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ANTIMICROBIAL SUBSTANCES FROM ENDOPHYTIC FUNGI IN TAMARIND (*Tamarindus indica*, Linn), MALAY APPLE (*Eugenia malaccensis*, Linn), RAMBUTAN (*Nephelium lappaceum*), AND INDIAN MULBERRY (*Morindacitrifolia*, Linn)

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Abstract

Endophytic fungi are known to produce useful substances including antibiotics and other active compounds. Endophytic fungi from local plants; Tamarind (*Tamarindus indica*, Linn), Malay apple (*Eugenia malaccensis*, Linn), Rambutan (*Nephelium lappaceum*), and Indian mulberry (*Morinda citrifolia*, Linn) were investigated for their ability to produce antimicrobial substances. Plant parts were sterilized cut, inoculated on Potato Dextrose Agar (PDA) plates, and incubated at 27^{O}C for weeks until the appearance of endophytic growth. It was then found that the highest total endophytic fungal count was observed from Tamarind (39.47%). Upon screening of antimicrobial activity, not all but MA1, MA5, RB2, BU1 and YB1 isolates showed growth inhibition activity. Antimicrobial production in liquid (PDB) and solid (PDA) condition were then compared, and results showed that in liquid condition of PDB, the fungi gave higher production. Extraction of antimicrobial substances by culturing the isolates and distilling the cell-free filtrate with chloroform (1:3) yielded partially-purified extract (PPE) with different degree of antimicrobial activity on *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus cereus*, and *Candida albicans.* Minimum Inhibitory Concentration (MIC) of these extracts in comparison with antibiotics as evaluated by broth microdilution technique showed that MA5 and YB1 gave the lowest value i.e. 1.709µg/mL, whereas BU1 gave the highest i.e. 437.5 µg/ml. RB2 gave similar MIC value with that of Chloramphenicol. As regard activity spectrum, all PPE were of broad type as it showed inhibition activity to all tested bacteria including *C. albicans*. Macroscopically, colonies of those isolates were white in color except for YB1, which was slightly reddish purple. Microscopically, all isolates showed aseptated hypha and non-sporulation in PDA. This primary study on local endophytic fungi and their plant sources is believed to be very useful for initiating further and advanced investigation with pharmaceutical application.

Key words: Antimicrobial, Endophytic fungi, Minimal Inhibitory Concentration (MIC), local plants

INTRODUCTION

Endophytic fungi residing in plant tissues are known by nature to produce active substance(s) with *in* vitroability to act as anti-cancer, antiviral, antibacterial, antifungal, anti-oxidant, plant growth hormones, insecticides and other biochemical agents (Worapong et al., 2001a-b; Strobel, 2003 & 2004; Agut & Calvo, 2004; Liu et al., 2007; Pandi et al., 2010; Ramos et al., 2010; Ahmad et al., 2011; Bhimba et al., 2012; McCutcheon & Moran, 2012; Raffaele & Kamoun, 2012; Teiten et al., 2013; Zilla et al., 2013; Yadap et al., 2014; Agusta et al., 2014; Wei et al., 2014; Hussain et al., 2007, 2009, 2011, 2014 & 2015; Gao et al., 2015; Syamsia et al., 2015) with possible applications as starting materials for pharmaceutical, industrial and agrochemical products (Sturz et al., 2000; Strobel, 2006; Hardoim et al., 2008; Kaul et al., 2012; Brader et al., 2014). As a world has faced the wide spread resistant bacterial strains due to wide and extensive use of antibiotics in the treatment of infectious illness (Kumarasamy et al., 2010; D'Costa et al., 2011; Kempf & Rolain, 2012; Nordmann et al., 2012), endophytic microoragisms in particular the fungi have become the alternative resources for new antimicrobials. For decades the exploration of these fungi has brought about promising achievement (Tan & Zou, 2001; Strobel, 2003, 2006; Zhang et al., 2006; Guo et al., 2008; Priti et. al., 2009; Aly et. al., 2011; Kharwar et. al., 2011; Radic &Strukelj, 2012; Zhang et. al., 2012) These antimicrobial-producing endophytes are as diverse as wide world forests, and so, they become unlimited sources of these microbial endophytes (Strobel, 2003, 2006; Debbab et al., 2012; Radic & Strukelj, 2012; Mousa &Raizada, 2013).

Tamarind (*Tamarindus indica*, Linn), ethnomedicinal dicotswidely used in tropical countriesand with claiming pharmacologically effective against various pathophysiological disorders (Komutarin et al., 2004; Maiti et al.,2004; Muthu et al., 2005; Sudjaroen et al., 2005; Ushanandini et al., 2006; Al-Fatimi et al., 2007; Havinga et al., 2010), especially its seed extract, which was demonstrated to have numerous therapical activities of infectious, immunological and physiological disorders (Ramos et al., 2003; Martinello et al., 2006; Hemshekhar et al., 2011;Razali et al., 2012).Malay apple (*Eugenia malaccensis* L.), an ethnomedicinal flowering tree native to Southeast Asian region, has been used in treating diseases and symptoms (Roosita et al., 2008). Its crude extracts and compounds showed anti-inflammatory, analgesic and antipyretic (Falcao et al., 2005), antifungal (Lima et al., 2006), hypotensive (Consolini et al., 1999), antihyperlipidemic (Ravi et al., 2005), hypoglycemic (Barbosa-Filho et al., 2005), and antioxidant (Velázquez et al., 2003) activities.Its phytochemicals include flavonoids (Mahmoud et al., 2001), tannins, terpenoids (Lunardi et al., 2001), and essential oils (Oliveira et al., 2005).Rambutan (*Nephelium lappaceum)* – common Southeast Asia tropical tree and known in Australia (Davidson et al., 2006; Jalikop, 2013),has been used in treating diabetes and high blood pressure (Kaushik et al., 2010), contained flavonoids, tannins and saponins (Dalimartha, 2003), and epigallocatechin-3-gallate (Palanisamy et al., 2011a). Its peel ethanol extract manifested actions of antimicrobial (Thitilertdecha et. al., 2008; Bhat & Al-daihan, 2014), anti-HSV-1 (Nawawi et al., 1999),antihyperglycemia (Waltner-Law et al., 2002; Palanisamy et al., 2011b; Lestari et al., 2014; Muhtadi et al., 2015),and low-cytotoxicity antioxidants (Tamimy, 2006; Okonogi et al., 2007; Tachakittirungrod et al., 2007; Tabata et. al., 2008; Palanisamy et al., 2008; Haruenkeit et al., 2010; Khonkarn et al., 2010; Thitilertdecha et al., 2010; Muhtadi et al., 2014).Indian mulberry (*Morinda citrifolia*) - popular ethnomedicinal and Southeast Asian-origin plants, now its powder and juice-form are well established in the US and elsewhere as dietary supplement for controlling arthritis, cancer, cardiovascular disease, inflammation, and as a general tonic (Pawlus & Kinghorn, 2007; Potterat & Hamburger, 2007; Pawlus et al., 2010). Its anthraquinones, fatty acid derivatives, flavonoids, iridoids, lignans, phenylpropanoids, saccharide derivatives, triterpenoids and other preparations were shown to have *in vitro* and *in*

vivo effect on cardiovascular disease and cancer (Wang & Su, 2001; Wang et al., 2002; Furusawa et al., 2003; Jasril et al., 2003; Wang et al., 2009, 2011; Nualsanit et al., 2012), fertility and inflammation (Hirazumi & Furusawa, 1999; Nualsanit et al., 2011), and infectious diseases (Saludes et al., 2002; Pawlus et al., 2010; Baque, 2011; Baque et al., 2011; Lv et al., 2011).

In the present study, tamarind, Malay apple, rambutan and Indian mulberry had been subjected to isolate endophytic fungi with antimicrobial activity against *Esherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Bacillus cereus* as well as *Candida albicans*. Efforts made included also preliminary characterization, MIC determination with reference to several antimicrobial agent. Subsequently, several endophytic fungi and partially putified extracts (PPEs) with efficacy in growth inhibition of test organisms, their characteristics, and MIC were expected to achieve.

RESEARCH METHOD

Plant materials. Plant samples were collected from sides of highway around Yala Capital District, Yala Province, Southern Thailand. Only disease-free leaf and branch stem of tamarind (*Tamarindus indica*, Linn.), Malay apple (*Eugenia malaccensis*, Linn.), rambutan (*Nephelium lappaceum*), and Indian mulberry (*Morinda citrifolia*, Linn.) were selected.

Test organisms.*E. coli*, *S. typhi*, *St. aureus* and *B. cereus*, and *C. albicans*, was supplied as a courtesy from Department of Microbiology, Faculty of Science, Prince of Songkla University, Hadyai, Songkhla Province, Southern Thailand. Bacteria were maintained on Nutrient Agar (NA, Merck, Germany) slant and yeast on PDA slants at 4^{O}C in refrigerator after confirming their purity.

Reference antimicrobial agents. Chloramphenicol (Cloman, Thailand), Streptomycin (M & H, Thailand), Rifampicin (Sigma, USA), Penicillin V (Sigma, USA), Tetracycline (Sigma, USA), and Nystatin (Cloman, Thailand) used as reference in minimal inhibition concentration (MIC) determination were purchased from suppliers in the locality. Their preparation was done as guided by manufacturers' description, and used as per requirement of experimental procedures.

Isolation of endophytic fungi. Leaf and stem samples were washed, surface-sterilized, blot dried, excised (~ 0.2 cm^3), and inoculated (40 pc/plate) using both aqueous agar (AA) and potato dextrose agar (PDA, Merck, Germany), supplemented by 0.3% (w/v) each of Chloramphenicol andAmpicillin sodium salt (Merck, Germany) (Nalini et al., 2014; Arnold et. al., 2000). Fungal colony from each segment developed following several days of incubation at 27^{O}C was subsequently transferred to antibiotic-free PDA for culture purifyingand identifying purposes. Morphological and reproductive structure and spore characteristics was determined(Barnett& Hunter, 1998; Domsch et. al., 2003; Leslie&Summerell, 2006; Mulloch, 2014).Culture stocks on PDA slants were maintained at 4^{O}C until further uses.

Preliminary Screening for antimicrobial-producing endophytes. Modified agar plate-based assay was employed in screening of endophyte isolates(Arasu et. al., 2009). Aseptically, hyphal plugs from maximal growth on PDAwasplaced on Mueller Hinton Agar (MHA, Merck, Germany)lawn of each test bacteria ($1x10^8$ cfu/mL)spread or PDA lawn of *C. albicans* ($1x10^4$ cfu/mL) spread. Clear zone surrounding the endophyte plugs developed after 24 hours at 27^{O}C incubation, was measured for inhibition activity evaluation.

Antimicrobial production and extraction. Selected endophytesMA1, MA5, RU2, BU1 and YB1 plugs were inoculated on 20 mL-PDA plates and in 500 mL-Erlenmeyer flasks containing 200 mL-PDB for 5-7 days at 27^{O}C without and with 150-rpm agitation (Rosa et. al., 2012;

Hayeewangoh, 2002). Both solid medium and fungal mycelium were harvested, and distillingextracted by soaking in chloroform(Sigma, USA) for 72 hours at 27^{O}C with 150-rpm agitation. Finally, resulting biomass as partially-purified extract (PPE) was filtered, vapour-dried using Rotary Evaporator (Buchi, Switzerland), kept in sterile capped bottles in refegerator until further use. Fungal biomass from 200-mL PDB was treated, filterred and distilled in the same manner as previously described.

Evaluation of Antimicrobial activity. , Antimicrobial activity was evaluated against test bacteria on MHA, and yeast on PDAby employing disc diffusion technique(Schwalbe et. al., 2007). Aseptically, sterile paper discs (6 mm-diameter) mounted with 10 µL of each endophyte extract (10 mG/mL), was firmly placed on the prepared test cultures, and incubated at 35^{O}C(28^{O}C for yeast) for 24-48 hours. Diameter of inhibition zone was measured.

Minimal Inhibition Concentration (MIC) of fungal extracts. MICs of each PPE and reference antimicrobial agent were determinedusing broth microdilution method (Pawthong et. al., 2012).Each PPE and reference agentin 96-well of polystyrene microtitre plates (Thermoscientific, USA)was serially 2-fold dilutedto make concentration ranged from 875 µG/mL to 0.43 µG/mL. Into each wells, 10 µL suspension of each test organism(cfu/mL: 1x10^{8} or 1x10^{4}) was added to diluted mixtures of PPE-reference agents (350 µL) and 2-strength growth media (350 µL). After incubation at 28^{O}C for 24-48 hours, growth inhibition was evaluated based on developed turbidity.

Morphological characteristics of endophytes. Each of MA1, MA5, RU2, BU1 and YB1endophyticisolates were characterized macroscopically and microscopically using conventional techniques including slide culture technique.

RESULT AND DISCUSSION

EndophyticDistribution. Counts of endophytic fungi recoveredfrom organ parts of tamarind, Malay apple, rambutan, and Indian mulberry were varied with the highest were from that of tamarind, 42.10%. Lowest count was recovered from rambutan and Indian mulberry, 18.32%each (Figure 1). Endophyte count from branches washigh, and from leaf stalk was low. Moderate count was observed from midrib, vein and stem sections of leaf (Figure 1). There was no uniformity in occurrence of fungal endophyte for every host plant (Figure 2). Higher number of fungal endophytes were recovered from tamarind branches (56.25%), and lower numbers were from midrib, veins and leaf stem of rambutan and Indian mulberry (28.5% each). Detailed result was shown in Figure 2.

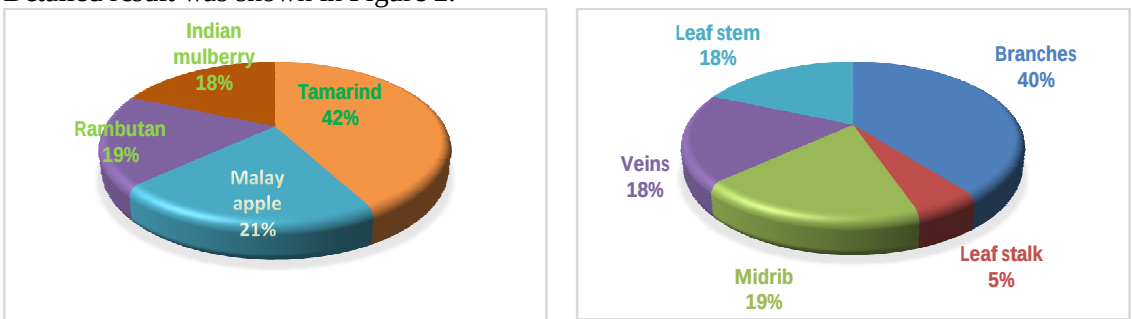


Figure 1. Percentage of endophytic fungi recovered from (Left) disease-free parts of tamarind, Malay apple, rambutan and Indian mulberry, and from (Right) different disease-free parts of host plants.

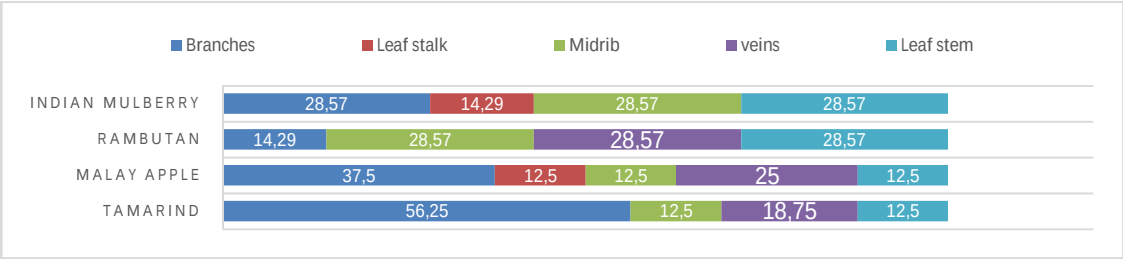


Figure 2. Distribution percentage of fungal endophytes in various organal parts of host plants, i.e. tamarind, Malay apple, rambutan, and Indian mulberry.

Varying distribution of endophytic fungi in different parts of plants was common, and determined by factors like ecology, nutrient availability, plant physiology and experimental parameters (Madigan et. al., 2015). Despite not many reports described this variation, Waenawae (2009) reported similar result of endophyte distribution in organ parts of cashew nut tree (*Anacardium occidenfale*) and common jujube (*Zizyphus mauritiana*, Lamk), cussud tree (*Cassia siamea*, Lamk), Khraimanpoo (*Glochidion sphaerogynum)*, and Longkong (*Lanseum domesticum correa*).Dolah (2005) reported varying percentage distribution in branches, and leaf stalk, midrib, vein and stem of endophytic fungi recovered from pomelo (*Citrus maxima, Merr*) and bullet wood (*Mimusops elengi*, Linn.). Sama (3013) and Bangosatoo (2013) comparatively described endophytic fungal distribution in parts of pomelo (*Citrus maxima, Merr*), bullet wood (*Mimusops elengi*, Linn.), guava (*Psidium guajava*), marian plum (*Bouea macrophylla*), Santol (*Sandoricum koetjape*), tamarind (*Tamarindus indica*, Linn), Malay apple (*Eugenia malaccensis*, Linn), Indian mulberry and (*Morinda citrifolia*, Linn).

In *Angelica sinensi*s root, stem and leaf, 3 separate collectionsof endophytic fungi recorded a total recovery of 206 isolates representing 22 species with 24.27%, 26.21% and 49.51% distribution (Shu et. al., 2013). Khan and colleagues (2010) reported the distribution of endophytic flora in medicinal *Withania somnifera* plant employing 643 segments (202 leaf, 391 stem, and 50 root) from 20 different plants, and claimed 20species within 12 genera of fungi, including 9 fungi from leaves, 20 from stems and 4 from roots.Leaf and stem segments of *N. arbor-tristis* were reported to give recovery of 19 endophytic fungal species in 15 taxa and 10 species in 9 taxa, with *Alternaria alternata* showed highest colonization in leaf tissues (15.0%), and *Cladosporium cladosporioides* mostly (12%)colonized stem tissues (Gond et. al., 2010). Occurrence of different groups of endophytes in halophytes from an estuarine mangrove forest was reported as their percentage occurrence and species richness (Suryanarayanan & Kumaresan, 2000).

Antimicrobial-producing endophytes. Results of preliminary screening of endophytic isolates for the antimicrobial production were shown in Table 1. There was neither all isolates exhibitinginhibition zone on growth lawn of any tested bacteria and yeast, norsingle isolate showed inhibition activity on all growth lawns of organisms.MA1, MA5, MC3, MC5, MD2, and ME4 tamarind isolatesdemonstrated varying degree of inhibition zones. Malay apple isolates showing inhibition zones were RA1, RA2, RB2, and RB5. Rambutan-related BU1 and BU4, and Indian mulberry-related YB1, YB2, and YE1 were also antimicrobial producers. PPEs with wider activity spectrum were from MC5, RA1, BU1, and YE1.

Table 1. Growth inhibition activity of fungal endophytes recovered from tamarind (T), Malay apple (MA), rambutan (R) and Indian mulberry (IM) against *E. coli* (EC), *S. typhi* (SA), *St. aureus* (ST), *B. cereus* (BA), and *C. albicans*(CA).

Endophyte Host-test organism	Endophyte isolates							
	Total	Antimicrobial producers			Antimicrobial non-producers			Antimicrobial Spectrum (organisms effective against)
		Total count	Designation	%	Total count	Designation	%	
T-EC	16	3	MA1, MA5, MC5	18.75	10	MA2, MA3, MA4, MA6, MA7, MA8, MA9, MC1, MC2, MC4	62.5	MA1 (2), MA5(2), MC5(3), MD2(3), MC3(1), ME4(2)
T-SA		2	MA5, MD2	12.5				
T-ST		3	MC3, MC5, MD2	18.75				
T-BA		2	MC5, ME4	12.5				
T-CA		3	MA1, MD2, ME4	18.75				
MA-EC	8	2	RB2, RB5	25.0	4	RA3, RA4, RB1, RB3	50.0	RB2(1), RB5(1), RA2(1), RA1(3)
MA-SA		1	RA2	12.5				
MA-ST		1	RA1	12.5				
MA-BA		1	RA1	12.5				
MA-CA		1	RA1	12.5				
R-EC	7	2	BU1, BU4	28.57	5	BU2, BU3, BB1, BB2, BF1	71.43	BU1(3), BU4(2)
R-SA		1	BU1	14.29				
R-ST		2	BU1, BU4	28.57				
R-BA		0		0				
R-CA		0		0				
IM-EC	7	2	YB2, YE1	58.57	4	YB3, YS3, YU1, YR2	57.14	YB2(2), YE1(3), YB1(1)
IM-SA		1	YE1	14.29				
IM-ST		1	YE1	14.29				
IM-BA		1	YB1	14.29				
IM-CA		1	YB2	14.29				

Percentage of antimicrobial producers in endophyte fungal population colonizing plant tissues was not only varied but also time-dependent. Waenawae (2009) reported the screening of endophytic fungi from *Anacardium occidenfale* and *Lanseum domesticum correa,* and isolated GU2M, GU2B, LA2P, LA1B, and RM1M with narrow spectrum of activity against only single test organism, i.e. *E. coli* or *St. aureus*. Dolah (2005) reported endophytic fungi isolation from pomelo (*Citrus maxima*, Merr) and bullet wood (*Mimusops elengi*, Linn) that only 8.33% of isolates wereantimicrobial producers, and of these only 1 was active against *St. aureus* and *E. coli*, but not *S. typhi, B. cereus* nor *C. albicans.* Casella and colleagues (2013) screened tropical leaf endophytes, and described 4 of 138 extracts (2.9%) possessing significant antibacterial activity against *S. aureus,* and 22 extracts (15.9%) were active against *C. albicans, S. flexnii, S. boydii, S. enteritidis, S. paratyphi, P. aeruginosa, C. freundii, M. morganii,* and *P. vulgaris.* This difference in distribution finding partly due to the nature of endophyte-host plant association, which in turn determined their natural trait (Madigan et. al., 2015). Producers fromTamarind (37.5%), Malay apple (50%), rambutan (28.57%), and Indian mulberry (42.86%) were all ethnomedicinal plants, contrasting with *A. occidenfale* and *L. domesticum correa* as well aspomelo and bullet wood, which were not. Such nature of association effected their distribution.

Antimicrobial production.PPEs from endophyte isolates grown on solid and in liquid media were **different** in quantities (data not shown) Overall, PDB-based production gave higher amount ofPPEs for ~ 20 to 110mg/200 mLof growing media. This difference effected the antimicrobial activity of the PPEs. Our finding showed that not all PPEs had inhibition activity against all test organisms. *E. coli* were sensitive to MA1, MA5, RB2, and BU1 with

varying degree. *S. typhi* were however found sensitive to MA5 and BU1, while *St. aureus* to BU1, and *B. cereus* to BU1 and YB1. As for *C. albicans*, it was only MA1 was effective (Table 2). As the actvity spectrum was concerned, BU1 had broader spectrum than those of MA1 and MA5. MA1 had wider range of actvity encompassing inhibition activity against fungi, *C. albicans*.

Table 2. Inhibition zone of extracts from endophyte grown in solid (A) and liquid (B) media of PDA and PDB evaluated by disc diffusion method against test organisms.

Test organisms/extracted media	Inhibition zones of endophyte extracts (mm) and the (difference)					Average difference (%)
	MA1	MA5	RB2	BU1	YB1	
E. coli/A	4.2 (-2.4)	12.9 (-6.3)	5.3 (-2.8)	3.5 (-3.7)	0 (0)	38.0
E. coli/B	6.6 (+2.4)	19.2 (+6.3)	8.1 (+2.8)	7.2 (+3.7)	0 (0)	
S. typhi/A	0 (0)	15.6 (-4.6)	0 (0)	2.4 (-4.3)	0 (0)	44.5
S. typhi/B	0 (0)	20.3 (+4.6)	0 (0)	6.7 (+4.3)	0 (0)	
St. aureus/A	0 (0)	0 (0)	0 (0)	6.3 (-9.4)	0 (0)	94.0
St. aureus/B	0 (0)	0 (0)	0 (0)	15.6 (+9.4)	0 (0)	
B. cereus/A	0 (0)	0 (0)	0 (0)	3.4 (-1.7)	8.6 (-7.7)	47.0
B. cereus/B	0 (0)	0 (0)	0 (0)	5.1 (+1.7)	16.3 (+7.7)	
C. albicans/A	16.3 (-1.8)	0 (0)	0 (0)	0 (0)	0 (0)	18.0
C. albicans/B	18.1 (+1.8)	0 (0)	0 (0)	0 (0)	0 (0)	

Many works on antimicrobial activity of endophytic fungi from plants were brought out, and claimed more or less similar degree of activity and spectrum. Bhimba& colleaques (2012) described the extract having activity against *E.coli, Pseudomonas, Enterococcus, Staphylococcus, and Bacillus* with zone inhibition ranged from 12 to 30 mm. Filtrate of endophytic fungal culture broth of *Talaromyces flavus, Mortierella hyaline, Paecilomyces variabilis, Penicillium* sp., which were isolated from ethnomedicinal host plants, *Potentilla fulgens, Osbeckia stellate, Osbeckia chinensis, Camellia caduca,* and *Schima khasiana* was reported to show antimicrobial activity against *B. cereus* (MTCC 430), *St. aureus* (MTCC 740), *E. coli* (MTCC 116), *S. typhi* (MTCC 733), and *C. albicans* (MTCC 227) with inhibition zone ranging from 8.60 mm to 32.67 mm (Bhagobaty & Joshi, 2012). Interestingly, those extracts were active against both bacteria and fungi, a case of which was in consistent with this current finding. Antibacterial activity of extracts from *Kigelia Africana*-associated endophytic fungi *Aspergillus flavus, Aspergillus* sp., *Curvularia lunata, Cladosporium sp.* and 3 unidentified were found active against *B. subtilis,* S*t. aureus* and *E. coli* with 14-37 mm inhibition zone diameter, and broader spectrum(Idris et. al., 2013). *Plectophomella*sp., *Physalospora*sp., and *Crataegus monogyna* extracts showed fungistatic and bacteriostatic activities with 1.0-30 mm inhibition zones (Hussain et. al., 2014). Moreover, , Yang and co-workers (2014) demonstrated by using agar diffusion assay that *Camellia sinensis*branch-associated endophytic fungus, *Alternaria alternata* displayed bacteriostatic effect against *St. aureus* and *B. subtilis* with 10–25 mm inhibition zones but insensitive to *E. coli*. *Ficus pumila* Linn-associated endophytic *Phomopsis* sp. was active against a panel of human and phytopathogenic microbes with 16-30.66 mm and 14-30 mm inhibition zones for test fungi and for test bacteria, respectively (Rakshith et. al., 2013).

Minimal inhibition concentration of antimicrobial extracts. MICs of PPEs from MA1, MA5, RU2, BU1 and YB1 with reference to antimicrobial agents were determined, and result showed that these PPEs and agents displayed varying MICs against the test organisms (Table 3).Overall, tamarind-associatedMA1 and MA5 gave lower MICs of 6.834 to 109.375 µg/mL, and 1.709 to 109.375 µg/mL, thus signifying higher inhibition effect on all test

organisms, including *C. albicans*. Malay apple-associated endophyte RB1 PPE exhibited moderate inhibition activity (µg/mL MIC ranged 54.688-437.5). Most effective bacteriostatic activity was demonstrated by Indian mulberry-associated endophyte PPE, YB1, whose MICs ranged from < 0.427 µg/mL to 3.418 µg/mL. Whereas, rambutan-associated BU1 showed highest MICs at 437.5 µg/mL. In addition, all PPEs had both bactericidal and fungical activity as seen by *C. albicans*sensitivity (µg/mL MICs, 6.836 to 437.5). Such MIC range was comparable with reference fungistatic Nystatin. Also, bacteriostatis of PPEs was comparable with reference agents, whose MICs ranged from 13.672 µg/mL to >875 µg/mL. As far as the efficacy of these PPEs were concerned, MA1 was found to have lower MIC than those of reference antimicrobial agents, whose MIC ranged 13.672 µg/mL to >875 µg/mL. For *E. coli*, MA5 and YB1 PPEs were the most effective (MIC, 1.709 µg/mL). Furthermore, YB1 was seen to be the most effective to all test bacteria (MIC range, < 0.428 µg/mL to 3.418 µg/mL).
MIC evaluation of endophyte biological substance(s) in comparison with the reference agents was very useful in determining their efficacy. That was why almost all reports on antimicrobial activity included MIC determination. Casella and colleaques (2013) reported MIC for *C. albicans* and *St. aureus* of crude extract from tropical leaf endophytes were ≤ 128 µg/mL. MIC ranging from 0.49 µg/mL to 15.625 µg/mL was reported by Powthong and co-workers (2012), who determined antimicrobial activities of endophytic fungi crude extract recovered from *Sesbania grandiflora (L.) Pers.* Against *St. aureus* ATCC 25923 *B. subtilis* ATCC6633, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *C. albicans*, *Cryptococcus neoformans* using broth microdilution method.

Table 3. MICs of the endophyte PPEs with reference to antimicrobial agents. NA standed for Not Applicable, i.e. tests were not performed.

Agents	**MIC (µg/mL)**				
	E. Coli	***S. typhi***	***St. aureus***	***B. cereus***	***C. albicans***
MA1	6.836	109.375	27.344	13.672	6.836
MA5	1.709	13.672	3.418	13.672	109.375
RB2	109.375	109.375	54.688	109.375	437.5
BU1	437.5	437.5	437.5	437.5	437.5
YB1	1.709	3.418	< 0.428	3.418	437.5
Chloramphenical	218.75	437.5	109.375	437.5	NA
Streptomycin	437.5	437.5	109.375	437.5	NA
Rifampicin	13.672	54.688	218.750	>875	NA
Penicillin V	109.375	27.344	13.672	>875	NA
Tetracycline	54.688	218.75	109.375	13.672	NA
Nystatin	NA	NA	NA	NA	27.34375

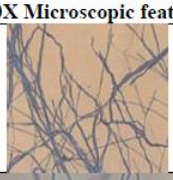
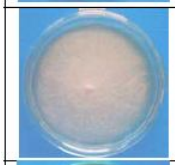
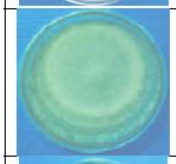
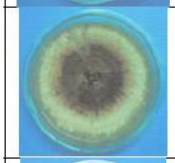
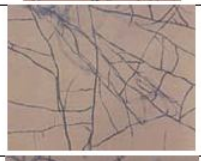
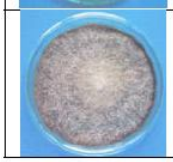
Macroscopic and microscopic characteristics of endophytes. Endophyte isolates MA1, MA5, RB2, BU1, and **YB1were** macroscopically and microscopically studies using conventional technique including slide culture, and it was found that these fungi on PDA medium were non-septate and did not produce any reproductive spores (Table 3). Consequently, it was unable and with doubt to identify the fungal identity based on the available characteristics. However, their characteristics were summarized in Table 3.

CONCLUSION AND SUGGESTION

Local plant-associated endophytic fungi were recovered in varying numbers from Tamarind, Malay apple, Rambutan and Indian mulberry. The highest numbers were from that of tamarind, 42.10%, and lowest **numbers** were from those of rambutan and Indian mulberry, 18.32%. In addition, those from branches were in high count,

contrasting with leaf stalks that were low count. There was neither uniformity in their distribution in every host plant, nor equality in their ability to produce antimicrobial substances. Hence, highest total endophytic fungal count was recovered from Tamarind (39.47%), and its associated MA1, MA5, MC3, MC5, MD2, and ME4 manifested varying inhibition zone sizes. Malay apple-associated RA1, RA2, RB2, and RB5, rambutan-associated BU1 and BU4, and Indian mulberry-associated YB1, YB2, and YE1 displayed unequal antimicrobial activity too. Extracts with wider spectrum included MC5, RA1, BU1, and YE1.Antimicrobial production in liquid (PDB) media gave higher yield than that of solid (PDA) condition. Partially-purified extract (PPE) that resulted from extracting and distilling using 1/3 chloroform showed different degree of antimicrobial activity against *E. coli*, *S. typhi*, *St. aureus*, *B. cereus*, and *C. albicans* with MICas lowest as 1.71 µg/ml. This signified the higher efficacy, and happened to be lower MICs than those of reference antimicrobial agents, whose MIC rangedfrom 13.671875 µg/mL to >875 µg/mL. All PPE showed broad type of spectrum. It was unfortunately unable to identify the isolates because their macroscopical and microscopical characteristics were not in line with those described in identification key of simple fungi. Therefore, we designated as unidentified isolates. This preliminary study on local endophytic fungi and their plant sources is believed to be very useful for initiating further and advanced investigation with pharmaceutical application

Table 3. Some macroscopic and ,icroscopic features of endophyte tamarind MA1 and MA5, Malay apple RB2, rambutan BU1, and Indian mulberry YB1.

Colony on PDA	Macroscopic feature		Microscopic feature	400X Microscopic feature
	5-day fully developed white colony on PDA appeared red-pink in color with loose hyphae covering all PDA surface.	MA1	Non-septated hyphae occurred singly or branches, and appeared loosely aggregated.	
	White colonies on PDA with huge mass of hyphae developed fully in 10 days.	MA5	Non-septated hyphae with branches and densely aggregated.	
	Creamy white colony with rough surface developed rapidly but matured within 9 days.	RB2	Non-septated hyphae and densely aggregated.	
	Greenish white colony developed in 5 days on PDA, and dark green color on maturation with full developed multilayered hyphae	BU1	Non-septated hyphae with small numbers of branches appeared resembling spider web.	
	Pinkish red colony on PDA with cotton texture developed in 5 days and bottom became black.	YB1	Non-septated and shorter hyphae appeared singly or branched.	

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