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I-CReST 2025: 038-015 – Wide-spectrum Antibacterial Endophytes Associated with Malaysian Medicinal Plant

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ABSTRACT

The rising prevalence of multidrug-resistant (MDR) bacterial infections has ignited a worldwide health crisis, rendering massive synthetic antibiotics ineffective and resulting in notable morbidity and mortality. As the antibiotic pipeline grows depleted, there is a greater need for novel antibiotic discovery, particularly from natural resources. Medicinal plants, which have long been utilized in conventional medicine, serve as a potential repertoire for endophytes, an untapped source of bioactive metabolites with antibacterial potential. Thus, the current study aimed to isolate potential antibacterial endophytes from various Malaysian medicinal plants and screen their antibacterial potential against a wide spectrum of MDR bacteria. A total of nine Malaysian medicinal plants and sixteen test bacteria were used in this study. Surface sterilization method was employed to isolate endophytic fungi from selected medicinal plants. The agar plugs diffusion assay was employed to screen the antibacterial potential of endophytic fungal isolates. The results revealed that 345 endophytic fungal isolates were successfully obtained in this study, with 62.6% demonstrating antibacterial potential against at least one test bacterium. The highest percentage of endophytic fungal isolates with antibacterial potential was recovered from *Terminalia catappa* (88.4%). *Staphylococcus aureus* was the most susceptible to endophytic fungal isolates, with 21.3% of inhibitory activity. The findings highlight Malaysian medicinal plants as a potential source of endophytic fungi capable of combating MDR bacteria, paving the path for the next generation of effective antibiotics.

Keywords: Multidrug-resistant bacteria; medicinal plant; endophytes; antibacterial potential

1. INTRODUCTION

The phenomenon of multidrug resistance (MDR) in bacteria has been reported since the commencement of antibiotic era, and as antibiotic usage and abuse have expanded, the issue is getting worse. Antibiotics have undoubtedly been a blessing to human society in the war against bacteria, saving many lives. For over 70 years, antibiotics have been prescribed to treat bacterial infections. These low-molecular-weight substances have been used not only for therapeutic purposes but also as preventive precautions in a range of industries, such as agriculture and animal husbandry [1]. However, the alarming growth in multidrug-resistant bacterial infections poses a serious threat to human health. The biofilms formation that has been frequently associated with MDR also complicating the infection control [2]. As a result, there is a growing demand for the development of newer and improved antibacterial drugs candidates with a fresh target and diversified chemical structure to combat MDR phenomenon. Nature source particularly medicinal plant provides plentiful supply of bioactive substances that possess outstanding healing effects against various infection. This valuable source is crucial for novel development of antibacterial leads and drugs as multidrug resistance grows tremendously. Recent attention has been shifted to the untapped group of microbes, endophytes, particularly endophytic fungi that inhabit asymptomatically inside the host plant tissues. Endophytes serve as a promising repertoire for biologically active and structurally diverse substances that could offer a new target resistance pathway to tackle MDR.

Medicinal plant that has long been used to alleviate pain, discomfort, inflammation and infection, could serve as an attractive host for the novel endophytic fungi exploration. The exploration of medicinal plants as a source of endophytic fungi with antibacterial potential has become more intense since the prime breakthrough of paclitaxel that becomes the world's first multibillion-dollar anticancer drug [3]. Since then, scientists realize the hidden potential of endophytic fungi that inhabiting in various parts of medicinal plants. Malaysia as one of the megadiverse countries in the world was endowed with amazingly diverse and greatest number of plants inhabit in its tropical rainforest. Therefore, it is expected to be a hidden treasure of numerous untapped medicinal plants that may anchor various endophytic fungi with broad-spectrum antibacterial potential. To date, there are very limited studies that have been carried out to investigate the potential of endophytes residing in Malaysian medicinal plants [4]. Previous studies have proved the existence of at least one endophytic microorganism in plants, with some plants harbouring hundreds of this beneficial microbial group [5]. Therefore, the current investigation was aimed to isolate the endophytic fungi from some common Malaysian medicinal plant and assessed their antibacterial potential against a broad-spectrum of human bacterial pathogens.

2. MATERIALS AND METHODS

2.1 Collection of Plant Samples

Fresh plant with an asymptomatic appearance (leaf, flower and rhizome) were collected from Jeli, Kelantan and stored in zip lock bags. The samples were then transported to the laboratory within 4 hours for further processes.

2.2 Endophytic Fungi Isolation

The plant samples were washed thoroughly under running tap water for dust and debris removal. The samples were then subjected to surface-sterilization process by adopting procedure proposed by Li *et al.* with some modifications [6]. The samples were soaked by sequential soaking in 75% ethanol (1 min) and 1% sodium hypochlorite (30 s), with rinsing thrice using sterile distilled water between solutions. The sterilized samples were dried using sterile filter paper before cutting aseptically with blade to the size of 5 x 5 mm2 pieces. The cutting of tissue pieces was then placed onto potato dextrose agar (PDA) plate incorporated with host plant powder (5 g/L) and chloramphenicol (200 mg/ L) to oppress the bacterial growth. The inoculated plates were incubated for up to 3 weeks at 30 0C and daily observation was done to check the emergence of fungal mycelia from the tissue cutting of plant samples. Small cuts of the fungal mycelia were repeatedly inoculated onto fresh PDA until a pure culture was obtained.

2.3 Preparation of Bacterial Inoculum for Antibacterial Susceptibility Test

A total of fifteen test bacteria were used in the current study consisting of six Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Lactobacillus acidophilus,* and *Staphylococcus epidermidis*) and nine Gram-negative bacteria (*Proteus mirabilis, Yersenia enterocolitica, Eschericia coli, Salmonella typhimurium, Acinobacter antratus, Pseudomonas aeruginosa, Shigella boydii, Aeromonas hydrophila,* and *Klebsiella pneumoniae*). A single bacterial colony from a 24-hour-old agar plate was inoculated into 5 mL of sterile physiological saline and mixed well to obtain cell suspension. The turbidity of the cell suspension was adjusted to match with 0.5 McFarland standards (approximately 1 x 10^8 CFU/ mL). The inoculums were then seeded on Mueller Hinton Agar (MHA) plate by employing swab method [7].

2.4 Agar Plugs Diffusion Assay

The antibacterial activity of endophytic fungi obtained from the isolated stage was assessed by adopting agar plug diffusion assay [8]. Preparation of agar plug was done by inoculating fungal mycelia onto fresh PDA plates incorporated with powdered host materials followed by incubation process at 30 ^{0}C for 14 days. The fungal cultures were then aseptically cut using a cork borer into plugs of 10 mm and 4 mm in diameter and thickness, respectively and placed on agar medium seeded with bacterial inoculum. Chloramphenicol served as a positive control in this investigation. The plates were then incubated at 37 ^{0}C for 24 hours and the formation of clear zone around the fungal plugs were observed and recorded. This step of investigation was conducted in triplicate.

2.5 Statistical Analysis

The investigations were carried out in three replicates (n=3) and the data collected were represented as mean ± standard deviation (SD). ONE-WAY ANOVA was used to analyse the data using SPSS 22.0. The difference between means was analysed using Duncan test with a significance level of $p<0.05$.

3. RESULT AND DISCUSSION

3.1 Endophytic Fungal Isolates Recovered from Malaysian Medicinal Plant

Medicinal plants are an excellent source for the isolation of endophytic fungi due to their therapeutic properties that have been extensively utilized in alternative medicine since ancient times. Malaysia is blessed with tropical rainforests that host a broad range of medicinal plants, providing a novel reservoir of potential endophytes for the natural antibiotics' discovery. In the current study, nine Malaysian medicinal plant has been selected for endophytic fungi isolation from various plant part and the result is tabulated in Table 1.

Table 1. Percentage of endophytic fungal isolates with antibacterial potential isolated from various plant part of Malaysian medicinal plant

Plant	Plant part	Total number of isolates	Number of isolates with antimicrobial potential	Percentage of isolates with antimicrobial potential
Piper betle	Leaf	30.0±1.0	25.0±1.0	83.3
Chromolaena odorata	Leaf	41.0±1.0	23.4±0.6	57.1
Cananga odorata	Flower	26.7±0.6	12.3±1.5	46.1
Clinacanthus nutans	Leaf	35.0±1.0	25.7±1.2	73.4
Syzygium polyanthum	Leaf	24.3±1.5	13.0±1.0	53.5
Zingiber zerumbet	Rhizome	27.7±1.5	19.0±2.0	68.5
Terminalia catappa	Leaf	51.7±1.6	45.7±1.2	88.4
Pandanus tectoris	Leaf	54.0±2.0	30.0±1.0	55.6
Ipomea pes-caprea	Leaf	47.7±1.2	17.3±0.6	36.3
TOTAL		338	211.7	62.6

A total of 338 of fungal isolates that has been successfully isolated from nine medicinal plant where 62.6% of these isolates possessed antibacterial potential. *Pandanus tectoris* yield the highest number of isolates (54.0±2.0) followed by *Terminalia catappa* (51.7±1.6) and *Ipomea pes-caprea* (47.7±1.2). Although *T. catappa* yielded the second highest number of fungal isolates, it possessed the highest percentage of fungal isolates with antibacterial potential (88.4%), followed by *Piper betle* (83.3%) and *Clinacanthus nutans* (73.4%). This finding indicated that *T. catappa* served as a potential host plant for endophytic fungi isolation with remarkable antibacterial potential. Previous studies have reported the various potential of biological activity of endophytic fungi isolated from various *Terminalia* species [9] - [10]. Previous study reported endophytic fungi *Trichoderma atroviride* and *Penicillium chermesinum* isolated from *T. catappa* demonstrated significant antifungal and antibacterial activities [11]. Endophytic fungi associated with *T. catappa* also capable to synthesize antimicrobial compounds consisting of volatile organic compounds such as butanoic acid, 3-methyl-, butanoic acid, 2-methyl- and propanoic acid, 2-methyl- [12]. The antimicrobial compounds secreted by endophytic fungi associated with *T. catappa* have made this plant widely used in alternative treatments for the treatment of cardiovascular, wound, sore throats, colds, abdominal pain, bacterial infections, diarrhoea, fever, ulcer, edema, leprosy, headaches hypertension and skin infections [13]. Therefore, *Terminalia* has been acknowledged as one of the potential genera of medicinal plant with remarkable antimicrobial activity.

3.2 Screening of Antibacterial Potential of Endophytic Fungal Isolates

Agar plugs diffusion assay was opted for the antibacterial potential determination of the isolates obtained from the isolation stage. The percentage of inhibitory activity of isolates against fifteen test bacteria was depicted in Table 2. The results revealed that endophytic fungal isolates associated with all the plant species were able to inhibit at least 6 out of 15 test bacteria used in the study (40%), with endophytic fungi associated with *C. odorata, C. nutans* and *T. catappa* showed the highest capability of inhibition as they can retard the growth of 11 out of 15 bacteria (73.3%).

Table 2. Percentage of inhibitory activity of endophytic fungal isolates from various Malaysian medicinal plant against test bacteria

Plant / Bacteria	*Piper betle*	*Chromolaena odorata*	*Cananga odorata*	*Clinacanthus nutans*	*Syzygium polyanthum*	*Zingiber zerumbet*	*Terminalia catappa*	*Pandanus tectoris*	*Ipomea pes-caprea*
Staphylococcus aureus	30.9	25.0	25.1	16.0	31.0	4.8	10.6	38.7	17.6
Bacillus cereus	26.9	16.6	16.7	8.0	15.3	9.5	23.4	19.3	11.8
Bacillus subtilis	19.2	8.3	8.3	12.0	15.3	14.2	29.7	12.9	23.5
Enterococcus faecalis	-	4.2	-	8.0	-	-	4.3	9.7	-
Lactobacillus acidophilus	-	-	8.3	-	-	9.5	6.4	-	5.9
Staphylococcus epidermidis	-	4.2	8.3	4	-	19.3	6.4	-	-
Proteus mirabilis	-	-	8.3	4	-	9.5	2.1	-	-
Yersenia enterocolitica	3.8	8.3	-	8	-	-	6.4	-	11.8
Eschericia coli	-	4.2	-	-	7.7	9.5	-	-	-
Salmonella typhimurium	-	8.3	-	4	7.7	-	2.1	3.2	-
Acinobacter antratus	7.7	-	8.3	-	-	14.2	4.3	-	5.9
Pseudomonas aeruginosa	-	4.2	-	8	15.3	-	-	6.5	-
Shigella boydii	-	4.2	-	-	-	9.5	4.3	-	-
Aeromonas hydrophila	3.8	-	-	8	-	-	-	-	-
Klebsiella pneumoniae	-	8.3	16.7	12	-	-	-	9.7	17.6

Endophytic fungi, especially those residing in medicinal plants, constitute a substantial source of bioactive metabolites with potentially unique modes of action. The special ability of endophytic fungi to secrete bioactive metabolites that closely mirror those of their host plant draws attention of many scientists to examine this untapped group of microorganisms [14]. Since *C. odorata, C. nutans* and *T. catappa* were widely used in alternative treatment due to their therapeutic values, endophytic fungi associated with these medicinal plants also believed to harbor similar healing potential. Endophytic fungi isolated from *C. odorata* has been reported to produce flavonoids and phenols with antibacterial and antifungal activities [15]. Previous isolation work of endophytic fungi from *C. nutans* also revealed that a total of 20 isolates with significant antibacterial potential were successfully obtained [16]. Similarly, endophytic fungi recovered from *T. catappa* also demonstrated promising antibacterial potential with MIC values ranging from 0.32 to 25 μg/mL [10].

S. aureus, B, cereus and *B. subtilis* were susceptible to all the endophytic fungal isolates associated with nine species of Malaysian medicinal plant. The least susceptible bacterium to the isolates was *A. hydrophila* since it only susceptible to the isolates from *P. betle* and *C. nutans*. This finding indicated different ability of inhibition by isolates towards Gram-positive and Gram-negative bacteria where Gram-negative bacteria was difficult to inhibit compared to Gram-positive bacteria. Cell wall structure of Gram-negative bacteria was more rigid and complex compared to Gram-positive bacteria with the presence of porin channels and outer membrane around cell wall that hinder the entry of any toxic substances [17].

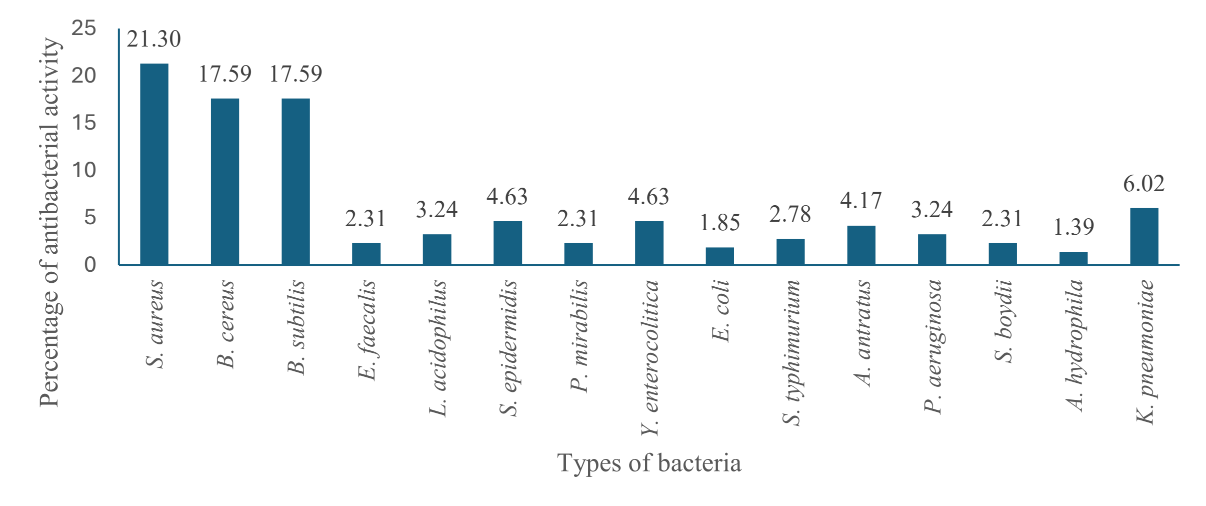

Fig. 1. Overall percentage of bacterial susceptibility to endophytic fungal isolates associated with Malaysian medicinal plant

The overall percentage of test bacterial susceptibility to isolates associated with selected Malaysian medicinal plant is depicted in Fig. 1. Based on this figure, Gram-postive bacteria, *S. aureus* was most susceptible to the isolates (21.30%), followed by *B. cereus* and *B. subtilis* with similar percentage (17.59%), whereas Gram-negative bacteria, *A. hdrophila* and *E. coli* were the least susceptible to the isolates with percentage of 1.39% and 1.85%, respectively. Similar findings from the previous studies also showed that endophytic fungal isolates demonstrated greater inhibition towards Gram-positive bacteria compared to Gram-negative bacteria [18] – [19]. *S. aureus* was also reported to be the most susceptible bacteria to the endophytic fungi isolated form *Artemisia sieberi*, consistent with the current findings. Prior research has suggested that, despite the remarkable antibacterial activities of bioactive metabolites of endophytic fungi, most of them are still ineffective against prevalent Gram-negative bacteria due to the presence of lipopolysaccharide outer membranes, which act as a permeability barrier that obstructs the entry of toxic substances or antibiotics into the bacterial cell [20] – [21].

4. CONCLUSION

The distribution of endophytic fungi in all plant species, particularly medicinal plants has been well documented. In the current study, nine Malaysian medicinal plant species that have long been utilized in traditional medicine were chosen for endophytic fungi isolation. All of these plants provided a bountiful source of endophytic fungi with promising antibacterial potential, offering an alternative solution for multidrug resistant problem. Since the study of endophytic fungi associated with Malaysian medicinal plant is still limited, the findings from the current investigation may help to fill in the gaps. However, further elucidation of antibacterial metabolites involving extraction, purification and identification should be carried out to quantify this valuable source for producing effective and safe drugs. Furthermore, research should also focus on scaling up the production of endophytic fungal secondary metabolites to meet demand for novel antibacterial drugs.

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