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EXPLORING THE ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF FUNGAL ENDOPHYTES IN BAEL AND CURRY LEAVES

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ABSTRACT

Endophytic fungi residing within plants have gained significant attention due to their potential for producing bioactive compounds and their influence on plant health. The present study is focused on the isolation, identification and biological potential of endophytic fungi from the leaves of *Murraya koenigii* and *Aegle marmelos.* Both tropical plants are known for its culinary and medicinal properties. Leaf samples were collected from healthy plants, and the endophytic fungi were isolated using standard techniques. Four endophytic fungal isolates were obtained from the *Murraya koenigii* leaves and *Aegle marmelos* leaves each. The identified endophytic fungi from curry leaves belonged to various genera, including *Daldinia, Colletotrichum, Hypoxylon, and Xylaria*. *Alternaria. Aspergillus and Fusarium* species were isolated and identified from Bael leaves. The fungal extracts were the evaluated for antimicrobial properties against a variety of bacteria and fungi. The extracts were also assessed for their radical scavenging capacity which contributes directly to its antioxidant activity using the DPPH method. This study has shed valuable light on the potential of endophytic fungi as antimicrobial and antioxidants.

KEYWORDS: Endophytic fungi, *Murraya koenigii, Aegle marmelos.*

INTRODUCTION

The intricate relationship between plants and the microorganisms residing within them has been a subject of growing fascination for researchers across diverse scientific disciplines. Among these microorganisms, fungal endophytes, which inhabit the internal tissues of plants

without causing apparent harm to their hosts, have gained prominence due to their multifaceted roles in plant health and potential for producing biologically active compounds.

Endophytic fungi (EF), as defined by Petrini and Fisher in 1990,[7] are communities of microorganisms that thrive within plant tissues without causing any observable infection or disease symptoms in the host plant. These endophytes are recognized for their ability to produce a diverse range of structurally unique bioactive natural compounds, resembling the secondary metabolites found in plants. These compounds encompass alkaloids, flavonoids, phenolic acids, quinones, steroids, terpenoids, xanthones, and various others, as noted by Tan and Zou in 2001,[10] Notably, endophytic fungi predominantly reside in above-ground plant structures, including leaves, stems, bark, petioles, and reproductive parts, setting them apart from the more extensively studied mycorrhizal symbionts, as highlighted by Faeth and Fagan in 2002.[4]

Bael (*Aegle marmelos*) and curry leaves (*Murraya koenigii*), two widely cherished and medicinally significant plants in South Asia, are no exception to hosting a rich diversity of fungal endophytes.

Murraya koenigii L. Spreng., popularly known as curry leaf tree is a tropical plant revered for its fragrant leaves employed in culinary practices. Alkaloids, flavonoids, and essential oils are among the many bioactive substances that *Murraya koenigii* leaves are abundant in, and these substances also give the leaves their flavour and medicinal potential. Among other things, these substances have been said to possess antioxidant, anti-inflammatory, antibacterial, and hepatoprotective activities. (Iyer D and Devi U, 2008).[4]

Bael (*Aegle marmelos* L. Correa) is another member of *Rutaceae* which has been extensively used in traditional medicine in South Asia. The leaves of this plant are aromatic and are a rich source of various phytochemicals. The leaves are rich in alkaloids, phenylpropanoids, terpenoids, and various other compounds. These leaves are known for their potential pharmacological effects, which encompass hypoglycaemic, anti-inflammatory, antimicrobial, anticancer, radioprotective, chemo-preventive, and antioxidative properties. (Yadav, N. P., & Chanotia, C. S. (2009)).[11]

Diverse population of fungal endophytes have been isolated from Bael and curry leaves which have led to exciting leads in the search for antimicrobials and antioxidants. These

endophytes can be used to produce antimicrobials against a plethora of pathogens and phytopathogens. Antimicrobials are a class of compounds or medications that inhibit the growth or kill microorganisms, including bacteria, viruses, fungi, and parasites, making them essential in the treatment of infectious diseases.

Antioxidants are molecules that help protect cells from oxidative damage by neutralizing harmful free radicals, potentially reducing the risk of various chronic diseases and contributing to overall health.

For the present study, the leaves of the above-mentioned plants were screened for fungal endophytes. After isolation of the fungal organisms, and identification of the isolates, further screening of these fungal isolates was carried out to assay their antimicrobial and antioxidant potential.

MATERIALS AND METHODS

Collection of plant material and authentication

Healthy leaves of *Murraya koengii* (L.) Spreng and *Aegle marmelo*s (L.) Correa plants were collected from local gardens of Mumbai, India. These plant samples were authenticated by Blatter Herbarium, St. Xaviers College, Mumbai and were found to match the herbarium sample of R. R. Fernandes, 3594 and Bole, 21 respectively.

Isolation of endophytic fungi

Fresh leaves, collected and processed within a 5-6 hour timeframe, were utilized for the extraction of endophytic fungi (EF). The leaves' surfaces were sterilized by treating them with a solution containing 4% free chlorine (Na-hypochlorite) and 70% ethanol, as per Petrini et al. in 1993, followed by multiple rinses with distilled water. Afterward, the leaves were dried with sterile tissue paper and then cut into small 5mm x 5mm pieces. These pieces were then positioned onto sterile potato dextrose agar (PDA) plates containing streptomycin (200µg/mL), following Strobel's method from 2003.[9] They were then incubated at 37℃ until mycelium growth was observed, which typically took around 10-15 days. Control plates were also incubated following the same surface sterilization protocol. To ensure the absence of surface contaminants that might appear after incubation, leaf imprints (both dorsal and ventral) were taken on Petri plates amended with streptomycin.

The identification of the isolated endophytic fungi was performed at NFCCI, Agharkar Research Institute in Pune.

Crude extraction of fungal metabolites

The pure fungal cultures, once isolated and identified, were inoculated into 100 ml of potato dextrose broth (PDB) and placed on a rotary shaker for a duration of one week. When sufficient visible mycelial growth became apparent in the flasks, the fungal mycelia were separated from the broth by passing it through muslin cloth. The resulting filtrate was quantified, and an equal volume of ethyl acetate was added to the filtrate. These mixtures were then subjected to two separations using a separating funnel, with the organic layer being collected. Simultaneously, the fungal mycelia were immersed in 100 ml of methanol for a week, after which they were macerated in methanol and filtered prior to collection. The two resulting filtrates were combined and utilized as the fungal extract, following the method outlined by Devi and Prabakaran in 2014.[3]

Antimicrobial Assay

The fungal extracts were air-dried at room temperature to eliminate any remnants of solvents. Subsequently, the dried extracts were reconstituted with sterile distilled water. To assess the minimum inhibitory concentration of these fungal extracts, experiments were conducted against a range of microorganisms, including Gram-positive bacteria (*S. aureus* MCMB-811, *Bacillus cereus* NCIM-2156), Gram-negative bacteria (*E. coli* MCMB-834, *Pseudomonas aeruginosa* MCMB-816), and fungi (*Candida albicans* NFCCI-5123, *Candida tropicalis* NFCCI-5327). These cultures were obtained from the Microbial Culture Collection and NFCCI at ARI in Pune.

The cultures were inoculated on nutrient agar slants and allowed to grow overnight at 37°C. Saline suspensions of the cultures were set to a standard of 0.5 McFarland.

The procedure involved preparing different concentrations of the fungal extracts, spanning from 0 µl to 50 µl, in a liquid growth medium like Mueller-Hinton Broth. Microdilution of these extracts with sterile distilled water was carried out in 96-well microtiter plates. For assessing microbial growth, a 0.02% Resazurin (w/v) solution was utilized as an indicator, following the methods proposed by Balouiri et al. 2016.[2]

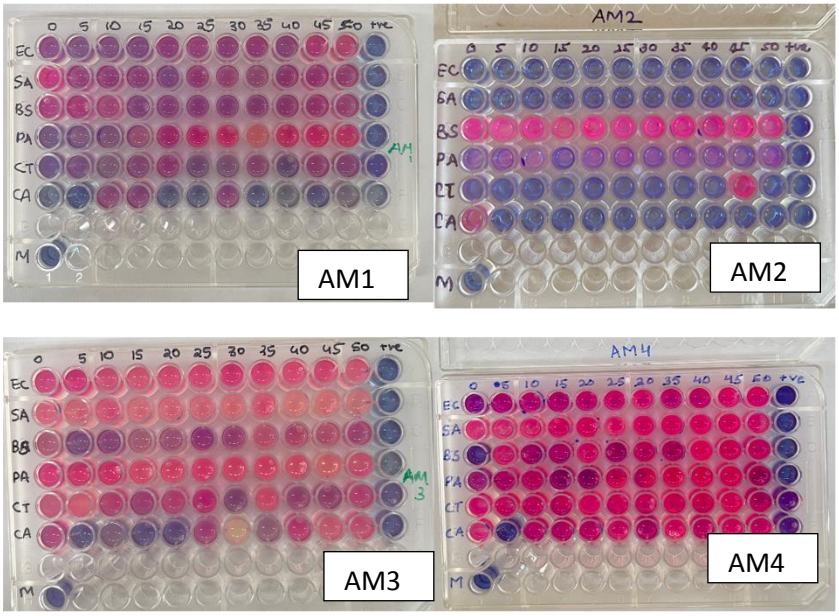
Antioxidant assay using DPPH

The fungal extracts' antioxidant potential was assessed through the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following the method outlined in Athavale et al. (2012)[1] with minor adjustments. The test samples were air-dried to eliminate excess solvent and concentration used for analysis was 20%. The extracts were subsequently mixed with methanol in varying dilutions, along with a fixed quantity of DPPH. These reaction mixtures were then left to incubate in darkness at room temperature for 60 minutes. The evaluation of antioxidant activity was based on the change in colour from deep violet to pale yellow, and it was also quantified using a spectrophotometer at 517 nm. Ascorbic acid, dissolved in methanol, served as the standard reference, while methanol was used as a blank. The calculation of DPPH radical scavenging activity as an antioxidant was carried out using the following formula

% radical scavenging activity = (Absorbance of control - Absorbance of test sample) x 100 / Absorbance of the control

OBSERVATIONS

Antimicrobial assay



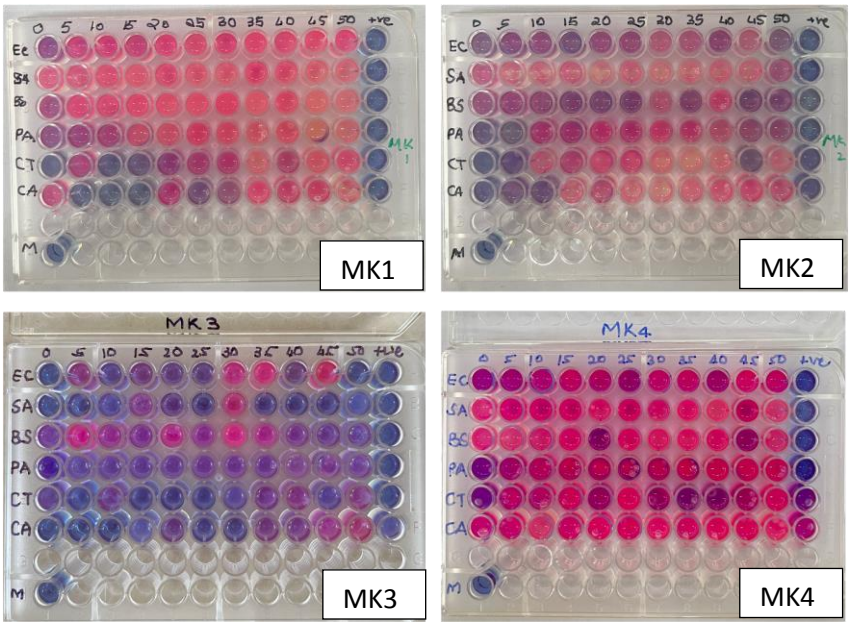


Figure 1: MIC plates showing antimicrobial activity of fungal extracts.

CODE	*S. aureus*	*E. coli*	*B. cereus*	*P. aeruginosa*	*C. albicans*	*C. tropicalis*
AM1	I	I	I	I	S	S
AM2	S	S	S	R	S	S
AM3	R	R	R	R	R	R
AM4	R	R	R	R	R	R
MK1	R	R	R	R	R	R
MK2	R	R	I	R	R	R
MK3	S	S	I	S	S	S
MK4	R	R	R	R	R	R
S= SENSITIVE		I=INTERMEDIATE		R= RESISTANT		

Antioxidant assay

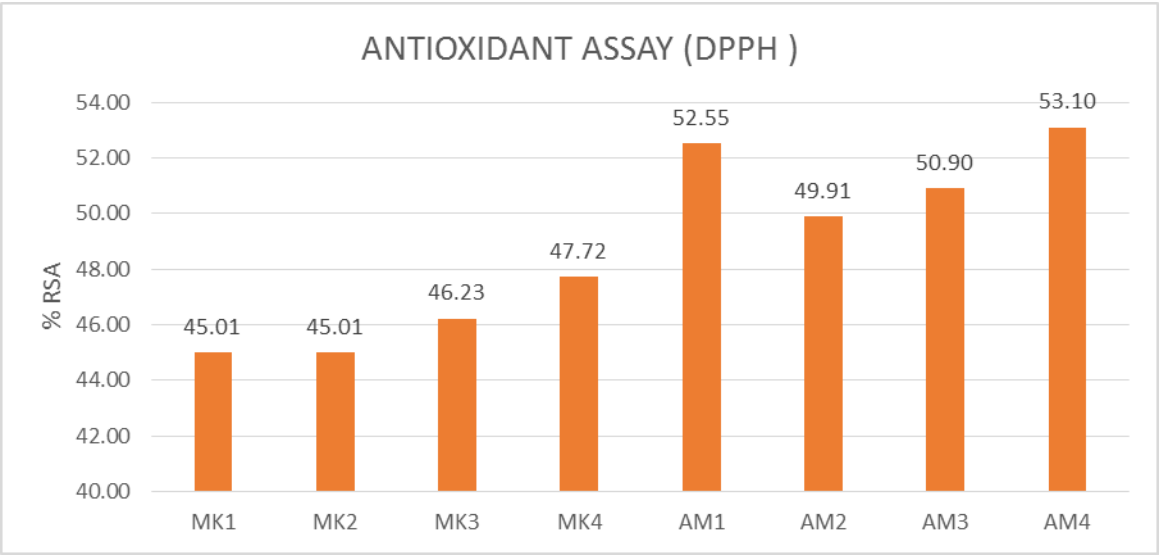


Figure 2: Antioxidant activity of fungal extracts using DPPH.

RESULTS AND DISCUSSION

Four endophytic fungi were isolated and identified from *Murraya koenigii* and *Aegle marmelos* each. These fungal endophytes were initially named as MK1, MK2, MK3, MK4 and AM1, AM2, AM3, AM4 respectively. The cultures were identified using ITS-sequencing and nucleotide BLAST.

The fungal strains of curry leaves were *Daldinia eschscholtzii* (Ehrenb.) Rehm (MK1), *Colletotrichum aenigma* B.S. Weir & P.R. Johnst (MK2), *Hypoxylon haematostroma* Mont. (MK3) and *Xylaria feejeensis* (MK4). The four endophytes isolated from bael were identified to be *Alternaria alternata* (AM1), *Aspergillus niger* (AM2), *Fusarium equiseti* (AM3), *Fusarium incarnatum* (AM4). The sequences were submitted in gene bank and their accession numbers were obtained.

The antimicrobial assay of these fungal extracts was carried using broth- dilution method. While *Fusarium equiseti* (AM3) and *Fusarium incarnatum* (AM4) did not show any antimicrobial activity at all, whereas *Alternaria alternata* (AM1) was found to be anti-fungal in nature as it could inhibit growth of *Candida species*. AM2 was found to inhibit the growth of *E. coli*, *S. aureus*, and *Candida species*. The antimicrobial activity of fungal extracts MK1, MK2 and MK4 of *Murraya* was found to be ineffective against the test cultures but MK3 showed most promising results as it could inhibit the growth of most of the test cultures.

The antioxidant assay was carried out using DPPH method to check for radical scavenging activity. It was observed that AM4 had maximum %RSA activity at 53.10% which was closely followed by AM1 and AM3 at 52.55% and 50.90% respectively. MK4 had maximum %RSA when compared to other isolates from the same host. The antioxidant capacity of these fungal extracts can provide greater immunity to host in case of biotic and abiotic stressors. These antioxidants can be further analyzed to isolate the specific groups responsible for this activity.

DISCUSSION

Endophytic fungi are diverse yet unexplored group of fungi which are troves of hidden treasures. These fungi are known to produce bioactive compounds which gained significance with the discovery of *Taxomyces andreanae* which was isolated from the Pacific yew tree. This endophytic fungus was found to produce the anti-cancer compound Taxol in higher quantities than its host. (Stierle, A., et al. 1995).[8]

Medicinal plants have been use for their antimicrobial and antioxidant properties throughout the ages in all parts of the world. So, the myco-endophytes of these plants have also adapted to a symbiotic relationship with the host to produce biosimilar compounds as the medicinal plant. *Aegle marmelos* is a revered and sacred plant all over India and has been utilised for its medicinal properties. *Murraya koenigii*, another member of the same family *Rutaceae* as *A. marmelos* has also been documented for its use in culinary and medicinal properties. These plants are commonly found and easily identifiable. These plants have been documented to harbour a myriad of endophytic fungi which have been isolated and characterised for various properties.

While AM1, AM2 and AM4 have been reported earlier by Gond et al (2007)[5] from Bael whereas AM3 was reported for the first time from the same host plant. *Murraya koenigii* was found to foster endophytic fungi which have not yet been reported from the same host. This variation of fungi could be because of the climatic conditions and geographical locations of the host habitat.

But the activity observed of the fungal extracts for antimicrobial as well as antioxidant have been promising.

CONCLUSION

This study on the antioxidant and antimicrobial potential of fungal endophytes in Bael and Curry leaves has unveiled encouraging insights into the bioactive compounds present in these plants. The results suggest that these endophytes play a crucial role in enhancing the medicinal properties of Bael and Curry leaves, contributing to their overall phytochemistry.

The antimicrobial activity observed suggests that these endophytes may serve as natural agents for controlling microbial infections, opening paths for the development of novel pharmaceutical and agricultural products. The antioxidant properties of the fungal endophytes indicate their potential in radical scavenging activity and preventing cellular damage which holds a potential in the field of therapeutics.

This work throws emphasis not only on the biological relevance of fungal endophytes in medicinal plants, but also on the importance of biodiversity for discovering novel sources of bioactive compounds. Further studies will aid in clarifying the exact processes behind these

bioactivities, establishing the way for the development of novel and sustainable solutions in health and agriculture.

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