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CHEMISTRY EDUCATION

BIOLOGY

B -01

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

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.

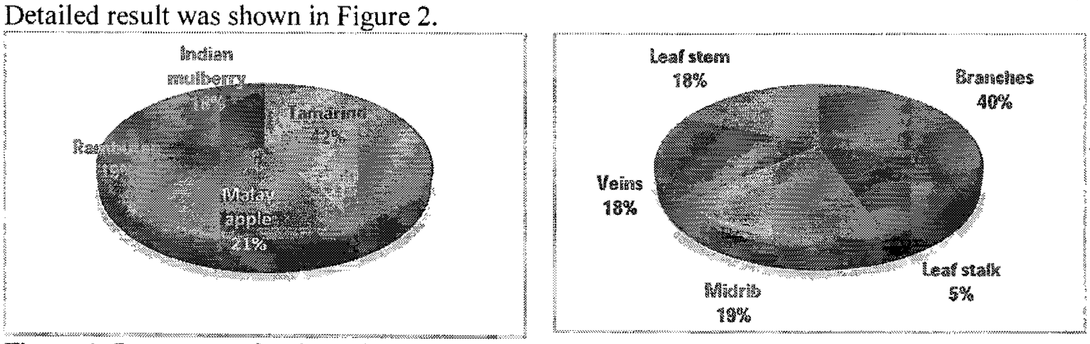

Figure 1. Percentage of endophytic fungi recovered from (Left) disease-free parts of tamarind, Malay apple, rambutan and Indian mulberry, and from (Right) different disease-free parts of host plants.

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,

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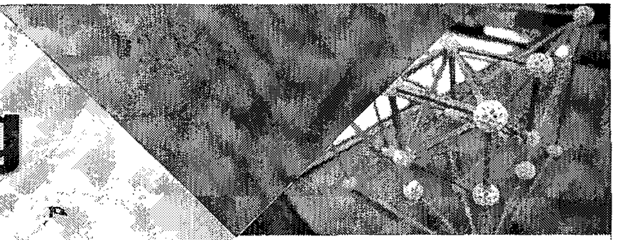

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CHEMISTRY EDUCATION

BIOLOGY

B -02

SYNERGISTIC EFFECT OF LOCAL GUAVA, NONI, CARAMBOLA AND KARIYAT EXTRACTS AND TETRACYCLINE IN INHIBITING THE GROWTH OF *Escherichia coli* AND *Salmonella* sp., CLINICALLY ISOLATED FROM YINGO HOSPITAL, NARATHIWAT PROVINCE, SOUTH THAILAND

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Abstract

The synergistic inhibition effect of the antibiotics Tetracycline with local guava, noni, carambola and Kariyat extracts on *in vitro* growth of diarrheal pathogens; *Escherichia coli* and *Salmonella* sp., clinically isolated from Yingo Hospital, Yingo District, Narathiwat Province, Southern Thailand was investigated using broth dilution technique. The solvents of herbal extraction included acetone, hexane, methanol, ethanol, and water. Results showed different degree of inhibition against *E. coli* and *Salmonella* sp. with the Minimum Inhibitory Concentration (MIC) values of 6.25 and 9.37 μg/mL, and the Minimum Bactericidal Concentration (MBC) values of 25 and 37.5 μg/mL, respectively. As for herbal extracts, growth inhibition of *E. coli* was observed with the MIC lowest value of 3.12 μg/mL (acetone-guava, hexane-guava, acetone–Kariyat extracts), and the lowest MBC of 25 μg/mL (acetone–kariyat extract). For *Salmonella* sp., it was found that the lowest MIC values was of 6.25 μg/mL (acetone-guava, methanol-guava, acetone-Kariyat, hexane-Kariyat, methanol-Kariyat, ethanol-Kariyat, methanol-carambola, ethanol-carambola, methanol-noni extracts), and the lowest MBC value was 50 μg/mL (acetone-noni, acetone-carambola, hexane-noni, hexane-carambola, hexane–guava, ethanol–noni, ethanol–guava extracts). Synergistic inhibiting effect of the tested herbal extracts with Tetracycline showed efficient results with the lowest MIC and MBC values for *E. coli* and *Salmonella*sp were of 0.78 μg/mL and 0.78 μg/mL, respectively. Synergistic effect of herbal extracts with Tetracycline was thus clearly shown, and therefore, of potential in clinical application after thorough and further detailed *in vitro* and *in vivo* investigation.

Key words: Synergy effect, Herbal plant extracts, MIC, MBC, Diarrheal pathogens

INTRODUCTION

Wide and extensive use of antibiotics in the treatment of infectious illness has largely caused emergence of resistant strains of infection. To name a few, these resistant strains included Methicillin-resistant and vancomycin-resistant nosocomial infection-causing *Staphylococcus aureus* (MRSA and VRSA) (Patterson, 2000; Centers for Disease Control and Prevention, 2001 & 2002; Chang et al., 2003), However, increase in efficacy of antimicrobial agents has been recently reported following in vitro use in combination with plant extracts

	MIC	TAC	MIC	
	MBC	AC	MBC	
	MBC	TAC	MBC	
	MIC	HC	MIC	
	MIC	THC	MIC	
	MBC	HC	MBC	
	MBC	THC	MBC	
	MIC	MC	MIC	
	MIC	TMC	MIC	
	MBC	MC	MBC	
	MBC	TMC	MBC	
	MIC	EC	MIC	
	MIC	TEC	MIC	
	MBC	EC	MBC	
	MBC	TEC	MBC	
	MIC	WC	MIC	
	MIC	TWC	MIC	
	MBC	WC	MBC	
	MBC	TWC	MBC	

Synergistic effect of antimicrobial agents on *in vitro* inhibition of microbial growth has been known for long but yet not fully established. Antibiotics-plant extract synergistic effect has, however, been reported. By being relied on case-to-case evaluation, the *in vivo* and clinical application is far limited because of difficulties relating to evaluation conditions and human or animal physiological factors (Bauman, 2015). Scattered reports on synergistic action of antimicrobial agents and plant extracts employing different pathogenic strains, extract and antibiotics types. In this study, synergy was observed in guava, kariyat, noni and carambola extracts used in combination with Tetracycline against clinical strains of *E. coli* and *Salmonella* sp., isolated from diarrheal patients admitted during 2014 to Yingo Hospital, Yingo District, Narathiwat Province, South Thailand. Finding results were more or less in consistent with previous studies on synergy. Adwan and colleagues (2009) reported *invitro* synergistic interaction of ethanolic seed extracts of *Rus coriaria,* and *Sacropoterium spinosum* as well as *Rosa damascena* flower extract with a number of antimicrobial drugs including oxytetracycline HCl, penicillin G, cephalexin, sulfadimethoxine and enrofloxacin against clinical isolates of methicillin-resistant *Staphylococcus aureus,* and suggested that those of competitive and protein synthesis inhibitors showed high synergism rate with plant extracts, while nucleic acid synthesis inhibitor showed no effect. Ahmed and co-workers (2009) investigated inhibitory effect of penicillin and tetracycline against *S. aureus* individually and in combination with ethanol leaf and stem extract of S*alvadora persica,* and found the highest synergistic effect on *S. aureus* growth upon exposing to Tetracycline-*S. persica* stem extract. Tetracycline-*S. persica* leaf extract effect stood second, while its stem and leaf extract with penicillin showed no effect. By using the time-kill and the Chekerboard methods, Aiyegoro and co-workers (2009) were able to showed varying degree of synergistic response from testing the acetone, chloroform, ethyl acetate and methanol extract of *Helichrysum longifolium* in combinationwith penicillin G sodium, amoxicillin, chloramphenicol, oxytetracycline, erythromycin and ciprofloxacin against a panel of referenced, clinical and environmental bacterial isolates. For Time-Kill method, the μg/mL (guava and carambola), and 37.5 μg/mL (kariyat and noni). Synergistic action exerted by Tetracycline-guava, kariyat, noni and carambola on growth of *E. coli* and *Salmonella* sp. was clearly observed with indifferent values of MICs below 0.39026 μg/mL and 0.5859 μg/mL. However, their MBCs for *E. coli* were ranged from below 0.39026 μg/mL to 3.125 μg/mL (for guava and carambola) and 6.25 μg/mL (noni). Whereas, their MBCs on *Salmonella* sp. were from below 0.5859 μg/mL to 4.6875 μg/mL. In summary, synergistic action of guava, noni, acarambola and kariyat extract with Tetracycline was achieved in our in vitro evaluation on growth of clinically resistant strains of E. coli and Salmonella sp., isolated from diarrheal patients, who were admitted to Yingo Hospital, Yingo District, Narathiwat Province, South Thailand.

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