**Isolation and Characterization of Endophytic Fungi from *Passiflora edulis*, *Mercurialis annua* and *Pouteria campechiana* Using MALDI-TOF and Evaluation of Antimicrobial Efficacy of Leaf Extracts**

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| **ABSTRACT:**  **Aim:** The present study aimed to isolate and identify the endophytic microflora of *Passiflora edulis*, *Pouteria campechiana* and *Mercurialis annua* plant leaves and to evaluate antimicrobial activity of their leaf extracts against pathogenic bacterial and *Candida* strains.  **Background:** Endophytes have extensive uses in agriculture and medicine and provide a host with various unknown advantages in addition to producing hormones that support plant growth and help the host resist abiotic stress. In addition, bioactive secondary metabolites are produced by endophytic fungi, some of which come from the host plants.  **Methodology:** The healthy plant leaves were subjected to surface sterilization. The leaf segments were cut aseptically (1cm x 1cm) and placed in Petri dishes containing Potato Dextrose Agar (PDA) and incubated at room temperature for 7 days. Identification was done based on macroscopic, microscopic characteristics and MALDI-TOF Analysis. Ethanolic leaf extracts were prepared and the antimicrobial activity of three plants were evaluated by using Disc diffusion and Agar well diffusion method.  **Results:** A total of 40 endophytic fungi were isolated from healthy leaves such as, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Talaromyces* spp, *Fusarium solani*, *Mucor* spp, *Rhizopus* spp and *Candida albicans*. The overall colonization rate of endophyte in all the 3 leaf segments was found to be 74.07%. The extracts of *P. edulis*, *P. campechiana* and *M. annua* exhibited considerable effectiveness in the agar well diffusion method compared to the disc diffusion method. *P. campechiana* leaf extracts exhibited significant antimicrobial activity against all the tested organisms such as *Escherichia coli*, *Klebsiella* spp, *Pseudomonas* spp, *Acinetobacter* spp, *Staphylococcus aureus* and *Candida* spp. **Conclusion:** This study highlights the diversity of endophytic fungal communities inhabiting *P. edulis*, *P. campechiana* and *M. annua*, as well as the antimicrobial property of ethanolic leaf extracts. These findings imply the potential of endophytic fungi as a source of bioactive compounds and the effectiveness of plant extracts in antimicrobial applications. |

***Keywords:*** *Endophytic fungi, Passiflora edulis, Pouteria campechiana, Mercurialis annua, antimicrobial activity, Ethanolic extract.*

1. INTRODUCTION

The term “endophyte” (endo-within, phyton-plant) means “in the plant.” A large number of endophytic microorganisms are found in plants as a reservoir [1]. Endophytes are resident microorganisms, usually fungi and bacteria, that reside inside plant hosts permanently or for a short duration [2]. Endophytic fungi inhabit the interior of healthy plants without any apparent harm and may synthesize bioactive compounds, which constitute an alternative for the control of human pathogens [3]. According to studies, endophytes enhance their host by protecting it against pathogens, pests, insects and even domestic herbivores [4]. Endophytes have extensive uses in agriculture and medicine and provide a host with various unknown advantages [5] in addition to producing hormones that support plant growth and help the host resist abiotic stress. In addition, bioactive secondary metabolites are produced by endophytic fungi, some of which come from the host plants [6]. Endophytes produce bioactive substances, that are unique to their host plants. These substances are important from ecological, biochemical and molecular aspects. A variety of species and a broad range of secondary metabolites are found in endophytic fungi [7]. In recent decades, research on endophytes has revealed that their significant role in promoting plants as endophytes has been shown to enhance nutrient uptake, stress tolerance, and disease resistance in the host plants, resulting in improved crop yields. Evidence shows that endophytes can provide improved tolerances to salinity, moisture, and drought conditions, highlighting the capacity to farm them in marginal land with the use of endophyte-based strategies. Furthermore, endophytes offer a sustainable alternative to traditional agricultural practices, reducing the need for synthetic fertilizers and pesticides, and in turn reducing the risks associated with chemical treatments [8].

***Passiflora edulis***, commonly known as passion fruit (Fig.1.a), is a vine species of the passion flower family, Passifloraceae [9]. The phytoconstituents found in Passiflora edulis, including cyanogenic chemicals, glycosyl flavonoids, phenols and alkaloids, are thought to be responsible for its health advantages. These are reported to be helpful in the treatment and prevention of conditions like cancer, diabetes, anxiety, asthma, osteoarthritis and cardiovascular illnesses [10,11]. ***Mercurialis annua***, commonly known as annual mercuryor French mercury (Fig.1.b), is a species of flowering plant belonging to the family Euphorbiaceae [12]. The plant which is used to treat rheumatism, dropsy, gall bladder, liver diseases and gynecological diseases is made into a homeopathic remedy and is also used in folk medicine as a diuretic and anti-syphilitic [13,14]. ***Pouteria campechiana,*** commonly known as the cupcake fruit, eggfruit or canistel[15](Fig.1.c), is rich in carbohydrates, amino acids, carotene, antioxidants including phenolics, polyphenolics, flavonoids and carotenoids, as well as minerals like calcium, phosphorus, iron, vitamins A and C. These attributes make the fruit beneficial for treating coronary issues, liver disorders and epilepsy [16-18].



(a) *Passiflora edulis* (b) *Mercurialis annua* (c) *Pouteria campechiana*

(Passion fruit) (Annual mercury) (Egg fruit)

**Fig.1. Pictures of selected plants used for fungal isolation**

Endophytic fungi play an essential role in the microenvironment of medicinal plants because they can colonize internal tissues without harming the host. These fungi are widely distributed and exhibit significant biodiversity, usually being specific to host plants and environmental conditions. Their presence is important because they produce bioactive secondary metabolites that enhance the host plants' medicinal properties [19]. Studies has shown that endophytic fungi from *P. edulis*, *P. campechiana* and *M. annua* can produce various bioactive compounds, such as alkaloids, terpenoids and polyketides with antibacterial, antioxidant and anticancer properties [20]. The antimicrobial properties of these plants come from the phytochemicals found in these plant tissues [21,13,22].

The present study aims to isolate and identify the endophytic microflora of *P. edulis*, *P. campechiana* and *M. annua* plant leaves by Matrix Assisted Laser Desorption- Ionization time of Flight-Mass Spectrometry (MALDI- TOF-MS) analysis and to evaluate antimicrobial activity of their leaf extracts against pathogenic bacterial and Candida strains. An attempt is made to analyze the antimicrobial efficacy of the isolated endophytes against various microbes.

**2. METHODOLGY:**

The present study was conducted in the Microbiology Research Centre Laboratory of a tertiary care Centre in Coastal Karnataka after obtaining ethical clearance (Reg. No. FMIEC/CCM/081/2023).

**2.1 Isolation of endophytic fungi:**

The isolation of endophytic fungi from P. edulis, P. campechiana and M. annua were carried out as described by Ezra D et al [23]. Pre-sterilized bags were used to collect the plants healthy leaves. Leaf samples were subjected to surface sterilization, initially with running tap water for 10 minutes, then with 1% Sodium hypochlorite followed by 70% ethanol for 1 minute and washed with sterile distilled water for 3 minutes [24]. The leaves were dried between sterile filter paper folds under aseptic conditions. After the surface sterilization leaf segments were cut aseptically into small pieces (1cm x 1cm) and placed in Petri dishes containing Potato Dextrose Agar (PDA). All the Petri dishes were incubated at room temperature for 3-7 days under dark conditions. The endophytic fungi that were isolated have been characterized and identified based on morphological and microscopic features of the spores and hyphae. For microscopic identification, colonies were teased and stained using Lactophenol cotton blue (LPCB). Doubtful colonies were identified using MALDI- TOF analysis (Bruker Daltonik, Germany).

**2.2 Analysis of Results:**

The Colonization frequency percentage (CF%) of the endophytic fungi was calculated as the number of segments colonized by an endophyte, divided by the total number of segments analyzed and expressed as percentage [25].

**Number of segments colonized by an endophyte**

**CF %= ---------------------------------------------------------------------- X 100**

**Total number of segments analyzed**

**2.3 Test organisms:**

Stockcultures of pathogenic bacteria and fungi such as *Escherichia coli*, *Klebsiella* spp, *Pseudomonas* spp, *Acinetobacter* spp, *Staphylococcus aureus* and *Candida* spp were collected from the Research Laboratory of our Institution.

**2.4 Preparation of leaf extract:**

Approximately 100g of the healthy leaves of *P. edulis*, *P. campechiana* and *M. annua* were rinsed with both running tap water and distilled water. The leaves were then dried in Hot air oven until the texture became crisp, these were ground into powder using blender. The powdered leaves were mixed with 200ml of 70% ethyl alcohol in a flask and left at room temperature for 48 hours. After filtering the mixture, under strict aseptic conditions, the filtrate was collected in a sterile beaker. The solvent was evaporated on the rotary evaporator. After complete evaporation, the extracts were dissolved in 10% Dimethyl sulfoxide (DMSO). The resulting extract was utilized to demonstrate antimicrobial efficacy [26,27].

**2.5 Screening of Antimicrobial assay of leaf extract:**

The antimicrobial activity of the leaf extract was evaluated by 2 methods: the disc diffusion method and the agar well diffusion technique. A sterile filter paper disc (Whatman No.1, 6mm diameter) was soaked in the leaf extract and placed on Muller Hinton Agar which was pre-inoculated with various pathogens. The plates were incubated at 37⁰C for 24 hours under aerobic conditions. Amoxiclav (AMC), Cefotaxime (CTX) were used as a positive control against bacterial pathogens and Caspofungin (CAS) for candida strains respectively.

**3. RESULTS:**

A total of 40 Endophytic fungi were isolated from 54 leaf segments of *P. edulis*, *P. campechiana* and *M. annua*. The endophytic fungi isolated in our study belonged to the class Eurotiomycetes, Sordariomycetes, Saccharomycetes and Zygomycetes. The isolates were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Talaromyces* spp, *Fusarium solani*, *Candida albicans*, *Mucor* spp and *Rhizopus* spp. The most commonly isolated species were belonging to the class Eurotiomycetes. The identification of fungal strains was based on macroscopic and microscopic observations of their cultural characteristics as well as Maldi-Tof Analysis **(Fig. 2)**.



**Figure:2** Morphology of the Endophytic Fungi isolated from *Passiflora edulis*, *Pouteria campechiana* and *Mercurialis annua*. A: *Aspergillus niger*, B: *Aspergillus flavus*, C: *Aspergillus fumigatus*, D: *Talaromyces* spp, E: *Fusarium solani*, F: *Candida albicans*, G: *Mucor* spp, H: *Rhizopus* spp

The overall colonization rate of endophyte in all the 3 leaf segments was found to be 74.07%. *M*. *annua* leaf segments showed a high Colonization frequency rate (88.9%) followed by *P*. *edulis* 77.8% and *P*. *campechiana* 55.6% (**Table 1**).

**Table 1.** Name and Colonization Frequency of Endophytic fungi isolated from leaf sample

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Class** | **Fungal Endophyte** | **Passiflora edulis** | **Pouteria campechiana** | **Mercurialis annua** |
|  |  | (18 segments) | (18 segments) | (18 segments) |
| Eurotiomycetes | Aspergillus niger | 4 | 2 | 6 |
| Aspergillus flavus | 0 | 1 | 2 |
| Aspergillus fumigatus | 3 | 1 | 2 |
| Talaromyces spp | 4 | 1 | 2 |
| Sordariomycetes | Fusarium solani | 2 | 3 | 0 |
| Saccharomycetes | Candida albicans | 1 | 2 | 1 |
| Zygomycetes | Mucor spp | 0 | 0 | 2 |
| Rhizopus spp | 0 | 0 | 1 |
|  | **Total** | **14** | **10** | **16** |
| **Frequency of colonization (CF%)** | | **77.8** | **55.6** | **88.9** |

The antimicrobial activity of ethanolic extracts from the leaves of three plants, was evaluated against bacterial and Candida strains using Disc diffusion and Agar well diffusion Techniques. The ethanolic leaf extracts of *P. edulis*, *P. campechiana* and *M. annua* exhibited considerable effectiveness in the agar well diffusion method compared to the disc diffusion method. *P. campechiana* leaf extracts exhibited the highest significant antimicrobial activity against all the tested organisms. However, *P. edulis* leaf extract showed no antimicrobial activity against any of the tested pathogens. Details of antimicrobial efficacy of the herbal leaf extract is given in **Table 2**.

**Table 2.** Antimicrobial efficacy of 3 leaf extracts against bacterial and fungal pathogens

|  |  |  |  |
| --- | --- | --- | --- |
| **Tested organisms** | **Disc diffusion method** | **Agar-well diffusion method** | |
| *Pouteria campechiana* | *Pouteria campechiana* | *Mercurialis annua* |
| **Gram Negative bacteria** | Mean zone of inhibition (mm) ± Standard deviation | | |
| *Escherichia coli* (10) | 11.5±2.07 | 18.7±1.16 | - |
| *Klebsiella pneumoniae* (10) | 16.1±2.85 | 23.4±4.65 | - |
| *Pseudomonas* species (10) | 10.4±1.58 | 19.1±1.66 | - |
| *Acinetobacter* species (10) | 10.2±0.92 | 16.2±1.55 | - |
| **Gram Positive bacteria** | Mean zone of inhibition (mm) ± Standard deviation | | |
| *Staphylococcus aureus* (10) | 10.9±1.97 | 18.7±3.77 | 9.8±8.5 |
| **Yeast/ Fungi** |  | | |
| *Candida* species (10) | - | 15.1±3.9 | - |

**DISCUSSION:**

Endophytic fungi represent one of the most diverse group of organisms, engaging in symbiotic relationships with higher life forms and yielding beneficial substances for their hosts [28]. Endophytic fungi grow in plant tissues, especially in the leaves and stems [1]. Variations in the occurrence of endophytic fungi and colonization frequency are influenced by environmental factors such as temperature, rainfall, atmospheric humidity and their impact on the host plant [16].

Our study investigated the diversity of endophytic fungi in three plant species, namely *P. edulis*, *P. campechiana* and *M. annua*, as well as the antimicrobial activity of ethanolic leaf extracts from these plants against various bacterial and Candida pathogens. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Talaromyces* spp, *Fusarium solani*, *Candida* *albicans*, *Mucor* spp and *Rhizopus* spp were the six different fungal species that have been identified. This highlights the predominance of Class Eurotiomycetes particularly Aspergillus spp. and Fusarium solani, within the endophytic fungal communities of the studied plant species. They indicate a strong ecological relationship between these fungi and their host plants. Several species have the ability to impact the health, growth and defense mechanisms of their host plants through their distinct contributions to the ecological dynamics within the plants [28]. The findings of Kharwar et al. (2010), who reported that *Aspergillus* spp, *Fusarium* and *Talaromyces* are commonly isolated endophytic species from medicinal plants, are consistent with our findings [29].

The overall colonization rate of endophytic fungi across all three plant species was found to be 74.07%. Notably, *P*. edulis (77.8%) and *P*. *campechiana* (55.6%) showed the lowest colonisation frequency rate, while *M*. *annua* had the highest frequency (88.9%). The results of the study have significant implications for pharmaceutical and ecological research. The high colonization rate of endophytic fungi (74.07%) across the three plant species indicates that these fungi are essential to the resilience and health of plants. Variations in plant physiology, leaf morphology, microenvironmental factors or inherent mechanisms against fungal colonization could be contributing factors to these variances in colonization rates.

Studies have shown that *P. edulis*, *P. campechiana* and *M. annua* have various biological activities, including antioxidant, anti-inflammatory, hypoglycemic, antibacterial and antifungal properties. These plants are significant sources of many biologically active compounds. For a long time, plants have served as sources of natural products for maintaining human health, especially in the past decade, with more intensive research into natural therapies. Phytochemical components of these plants, such as flavonoids and triterpenes, are primarily responsible for their antibacterial properties [30-32]. Using the disc diffusion and agar well diffusion methods, our study also evaluated the antibacterial efficacy of ethanolic leaf extracts from the three plant species against bacterial and Candida strains. The leaf extracts showed significant antibacterial activity with the agar well diffusion method compared to the disc diffusion method. The difference in efficacy between the two methods may be due to differences in the volume of extracts added in both techniques. This study also implies that *P. campechiana* leaves would be an excellent candidate for developing natural antimicrobial agents as new therapeutic options.

**CONCLUSION:**

This study investigates the diversity of endophytic fungal communities inhabiting *P. edulis*, *P. campechiana* and *M. annua*, as well as the antimicrobial properties of their ethanolic leaf extracts. These findings highlight the potential of endophytic fungi as a source of bioactive compounds and the effectiveness of plant extracts in antimicrobial applications, which could be further explored for developing natural antimicrobial agents for pharmaceutical applications.

**ETHICAL APPROVAL**

Ethical clearance was obtained from Institutional ethics committee of Father Muller Medical College and hospital, Kankanady, Mangalore. (Reg. No. FMIEC/CCM/755/2022).

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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