus cereus*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The extract was evaluated for its antifungal activity against *Candia albicans*, *Saccharomyces cerevisiae*, and *Trichophyton interdigitale*.

***In-vitro* Antibacterial Screening of the Crude Extract:** The crude extract of *Fusarium oxysporum* exhibited a broad antibacterial spectrum against the tested bacteria at the extract inoculum volume of 30 µl from 20-80 mg/ml concentration of the extracted stock solution **Table 1**. The extract obtained from the fungus shown a large zone of inhibition against all the tested bacteria from 40-80 mg/ml. There was no inhibition zone observed for the control plate inoculated without the fungal extract.


**FIG. 8 AND 9: *FUSARIUM OXYSPORUM* CULTURE FERMENTATION ON RICE MEDIUM AND POTATO DEXTROSE BROTH MEDIUM** (8: SSF; 9: SMF).

**TABLE 1: ANTIBACTERIAL ACTIVITY SCREENING OF *F. OXYSPORUM* EXTRACT BY AGAR WELL DIFFUSION**

| | The Mean Inhibition Diameter Zone Against Tested Bacteria (mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Conc (mg/ml)** | ***B. subtilis*** | ***E. coli*** | ***E. faecalis*** | ***K. pneumoniae*** | ***P. vulgaris*** | ***P. aeruginosa*** | ***S. aureus*** |
| 20.00 | 11.33±0.58 | 10.00±1.00 | 8.67±1.15 | 7.67±0.58 | 6.67±0.58 | 8.33±0.58 | 10.33±0.58 |
| 40.00 | 15.00±1.00 | 13.33±0.58 | 12.67±0.58 | 10.33±1.53 | 9.00±1.00 | 10.67±0.58 | 14.00±1.00 |
| 60.00 | 17.67±0.58 | 17.33±0.58 | 15.33±0.58 | 13.00±1.00 | 11.67±0.58 | 12.00±1.00 | 16.67±1.15 |
| 80.00 | 20.67±0.58 | 22.00±1.00 | 18.00±1.00 | 16.00±1.00 | 15.00±1.00 | 14.00±0.00 | 19.67±0.58 |
| Chlo30 | 29.33±1.15 | 30.00±1.00 | 28.33±0.58 | 24.00±0.00 | 21.00±1.00 | 27.67±0.58 | 28.00±0.00 |

*All measurements were in triplicate and expressed in Mean ± SD; Chlo30: Chloramphenicol 30 µg/disk; [a]the antibacterial activity of the crude extract is significant at $p<0.05$.

The crude metabolite inhibited the growth of all the tested bacteria at 30 µL inoculum volume with mean inhibition diameter ranged from 6.67 ± 0.58 mm to 22.00 ± 1.00 mm. At 20 mg/ml concentration, the extract shown small inhibition zone diameter for *P. vulgaris* (6.67±0.58 mm), *K. pneumoniae* (7.67 ± 0.58 mm), *P. aeruginosa* (8.33 ± 0.58 mm), and *E. faecalis* (8.67 ± 1.15 mm). This implies the low antibacterial activity of the extract at 20 mg/ml [42].

The bioactive metabolite exhibited high inhibitory activity with zone inhibition diameter from 12.67 ± 0.58 mm to 15.00 ± 1.00 mm at 40 mg/ml except for *P. vulgaris* (9.00 ± 1.00 mm), *K. pneumoniae* (10.33 ± 1.53 mm), and *P. aeruginosa* (10.67 ± 0.58 mm). *E. coli* was the most sensitive to the metabolite with the highest zone of inhibition (22.00 ± 1.00 mm) followed by *B. subtilis* (20.67 ± 0.58 mm), *S. aureus* (19.67 ± 0.58 mm), *E. faecalis* (18.00 ± 1.00 mm) and *P. vulgaris* (15.00 ± 1.00 mm) at 80 mg/ml.

The endophytic *Aspergillus* species presented an antibacterial activity with different concentrations [43]. In a different study, the endophytic *Aspergillus* sp. and *Penicillium* produced antibacterial activity against the *E. coli* with growth inhibition diameter of 4-11 mm and 5 mm respectively [44]. The endophytic fungi isolated from *Mitrephora wangii* flower showed antibacterial activity against human pathogens with inhibition zones in the range of 7.4 to 13.4 mm in diameter [45]. Whereas our endophytic *F. oxysporum* revealed the antibacterial activity with a 10-22 mm zone of inhibition against *E. coli*.

Raina *et al.* [46] isolated endophytic bacteria and fungi from the medicinal plant *Picrorhiza kurroa*. From their fungal endophytes, the *Fusarium* sp. revealed potent antimicrobial activity against *E. coli* and *P. aeruginosa* with an inhibition zone of 4.33 mm to 13.33 mm but inactive against *S. aureus*. The antibacterial activity of crude extract from the endophytic fungi tested against *E. coli*, *K. pneumonia*, and *S. aureus* with the inhibition zone of 9 mm to 24 mm [47]. The endophytic *Fusarium napiforme* in this study showed antibacterial activity with the growth inhibition zone of 7.67 ± 0.58 mm to 22.00 ± 1.00 mm against the tested bacterial pathogens. The *in-vitro* antagonistic activity of the endophyte *Thielaviopsis basicola* extract TBXA1 and TBXA2 showed a prominent antagonistic activity against bacterial pathogens [48]. These extract produced inhibition against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*. Mishra *et al.* [49] reported the broad antimicrobial activity of endophytic fungi isolated from *Melastoma malabathricum* L. with the growth inhibition zone ranging from 1.8 mm to 16.83 mm. Our endophytic extract showed similar antagonistic activity with the zone of inhibition from 14 mm to 22 mm in a dose-dependent antagonism.

The endophytic *Aspergillus fumigatus* extract exhibited a broad spectrum of antimicrobial activity against human pathogenic microbes [50]. Similarly, the antimicrobial evaluation of our endophyte isolate *F. oxysporum* showed significant antibacterial activity at $p<0.05$. This fungus can be a reliable resource for pharmaceutical applications. Therefore, further, bioactivity screening in the purified compound of the fungal metabolite would better compliment in the discovery of novel antimicrobial therapeutic agents.

***In-vitro* Antifungal Screening of the Endophyte Extract:** The extract of *F. oxysporum* has selectively inhibited only *S. cerevisiae* with the inhibition zone from 5.33 mm to 12.67 mm **Table 2**. There was no growth inhibition in the control plate inoculated with test fungi without the endophyte extract. For *C. albicans* and *Trichophyton interdigitale*, it has shown no inhibition in **Table 2**. Nevertheless, the little selective antifungal effect of the isolate crude

extract may not imply the absence of bioactivity. Further purification of the endophytic crude extract may result in strong inhibitory activity.

**The MIC Determination of the Extract:** The MIC of the fungal extract was evaluated against the tested bacteria at a concentration of 10 mg/ml to 0.039 mg/ml in nine series of dilution. The extract had presented MIC from 0.156 mg/ml to 5 mg/ml against the tested bacteria **Table 3**. The extract was strongly inhibitory against *E. faecalis* and *P. vulgaris* with MIC of 0.156 mg/ml followed by *E. coli* and *P. aeruginosa* (0.625 mg/ml MIC for both), *B. subtilis* (1.25 mg/ml), *S. aureus* (2.5 mg/ml) and *K. pneumoniae* (5 mg/ml) [51, 52].

**TABLE 2: ANTIFUNGAL SCREENING OF CRUDE EXTRACT BY AGAR WELL DIFFUSION**

| Antifungal Mean Inhibition Zone Diameter (mm)* | | | | | |
|---|---|---|---|---|---|
| Extract (mg/ml) | 20 | 40 | 60 | 80 | FLC[25] |
| *C. albicans* | NA | NA | NA | NA | NA |
| *S. cerevisiae* | 5.33 ± 0.58 | 6.67 ± 0.58 | 9.33 ± 0.58 | 12.67 ± 0.58 | 30.00 ± 5.00 |
| *T. interdigitale* | NA | NA | NA | NA | NA |

*NA: No activity observed; FLC[25]: Fluconazole 25 mcg/disc; All measurements were in triplicate and expressed in Mean ± SD. SD = standard deviation

**TABLE 3: MINIMUM INHIBITORY CONCENTRATION OF THE CRUDE EXTRACT**

| MIC of Extract Against the Tested Bacteria (mg/ml)* | | | | | | |
|---|---|---|---|---|---|---|
| ***E. fecalis*** | ***K. puemoniae*** | ***P. aeruginosa*** | ***B. subtilis*** | ***E. coli*** | ***P. vulgaris*** | ***S. aureus*** |
| 0.156 | 5.0 | 0.625 | 1.25 | 0.625 | 0.156 | 2.5 |

*All assays were performed in triplicate

The MIC of the crude extract determined for *P. vulgaris* agreed with the study reported by Valgas *et al.* [39]. Sharma *et al.* [53] and Stanković *et al.* [54] have reported the MIC from 25 to 6.25 mg/ml and from 100 to 6.3 mg/ ml by tube dilution and microwell dilution assays.

The endophyte, *Botryodiplodia theobromae*, inhibited bacterial growth at 0.06 and 0.15 mg/ml in the study reported by Zaher *et al.* [45]. The extract of *F. napifomre* depicted strong MIC value in comparison to report by Ariffin *et al.* [55] against some bacteria tested. The crude extract showed significant antibacterial activities against the tested pathogenic bacteria. Currently, antibiotic resistance is dangerously increasing to alarming levels in all parts of the world. There are several reports in the literature of compounds isolated from endophytic fungi having antimicrobial activity [56]. The endophytic fungi isolated from *Bauhinia guianensis* revealed the antimicrobial activity of extracts with the MIC range from 78-2500 µg/ml [55]. *Fusarium* sp. identified from the bark of *Cinnamomum mercadoi* revealed the antibacterial activity with MIC values in the range of 2.1 - 4.2 mg/ml [56]. In this study, the *F. oxysporum* crude extract showed stronger antibacterial activity with the MIC of 0.165 to 5.0 mg/ml against the tested bacteria **Fig. 10**. Therefore, the endophyte fungal isolate *F. napiforme* could be the ideal candidate for the development of potent antibacterial drugs **Table 2**.

**Minimum Bactericidal Concentration (MBC) of the Extract:** The MBC of the crude extract was evaluated from two dilution units above and below the MIC of the extract against the tested bacteria **Fig. 10**. The crude extract was bactericidal in the range of 10 mg/ml to 0.625 mg/ml. The lowest MBC of the extract that exhibited the complete inhibition of the bacterial growth was 0.625 mg/ml for *P. vulgaris* **Table 4**.

**TABLE 4: MINIMUM BACTERICIDAL CONCENTRATION OF THE EXTRACT**

| MBC of Extract Against Tested Bacteria (mg/ml)# | | | | | | |
|---|---|---|---|---|---|---|
| ***E. faecalis*** | ***K. puemoniae*** | ***P. aeruginosa*** | ***B. subtilis*** | ***E. coli*** | ***P. vulgaris*** | ***S. aureus*** |
| 2.5 | 10.0 | 2.5 | 5.0 | 1.25 | 0.625 | 5.0 |

#All assays were performed in triplicate

*Psidium guajava* harbors different types of symbiotic microorganisms of which fungi are the predominant one. The mycobiont from different genera establishes various types of association with the plant.

The association takes a different degree of form such as phyllospheric, [58] mycorrhizal, [59] plants pathogenic and endophytic [60].

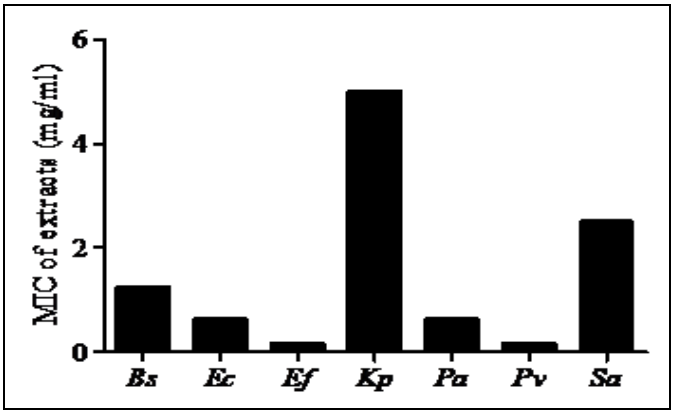


**FIG. 10: THE MIC OF F. OXYSPORUM SHOWING ANTIBACTERIAL ACTIVITY AGAINST BACTERIAL PATHOGENS.** BS: *B. subtilis*; Ec: *E. coli*; Ef: *E. faecalis*; Kp: *K. pneumoniae*; Pv: *P. vulgaris*; Sa: *S. aureus*

The genus *Fusarium* (Ascomycota, Hypocreales, Nectriaceae) isolated from *Psidium guajava* forms a different association with the host plant as mycorrhizae, [59] plants pathogenic and endophytic [61, 62]. The *Fusarium* sp. reported in our observation was endophytic and synthesized strong antimicrobial secondary metabolites.

The endophytic fungi from *Psidium* species produce natural products with different biological activities [63-67]. The natural products have been known for their enormous potencies such as antimicrobial agents, [68, 62] anticancer, [69] and herbicides [70]. The result of our study indicated that the endophytic *F. oxysporum* extract from *P. guajava* showed broad-spectrum antimicrobial activities against the tested bacterial pathogens.

The efficacy of drugs in the market in today's world is being tempted due to the drug resistance developed by the pathogens. This scenario demands a continual investigation of therapeutically lead compounds from the natural sources to overcome the resistance. Endophytic fungi are one of the natural reservoirs to discover anti superbug agents. The extract of endophyte *F. oxysporum* revealed a potent antimicrobial activity against gram-negative and gram-positive bacteria tested. The result of this study proves that endophyte fungus from the medicinal plant can be ideal candidates for natural therapeutic agent development.

Even though the crude extract has shown inhibition against only *S. cerevisiae,* this may not indicate the complete absence of bioactivity to other pathogenic fungi.

Thus, further, bioactivity screening of the *F. oxysporum* on purified extract is needed for more potent chemotherapeutic agent development. The *Psidium* is very less investigated for its endophytic microorganisms, unlike many other medicinal plants, and it is more worthy to further extend the natural product search from its endophytes.

**CONCLUSION:** The current drug discovery move largely relies on natural products due to its nil or low toxicity, unlike synthetic chemical products. Medicinal plants are heavily exploited by pharmaceutical companies for the formulation of therapeutic agents, which leads to plant extinction. This can be countered by the use of endophytic microorganisms in the development of natural products. Recently, there are increasing trends in scientific research on endophytic fungi for the bioprospecting of natural products.

From this study, it is deduced that the endophytic fungus *F. oxysporum* extract has shown broad antagonism against gram-positive and gram-negative bacteria, and *S. cerevisiae*. Therefore, the fungus *F. oxysporum* could be the ideal resource for the formulation of therapeutic agents against pathogenic microorganisms.

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