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Isolation of Endophytes with Antimicrobial Activity from Selected Indigenous Medicinal Plants against Amoxicillin-Resistant Environmental Bacteria

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Abstract

The continuous discovery of novel antimicrobial compounds and antibiotics-producing microorganisms is an obligatory process to overcome the antibiotic resistance of pathogenic bacteria. Antibiotic resistance arises as a consequence of the misuse of antibiotics. Thus, the main objective of this study is based on the isolation of endophytic bacteria and fungi with antimicrobial activity against amoxicillin-resistant environmental bacteria. The endophytes were isolated from the selected indigenous medicinal plants, namely *Acalypha indica* (Kuppameniya) and *Cyanthillium cinereum* (Monarakudumbiya). Fresh samples of the selected plants were collected from their natural habitats, and the plant parts were surface-sterilised using 70% ethanol. The endophytic bacteria and fungi were isolated on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) media respectively at room temperature. The isolated endophytic bacteria and fungi were screened for their antimicrobial activity against previously isolated amoxicillin-resistant (AR) environmental bacteria, namely *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterobacter ludwigii* and *Enterobacter pyrinus*, on Mueller-Hinton Agar (MHA) media. As per the results, a prominent inhibition of the AR bacteria *Staphylococcus aureus* was exhibited by four endophytic isolates (KpR-02B, MnS-01B, KpS-01F and MnR-04F). The AR bacteria *Enterobacter ludwigii* was prominently inhibited by two endophytic isolates (MnS-01B and MnR-04F), while *Enterobacter pyrinus* was also observed to be prominently inhibited by two endophytic isolates (MnF-03B and MnR-04F). Therefore, the indigenous medicinal plants, *Acalypha indica* (Kuppameniya) and *Cyanthillium cinereum* (Monarakudumbiya), can be considered as a potential source of antimicrobial compound-producing endophytes against amoxicillin-resistant environmental bacteria, which can be utilised to develop a novel antibacterial drug against antibiotic-resistant pathogenic bacteria.

Keywords: Antimicrobial compounds, Medicinal plants, Endophytes, Antibiotic-resistance

1. Introduction

Endophytes are microorganisms that colonise the endospheric domain of the plant microbiome. Endophytes are present in the intracellular regions of the roots, stems, leaves, and flowers of plant tissue.[1] Endophytes have a mutualistic relationship with the host plant.[2] Endophytes can be either bacteria, actinomycetes, or fungi. Most endophytic bacteria have been found to belong to the phyla Proteobacteria, Actinobacteria, and Firmicutes, while most endophytic fungi belong to the phyla Ascomycota and Basidiomycota.[3,4]

Endophytes are essential in maintaining the quality of the soil as they can solubilize nutrients without disturbing the microbial community of the soil, remove pollutants from the soil, and are also involved in soil mineral cycling.[5] Endophytes also produce metabolic compounds which benefit the survival of the host plant.[6] Endophytes promote plant growth by increasing the uptake

of nutrients (nitrogen, phosphorus, and iron), producing plant growth promoters (PGP) such as auxins, ethylene and gibberellins.[7]

Endophytes help to defend the host plant by providing resistance against biotic and abiotic stresses of the environment.[8] They protect the host plant from pathogenic microorganisms by synthesising various bioactive compounds, such as alkaloids, peptides, phenols, terpenoids and aliphatic compounds.[9] The bioactive secondary metabolites produced by endophytic bacteria include substances that possess antibiotic, antifungal, antiviral, anticancer, antiarthritic and antidiabetic properties.[10] Therefore, these secondary metabolites can be utilised to develop novel drugs for therapeutic purposes.

Antibiotic resistance has posed a significant threat globally in recent years.[11] Antimicrobial resistance is the ability that microorganisms such as bacteria and fungi develop to overcome the mechanisms of antibiotics designed to kill or inhibit their growth.[12] Some of the most common antibiotic-resistant (AR) bacterial strains include MRSA (Methicillin-resistant *Staphylococcus aureus*) and VRE (Vancomycin-resistant *Enterococcus faecalis*).[13] The misuse and overuse of antibiotics have greatly contributed to the development of AR bacteria, leading to challenging consequences in the agricultural, medical and pharmaceutical sectors.[14]

Amoxicillin is a broad-spectrum antibiotic that is commonly used to treat bacterial infections in both humans and animals.[15] Amoxicillin is rapidly degraded in the water by biotic and abiotic environmental stress factors, resulting in different intermediate compounds which are more toxic and resistant to degradation.[16] Amoxicillin cannot be completely removed from the environment as it is hydrophobic and lipophilic.[17] Over time, the bacterial strains present in the environment develop antibiotic resistance genes (ARGs) and can eventually lead to disease outbreaks.[18] Therefore, antibiotic contamination in wastewater has significantly increased the risk of environmental bacteria developing resistance to antibiotics.[19]

Based on previous studies conducted, medicinal plants and crops have been targeted as the most potential source to screen for novel secondary metabolites produced by endophytic microbes.[20,21] The antimicrobial compounds produced by endophytes are eco-friendly and non-toxic to humans.[22] Hence, there is an immense potential for having an endophytic microbial flora which produces important secondary metabolites associated with these medicinal plants.

Several antimicrobial compounds are produced by endophytic bacteria. Ecomycins and Pseudomycins have antifungal properties, while Munumbicins, Kakadumycin, and Xiamycins have effective antibiotic activities against several bacterial strains such as MRSA, VRE, and MDR-TB (multidrug-resistant tuberculosis).[23] The continuous discovery of novel antimicrobial compounds against AR bacteria has attracted immense interest among scientists. Therefore, the natural bioactive compounds produced by endophytes, which exhibit antimicrobial activity against amoxicillin-resistant environmental bacteria, can be beneficial in developing novel drugs to combat the issue of antibiotic resistance.

Acalypha indica, commonly known as Kuppameniya, is a plant traditionally known to have medicinal properties which have made it useful in treating rheumatoid arthritis and respiratory problems, and also help in wound healing.[24] *Acalypha indica* was found to possess various secondary metabolites, such as flavonoids, saponins, alkaloids, catechols and phenolic compounds, with antimicrobial, antioxidant, anti-inflammatory, anti-diabetic and anti-cancer properties.[25] Therefore, the Kuppameniya plant is proven to be a valuable medicinal plant to screen for secondary metabolites with pharmacological benefits.

Cyanthillium cinereum, which is known as Monarakudumbiya, is a plant traditionally used to treat arthritis, rheumatism, and conjunctivitis, and also helps in wound healing.[26] *Cyanthillium cinereum* is known to

possess therapeutic properties against various maladies such as asthma, cough, diarrhoea, cholera, malaria, cancer, and night blindness.[27] According to previous studies that were conducted, this plant is known to possess bioactive compounds such as alkaloids, flavonoids, sterols, esters and terpenoids.[28,29] Hence, Monarakudumbiya is a plant of significant medicinal importance with possible therapeutic potential against AR microorganisms.

Thus, the main objective of this study is to isolate endophytic bacteria and fungi from selected indigenous medicinal plants, *Acalypha indica* and *Cyanthillium cinereum*, with potential antimicrobial activity against previously isolated amoxicillin-resistant environmental bacteria.

2. Methodology

2.1 Collection of samples. Whole plants (containing roots, stems, leaves and flowers) of *Acalypha indica* and *Cyanthillium cinereum* were collected from their natural habitats (home gardens and roadsides). The plant samples were placed in clear plastic zip-lock bags and were transported to the laboratory within 24 hours of sampling under refrigerated conditions.

2.2 Enrichment of plant endophytes. About 1g of each plant part (roots, stems, flowers and leaves) of both plants were separately surface sterilised using distilled water and 70% ethanol. Each of the disinfected plant parts was separately ground aseptically in 10ml of 0.9% NaCl solution using a sterile mortar and pestle. About 5ml of each crude extract was transferred aseptically into quarter-diluted nutrient broth (NB) media flasks. The flasks were incubated at room temperature for about 24 to 48 hours on the shaking incubator at 120 rotations per minute (rpm) speed.

2.3 Preparation of serial dilutions. After incubation, each of the enriched NB cultures was serially diluted up to 10^{-6} dilution by adding sterile 0.9% NaCl solution.

2.4 Isolation of endophytic bacteria.

An aliquot of 100µl of the serially diluted cultured broth of each sample was transferred onto separate nutrient agar (NA) media plates and inoculated evenly via spread-plate technique. Following incubation at room temperature for about 24 to 48 hours, the morphologically distinct bacterial colonies were identified and isolated by further inoculation on NA plates via quadrant-streak plate technique to obtain pure cultures.

2.5 Isolation of endophytic fungi. Surface-sterilised plant parts were aseptically cut into small pieces of approximately the same size and separately placed on potato dextrose agar (PDA) media plates. Following incubation at room temperature for about 72 hours, the morphologically distinct fungal colonies were identified and isolated by further inoculation on new PDA plates to obtain pure cultures.

2.6 Preparation of AR environmental bacterial cultures. The previously isolated AR environmental bacteria, namely *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterobacter ludwigii* and *Enterobacter pyrinus*, from the stock cultures were enriched by aseptically mixing a loopful of the bacterial inoculum into separate NB media flasks. The flasks were incubated at room temperature for about 24 to 48 hours on the shaking incubator at 120rpm speed. After incubation, a loopful of each NB culture was inoculated on new NA plates via the quadrant-streak plate technique. The plates were then incubated at room temperature for about 24 to 48 hours.

2.7 Antimicrobial assay for endophytic bacteria. Equalized AR environmental bacterial solutions were prepared for the absorbance value of 0.35 at 395nm wavelength. The equalized solutions of AR environmental bacteria were evenly spread on Mueller-Hinton agar (MHA) plates using sterile cotton swabs to prepare the bacterial lawns. Then each endophytic bacterial isolate was separately inoculated at the centre of AR environmental bacterial lawns. Following incubation at room

temperature for about 24 to 48 hours, the plates were observed for clear zones around each endophytic bacterial colony and the results were recorded. Likewise, the antimicrobial assay was separately performed for each AR environmental bacteria.

2.8 Antimicrobial assay for endophytic fungi. Equalized AR environmental bacterial solutions were prepared for the absorbance value of 0.35 at 395nm wavelength. The equalized solutions of AR environmental bacteria were evenly spread on MHA plates using sterile cotton swabs to prepare the bacterial lawns. Then each endophytic fungal isolate was separately inoculated at the centre of AR environmental bacterial lawns. Following incubation at room temperature for about 72 hours, the plates were observed for clear zones around each endophytic fungal colony and the results were recorded. Likewise, the antimicrobial assay was performed for each AR environmental bacteria.

3. Results

3.1 Isolation of endophytic bacteria.

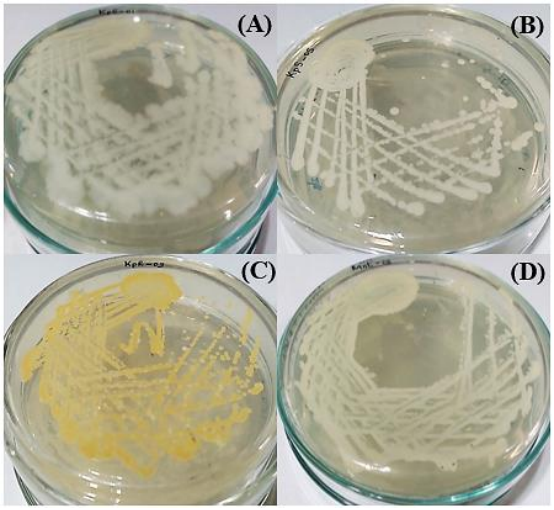

Figure 1. Pure cultures of endophytic bacteria. (A) KpS-01B isolated from the stems of *Acalypha indica* plant. (B) KpS-05B isolated from the stems of *Acalypha indica* plant. (C) KpR-03B isolated from the roots of *Acalypha indica* plant. (D) MnL-05B isolated from the leaves of *Cyanthillium cinereum* plant.

Growth of bacterial colonies was observed after incubation for 24 to 48 hours at room temperature. The bacterial colonies appeared to be white, yellow or cream coloured, with smooth shiny surfaces. Endophytic bacteria with different morphological characteristics were observed, with entire, undulate or lobate margin, circular or irregular form, and raised, flat or convex elevation (Figure 1).

3.2 Isolation of endophytic fungi.

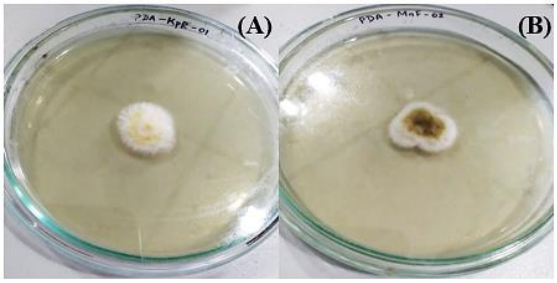

Figure 2. Pure cultures of endophytic fungi. (A) KpR-01F isolated from the roots of *Acalypha indica* plant. (B) MnF-02F isolated from the flowers of *Cyanthillium cinereum* plant.

Growth of fungal colonies was observed after incubation for about 72 hours at room temperature. The fungal colonies were observed to be of different colours, including white, grey, yellow, pink, green, blue, black and purple. Endophytic fungi with different morphological characteristics were observed, with woolly, powdery, cottony or granular texture (Figure 2).

3.3 Antimicrobial assay for endophytic bacteria.

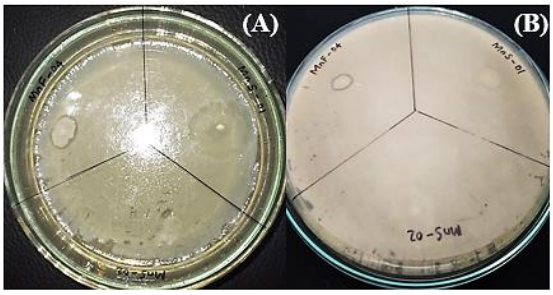

Figure 3. Antimicrobial activity of endophytic bacterial isolates. (A) MnS-01B isolated from

the stems of *Cyanthillium cinereum* plant against *Enterobacter ludwigii*. (B) MnF-04B isolated from the flowers of *Cyanthillium cinereum* plant against *Enterobacter pyrinus*.

Endophytic bacterial isolates, namely KpR-02B, MnS-01B and MnF-03B, showed prominent inhibition against the tested AR environmental bacteria after incubation for 24 to 48 hours at room temperature (Table 1).

3.4 Antimicrobial assay for endophytic fungi.

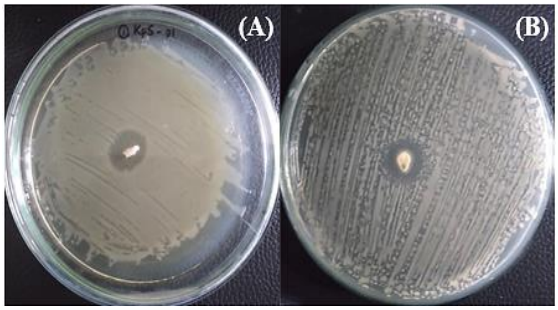

Figure 4. Antimicrobial activity of endophytic fungal isolates. A) KpS-01F isolated from the stems of *Acalypha indica* plant against *Staphylococcus aureus*. B) MnR-04F isolated from the roots of *Cyanthillium cinereum* plant against *Staphylococcus aureus*.

Endophytic fungal isolates, namely KpS-01F and MnR-04F, showed prominent inhibition against tested AR environmental bacteria after incubation for about 72 hours at room temperature (Table 1).

Table 1. Prominent antimicrobial activity.

AR environmental bacteria	Endophytes that exhibited inhibition
Staphylococcus aureus	KpR-02B MnS-01B KpS-01F MnR-04F
Enterobacter ludwigii	MnS-01B MnR-04F
Enterobacter pyrinus	MnF-03B MnR-04F

4. Discussion

Equalising the concentration of each bacterial solution to a specific absorbance of 0.35 before preparing the bacterial lawn ensured that fair results were obtained when screening the isolated endophytes for antimicrobial activity. However, other factors, such as the incubation period, the volume of bacterial solution spread on each MHA plate and the amount of each endophytic isolate inoculated on the AR environmental bacterial lawn, may also affect the results obtained by the antimicrobial assay.[30,31]

The presence of zones of clearance around the endophytic isolates indicated the inhibition of the growth of the AR environmental bacteria (Table 1). The growth of AR environmental bacteria was suppressed due to the production of antimicrobial compounds by the endophytic isolates.[32] Weak inhibitory zones may be due to small quantities of antimicrobial compounds produced by the endophytes.[33] Phytochemicals from the plant extracts exert inhibitory effects against the AR bacteria by various mechanisms of action, such as impairing the function of the bacterial cell membrane, inhibiting the synthesis of bacterial cell walls and disrupting the synthesis of nucleic acids.[34] Polyphenols are considered as the phytochemicals with the most potent antibacterial and antifungal activities.[35] The mechanisms of action by which AR bacteria can resist antibiotics are by producing enzymes which chemically modify, deactivate or destroy the antimicrobial compounds, by modifying the cellular target sites, and by reducing the intracellular accumulation of the antimicrobial compounds.[36,37]

Prominent inhibition by the endophytic isolates was not observed against the amoxicillin-resistant bacteria *Acinetobacter baumannii*, although some positive results were obtained. This implies that of the four different amoxicillin-resistant environmental bacteria which were screened, *Acinetobacter baumannii* exhibited the most resistance to the antimicrobial activity of the endophytic

bacterial and fungal isolates. *Acinetobacter baumannii* is known to possess various resistance mechanisms against antimicrobials, such as hydrolysis of β-lactamases, overexpression of efflux pumps, reduction of porin permeability, mutations in target genes and modification of antibiotic targets.[38,39]

The endophytic isolates displayed the highest number of prominent zones of clearance against the amoxicillin-resistant bacteria *Staphylococcus aureus*. This demonstrates that of the four different AR environmental bacteria which were analysed, *Staphylococcus aureus* exhibited the highest susceptibility to the antimicrobial activity of the endophytic bacterial and fungal isolates. Previous studies have also reported findings of endophytes displaying antagonist action against *Staphylococcus aureus* by inhibiting its protein expression and nucleic acid synthesis.[40,41,42]

Furthermore, two endophytic isolates (MnS-01B and MnR-04F) exhibited prominent antimicrobial activity against the AR bacteria *Enterobacter ludwigii*, and two endophytic isolates (MnF-03B and MnR-04F) also showed prominent inhibition of the AR bacteria *Enterobacter pyrinus*. Hence, the results of this study indicate that these endophytic isolates have the potential to inhibit the activity of AR environmental bacteria.

The previous studies conducted on endophytes isolated from *Acalypha indica* and *Cyanthillium cinereum* plants also reported several antimicrobial compound-producing bacteria and fungi against human pathogenic bacteria and antibiotic-resistant bacteria. The endophytic fungi *Trichoderma harzianum* isolated from *Acalypha indica* is known to have antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella*.[43,44] The endophytic fungi *Colletotrichum cobbittiense* isolated from *Cyanthillium cinereum* has been found to produce bioactive substances, such as saponins, terpenoids and flavonoids, which have effective antimicrobial activity.[45,46]

Based on this study, the endophytic bacterial isolate which exhibited the most prominent antimicrobial activity was MnS-01B, which was extracted from the stems of *Cyanthillium cinereum* plant, and the endophytic fungal isolate which exhibited the most significant antimicrobial activity was MnR-04F, which was extracted from the roots of *Cyanthillium cinereum* plant. This proved *Cyanthillium cinereum* to be an excellent source to isolate bioactive compounds with effective antimicrobial activity against specific AR environmental bacteria. According to findings from a previous study, the bioactive compounds in the crude extract of *Cyanthillium cinereum* were known to have significant antibacterial activity against AR bacteria such as *Escherichia coli* and *Staphylococcus aureus*.[26] Several phytochemical compounds such as phenols, saponins, flavonoids and tannins present in *Cyanthillium cinereum* were responsible for the effective antimicrobial activity.[47]

The endophytic isolates from *Acalypha indica* also provided prominent results, proving it to be a beneficial source for extracting novel antimicrobial compounds. However, the difference in results for *Acalypha indica* compared to *Cyanthillium cinereum* could have been due to experimental errors. Therefore, further evaluation of the antimicrobial activity of endophytes isolated from *Acalypha indica* is suggested. According to findings from previous studies, the phytochemicals in the crude extract of *Acalypha indica*, including alkaloids, glycosides and phenolic compounds, were found to exhibit prominent antibacterial activity against various AR bacteria, such as *Staphylococcus aureus* and *Salmonella typhi*, as well as against several other gram-positive bacteria.[48,49]

As future perspective, the microbial isolates which showed antimicrobial activity against AR environmental bacteria need to be identified using conventional and molecular biological methods. The biochemical characterisation of the endophytes can be carried out by performing biochemical tests such as catalase test, endospore staining, capsule staining, gelatinase test, Simmon's citrate test and triple sugar iron test.[50,51] The

microscopic characterisation can be done using simple staining and Gram's staining techniques.[52] The molecular biological characterisation can be done by sequencing of 16S rRNA gene of potential bacterial isolates and 18S rRNA gene sequencing of potential fungal isolates.[53]

Moreover, the antimicrobial compounds produced by the endophytes can be identified and quantified by separation techniques, such as LC (liquid chromatography), GC (gas chromatography) or CE (capillary electrophoresis), coupled with detection systems, such as MS (mass spectrometry), NMR (nuclear magnetic resonance) or FTIR (Fourier transform infrared spectroscopy).[54] Further metabolomic studies can help to determine the metabolic stability, therapeutic efficacy and toxicological profiles of the compounds.[55] Therefore, these antimicrobial compounds can be designed and developed into novel antibiotic agents against pathogenic amoxicillin-resistant bacteria.[56]

5. Conclusion

This study aimed to isolate endophytes with antimicrobial activity from *Acalypha indica* and *Cyanthillium cinereum* plants against Amoxicillin-resistant environmental bacteria. As per the results of this study, prominent inhibition against *Staphylococcus aureus* was exhibited by four endophytic isolates (KpR-02B, MnS-01B, KpS-01F and MnR-04F), while *Enterobacter ludwigii* was prominently inhibited by two endophytic isolates (MnS-01B and MnR-04F) and *Enterobacter pyrinus* was also inhibited by two endophytes (MnF-03B and MnR-04F). Therefore, *Acalypha indica* and *Cyanthillium cinereum* plants can be considered as a good source of antimicrobial-compound producing endophytes against AR environmental bacteria.

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