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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

(Adwan et al., 2008; Chang et al., 2007; Horiuchi et al., 2007; Ahmad & Aqil, 2007; Betoni et al., 2006; Esimone et al., 2006; Ebezim et al., 2006; Yang et al., 2005; Aqil et al., 2005; Braga et al., 2005; Darwish et al., 2002). The so-called synergistic effects can be produced if the constituents of an extract affect different targets or interact with one another in order to improve the solubility and thereby enhance the bioavailability of one or several substances of an extract (Wagner & Ulrich-Merzenich, 2009). Combining use of antimicrobials from microorganism and plant origin offers advantages like increase of efficiency, reduction of undesirable effects, increase in stability or bioavailability of the free agents and obtaining an adequate therapeutic effect with relatively small doses, when compared with a synthetic medication (Inui et. al., 2007) In addition, use of herbal medicines has been known and regular practised for thousands of years. The advantages of this type of therapeutics include good availability, local cultural aspects, individual preferences, the increasing demand for natural and organic products, and the already validated synergistic effects of herbal medicines (Carmona & Pereira, 2013). Synergistic effect of some plant extracts with antibiotics including Tetracycline and others against gram-positive and gram-negative has previously been reported (Nascimento et al., 2000; Adwan et al., 2010; Hussin & El-Sayed, 2011; Subramaniam et al., 2014; Veras et al., 2012; Mandal et al., 2010; Chusri et al., 2014: Adwan & Mhanna, 2008; Hemaiswarya & Doble, 2009; Kyew et al., 2013). With numerous reports on the effective plant extracts or chemicals against bacterial pathogens, here we investigated the synergistically antibacterial effect of Tetracycline and the acetone, hexane, methanol, ethanol, and water-extracts of local guava, Kariyat, noni, and carambola against strains of *Escherichia coli* and *Salmonella* sp., possibly pathogenically resistant strains, which were clinically isolated from diarrheal patients, who were admitted to Yingo Hospital, Yingo District, Narathiwat Province, South Thailand.

RESEARCH METHOD

Herbal plants. Leaves of Guava (*Psidium guajava* Linn.), noni (*Morinda citrifolia* Linn.), and carambola or star fruit (*Averrhoa carambola*Linn.) were obtained by the courtesy of villagers of Kubang Badak, Phase No. 4, Lubok Bayah Sub-district, Yingo District, Narathiwat Province, South Thailand. Kariyat (*Andrographis paniculata*Wall ex Ness) was, however, supplied as a contribution from Royal Phikulthong Agriculture Center, Narathiwat Province. Only healthy, clean and mature herbal plant leaves and Kariyat stems were selected and separately filled in sterilized plastic bag, and transported to the Laboratory of Microbiology Program, Center of Science and Applied Science Building, Faculty of Science, Technology and Agriculture, Yala Province, South Thailand. In the Laboratory, these plant parts were drained for cleaning using tap water, and followed by sterilized distilled water. They were cut into pieces and dried in hot air oven before these parts were blended. Dried herbal plant parts were stored in the sterilized plastic bags until further use of investigation.

Herbal plant extraction. For each herbal plant, 150 g of dried and grinded parts was soaked into 500 mL of each solvents, i.e. water, ethanol (Avantor, USA), methanol (Avantor, USA), acetone (Avantor, USA) and hexane (Merck, USA) in 2.5 L-bottles. The solvent-herbal plant mixtures were then kept shaking at 40^{O}C, 160 rpm using water bath shaker (Digital Orbital Shaker, Korea) for 2 days. The solution of each extract was filtered using Whatman No.1 filter paper, and concentrated using Rotary Evaporator (Shenshun R2OSD, China). Complete dried extracts were achieved using water bath (Memmert, Germany). The extracts were filled in sterilized universal bottle, and they were stored in refrigerator at 4^{O}C for further evaluation.

Tested Microorganisms. *Escherichia .coli* and *Salmonella* sp., clinically isolated from diarrheal patients during their treatment were given as a courtesy of the Unit of Medical Technology, Department of Pathology, Narathiwat Ratchanakharin Hospital, Capital Province, Narathiwat, South Thailand. Cultures were prepared on Brain-heart Infusion Agar (BHIA)

(Difco, USA) in parafilm-sealed disposable plastic petri disc, bottled with tightly sealed and transported in refrigerated condition to the Laboratory of Microbiology, Microbiology Program, Department of Science, Center of Science and Applied Science Building, Yala Rajabhat University, Yala, South Thailand. These clinical strains of bacteria were sub-cultured in BHI broth (BHIB) for 18 hours at 37^{O}C, re-tested for their purity using biochemical characteristics, and stored in BHIA slants at 4^{O}C until further use. To standardize the tested bacteria, they were grown in BHIB for 18 hours at 37^{O}C, read and adjusted their count with Mc Farland standard No. 0.5 ($5x10^{5}$ cfu/mL) using UV-VIS Spectrophotometer ((LIBRA S32, England)).

Antibiotics. Tetracycline used in this test was that of Oxytetracycline (H. K. Pharmaceutical), and the stock solution was prepared by diluting in sterilized phosphate buffer saline (PBS), pH 7.2 to make up the starting concentration of 25,000 g/mL. For testing the Minimal Inhibition Concentration (MIC), its serial 2-fold dilution was made using sterilized Mueller Hinton Broth (MHB) (Himedia, India) as a solvent (EUCAST, 2003).

MIC Determination of Tetracycline and herbal plant extracts (HPEs). Minimal inhibition concentration of Tetracycline was determined using tube dilution method (Anu Kiruthika, 2011; EUCAST, 2003), and done in duplicate. Initially, stock solution of Tetracycline was serially 2-fold diluted using sterilized MHB making starting tube with concentration of 100 µg/mL and 150 µg/mL, making the last tubes at 0.512 µg/mL, and 0.5859 µg/mL as for *E. coli* and *Salmonella* sp, respectively. For determination of the MIC, the working mixture contained 0.1 mL tetracycline, 0.1 mL bacterial inoculum and 1.0 mL sterilized MHB. After 24-hour incubation at 37^{O}C in incubator, all tubes were read for growth turbidity. In determining the minimal bactericidal concentration (MBC), a loopful of 24-hour resulting culture was streaked onto BHIA, and growth observed after 24 hours of incubation at 37^{O}C was read and determined.For each herbal plant extract (HPE), 2 mg was diluted in 10 mL sterilized distilled water, and proceeded with serially 2-fold dilution, and lasting at 0.7813 µg/mL. In determining MICs and MBCs, the same volume and condition used with tetracycline was applied.

Synergistic effect of extracts-antibiotics. The starting concentration for tetracycline and each HPE employed was those of MIC values. They were serially 2-fold diluted further using sterilized PBS, pH 7.2. Inoculum mixture for this evaluation test composed of 0.1 mL each of diluted tetracycline and HPE, 1.0 mL of MHB, and 0.1 mL of standard bacterial culture. All tests were done in duplicate, and incubation was made at 37^{O}C for 24 hours. For determining MBC, the same conditions for tetracycline and extracts were applied.

RESULT AND DISCUSSION

Quantity yield of dried HPEs. With herbal plants of 150 g in each solvent of 500 mL, different quantities of dried extracts were obtained (Figure 1). Guava leaf extracted by hexane gave higher yield than other solvent. Whereas, noni and carambola highest extraction yield were achieved using water. Solvent for higher extraction yield for kariyat was methanol. As regard to the efficiency of each solvent in herbal plant extraction, it was no mean to conclude that a particular solvent was best solvent for all types of herbal plants.Since each herb plant varies in composition, the yield of extract was also different. Moreover, water content in herbal plants effected the total yield of extract. Other contents are alkaloids, phenolic compounds, flavonoids, terpenoids, saponins, cardiac glycosides, and proteins, whose quantities are varied with types of plants (Khanam, 2015).

MIC of Tetracycline and HPEs. In determining the minimal inhibition concentration of Tetracycline and extracts of guava, kariyat, noni and carambola using the clinical strains of *E. coli* and *Salmonella* sp., it was found that MICs of Tetracycline for *E. coli* and *Salmonella* sp.

were 6.25 µg/mL and 9.37 µg/mL, respectively. However, the MBCs of Tetracycline were 25 µg/mL and 37.5 µg/mL. Individually, HPEs showed different MIC and MBC values even within a type of plant but different solvent (Table 1). Overall, acetone was seen to be the most efficient solvent for antimicrobial extraction, contrasting with water, which showed least efficient. For instances, acetone-extracted guava and kariyat gave the lowest values of MIC at 1.5623 µg/mL, with those of noni and carambola at 3.126 µg/mL. Next efficient solvents for antimicrobial extraction were hexane, methanol and ethanol. Values of MBC for water-extracted HPEs were more than 200 µg/mL.

Synergistic effect of HPE-Tetracycline. With fixed concentration of Tetracycline at 6.25 µg/mL for *E. coli* and 9.37 µg/mL for *Salmonella* sp., the HPEs were evaluated for synergistic antimicrobial activity, and it was observed that synergy was achieved as it showed by the reduction of all MIC and MBC values (Table 2)

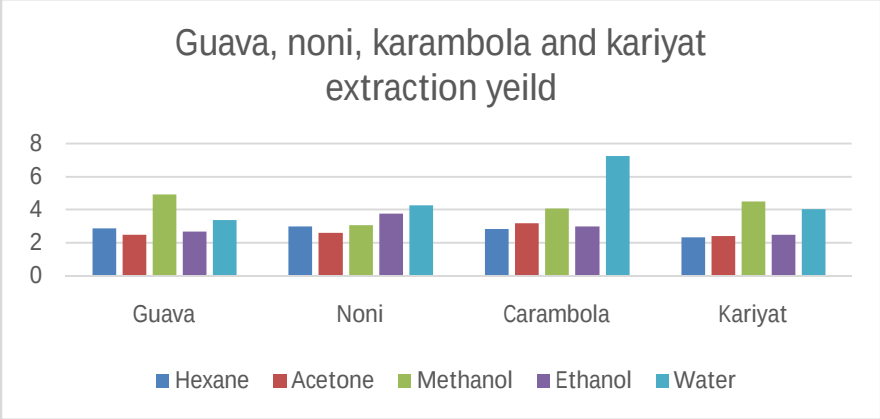

Figure 1. Quantity of HPEs (in gram) resulted from extraction process using hexane, acetone, methanol, 95% ethanol, and distilled water as the solvents for extraction

Regardless of the extracting solvents used, MICs of all extracts were lower than 0.78125 µg/mL. Whereas, the extract MBCs were also reduced in varying degree. When the MIC and MBC values of individual HPEs were compared with those of Tetracycline-HPEs, synergistic effect with varying degree was observed. As for guava, for examples, synergistic MIC decreased at least 2 fold and more, so did the MBC, which decreased with magnitude of up to 8 fold. Similarly, kariyat, noni and carambola MICs were found decreasing with magnitude of 2 fold or even more. Magnitude decrease of MBCs found with those of kariya, noni and carambola ranged from 2 to 128 folds (Table 2).

Table 1.Minimal Inhibition and Bactericidal Concentrations (MIC and MBC) of Tetracycline and HPEs evaluated on growth of *Escherichia Coli* and *Salmonella* sp. Extracts [(a)]represent guava (G), kariyat (K), noni (N) and carambola (C) extracted using acetone (A), hexane (H), methanol (M), ethanol (E) and water (W). Numbering 1 to 9 referred to serial tubes and their respective petri plates for MIC and MBC, with concentration of Tetracycline (T) for *E. coli* (from 100 µg/mL and its 2-fold gradual decrease to 0.39 µg/mL) and for *Salmonella* sp. (from 150 µg/mL to 0.58 µg/mL) and extracts (from 200 µg/mL to 0.78 µg/mL). Shaded boxes represented for no growth in tubes and petri plates, and unshaded boxes for growth. ND referred to "Not done", i.e. test was not performed.

	9	8	7	6	5	4	3	2	1	MIC/MBC results in serial tubes/plates of Tetracycline and HPEs [(a)]			1	2	3	4	5	6	7	8	9	
Escherichia coli										MIC	T for *Escherichia coli*	MIC	ND									*Salmonella* sp.
										MBC		MBC	ND									
	ND									MIC	T for *Salmonella* sp.	MIC										
	ND									MBC		MBC										
										MIC	AG	MIC										
										MBC		MBC										
										MIC	HG	MIC										
										MBC		MBC										
										MIC	MG	MIC										
										MBC		MBC										
										MIC	EG	MIC										
										MBC		MBC										
										MIC	WG	MIC										
										MBC		MBC										
										MIC	AK	MIC										
										MBC		MBC										
										MIC	HK	MIC										
										MBC		MBC										
										MIC	MK	MIC										
										MBC		MBC										
										MIC	EK	MIC										
										MBC		MBC										
										MIC	WK	MIC										
										MBC		MBC										
										MIC	AN	MIC										
										MBC		MBC										
										MIC	HN	MIC										
										MBC		MBC										
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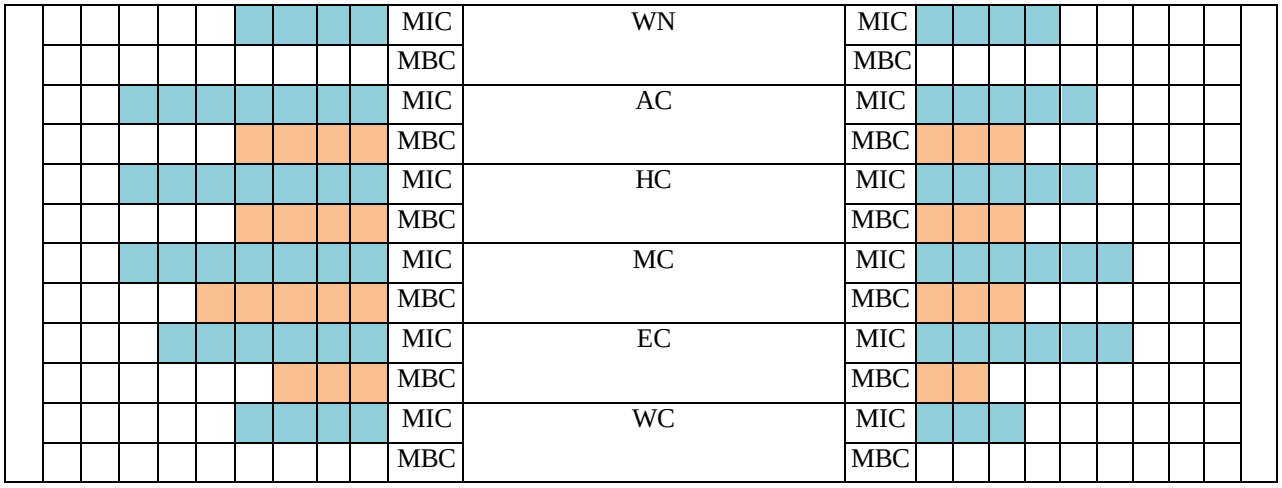
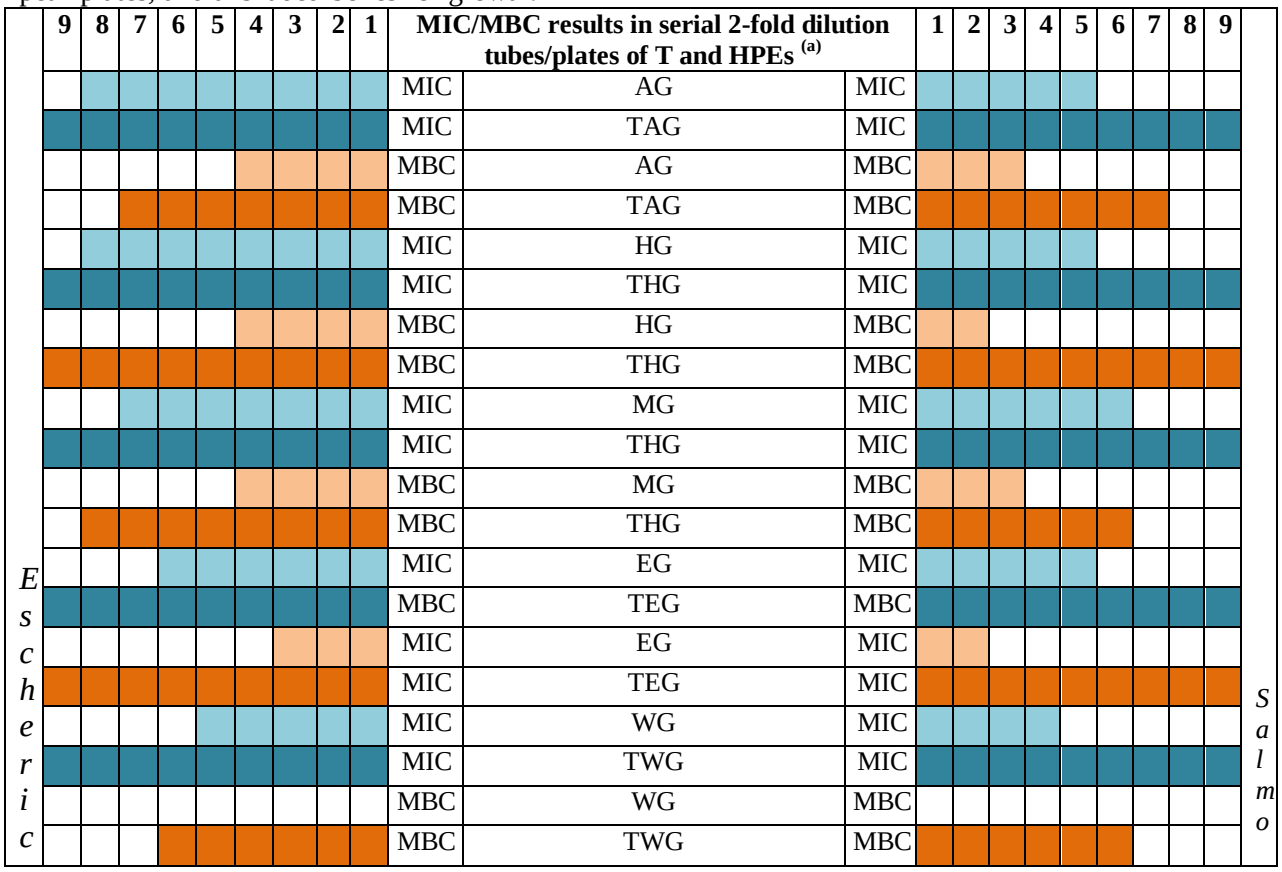


Table 2.Individual effect of HPEs as compared with Tetracycline-HPEsynergistic effect on Minimal Inhibition and Bactericidal Concentration (MIC and MBC) values evaluated on growth of *Escherichia Coli* and *Salmonella* sp. Extracts [(a)]represented guava (G), kariyat (K), noni (N) and carambola (C) extracted using acetone (A), hexane (H), methanol (M), ethanol (E) and water (W). Numbering 1 to 9 referred to serial 2-fold dilution tubes and their respective petri plates for MIC and MBC, and representing varying concentration of extracts (from 200 µg/mL to 0.78µg/mL), and fixed concentration of Tetracycline (T) of 6.25 µg/mL (for *E. coli*), and 9.37 µg/mL (for *Salmonella* sp.). Shaded boxes represented for no growth in tubes and petri plates, and unshaded boxes for growth.



hiacoli										MIC	AK	MIC									*nellasp.*
										MIC	TAK	MIC									
										MBC	AK	MBC									
										MBC	TAK	MBC									
										MIC	HK	MIC									
										MIC	THK	MIC									
										MBC	HK	MBC									
										MBC	THK	MBC									
										MIC	MK	MIC									
										MIC	TMK	MIC									
										MBC	MK	MBC									
										MBC	TMK	MBC									
										MIC	EK	MIC									
										MIC	TEK	MIC									
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										MIC	TWK	MIC									
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										MIC	TWN	MIC									
										MBC	WN	MBC									
										MBC	TWN	MBC									
										MIC	AC	MIC									

									MIC	TAC	MIC								
									MBC	AC	MBC								
									MBC	TAC	MBC								
									MIC	HC	MIC								
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									MIC	WC	MIC								
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									MBC	WC	MBC								
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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

extent of response for synergy, indifference, and antagonism were 65%, 28.33% and 6.67%, respectively. In Checkerboard method, results were 61.67% synergistic, 26.67% indifference, and 11.66% antagonistic.

Synergy was also reported for norfloxacin, tetracycline and erythromycin with ethanol peel extract of *Mangifera indica* L. against *S. aureus* strains (Souto de Oliveira et al., 2011). Individually, the extract displayed no significant antibacterial activity (MIC ≥ 2048 µg/mL), but with Tetracycline or Erythromycin, it modulated the activity of antibiotics (MIC = 512 µg/mL). With gentamicin, cephalothin, ceftriaxone and nystatin against 13 microbial species, Toroglu (2011) showed *in-vitro* synergistic effects of different spices and herbs i.e *Rosmarinus officinalis, Coriandrum sativum, Micromeria fruticosa* L., *Cumium cyminum, Mentha piperita,* and consequently suggested dual actions of essential oils in tested spices and herbs that on one hand protected some bacterial strains, and on the other hand upon combining with antibiotics, drug resistance was reduced. Using Checkerboard technique, Adikwu and co-workers (2010) investigated synergy effect resulted from *in vitro* interactionof erythromycin and methanol *Euphorbia hirta* leaf extract on growth of clinical *S. aureus* isolates. *S. aureus* showed sensitivity to the extract and erythromycin alone with MICs of 25 mg/mL and 0.005 mg/mL, respectively. Combination of erythromycin and *E. hirta* in the ratios of 9:1, 8:2, 7:3, 6:4, 3:7, 2:8, and 1:9 resulted in synergistic effect against *S. aureus*.

Doxycycline and ofloxacin with ethanolic *Vangueria spinosa* leaf extract were reportedto showed synergistic effect on pathogenic bacteria (Chatterjee et al., 2009). Synergistic action of oxytetracycline and methanolic extract of *Thespesia populnea* on 12 different Gram positive and Gram negative bacteria showed MIC values ranging from 62.5 µg/mL to 1000 µg/mL (Saravana Kumar et al., 2009). Standard antibiotics and ethanolic *Ficus exasperata* leaf extract synergistically actedon growth of *E. coli* and *S. albus* with MIC values of 300 mg/mL and 700 mg/mL, respectively (Odunbaku et al., 2008).

On mechanisms of antibiotics-plant extract synergy, the precise actions are still unknown. But it is believed that this synergy is effected by a multitarget action of compounds on a molecular level or partly by an improved resorption rate and a change of pharmacokinetic (Wagner, 2006). However, advance in the field of drug synergy research may lend with standardized plant extracts a new legitimacy and may open the chance to use extract combinations for the treatment of diseases which previously have been reserved for chemotherapeutics only. In addition, the understanding of the molecular mechanisms of synergy would pave a new strategy for the treatment of infectious diseases, overcome drug-resistant pathogens, and decrease the use of antibiotics and hence the side effects created by them.

CONCLUSION AND SUGGESTION

Diarrheal pathogens and possibly the resistant strains; *Escherichia coli* and *Salmonella* sp., clinically isolated from Yingo Hospital, Yingo District, Narathiwat Province, South Thailand showed indifference in sensitivity to Tetracycline and local HPEs of guava, noni, carambola and kariyat. MIC and MBC of Tetracycline for *E. coli* was 6.25 µg/mL and 25 µg/mL. Whereas for *Salmonella* sp., were 9.37 µg/mL MIC and 37.5 µg/mL MBC. Individually, each HPE showed varying degree of growth inhibition. For guava, kariyat, noni, and carambola on *E. coli*, MIC ranged from 6.25-0.7813 µg/mL, 6.25-0.7813 µg/mL, 3.125-1.5625 µg/mL, and 12.5-1.5625 µg/mL, respectively. Their MICs on *Salmonella* sp., were ranged 18.75-4.6875 µg/mL, 18.75-4.6875 µg/mL, 9.35-4.6875 µg/mL, and 37.5-4.6875 µg/mL. As for MBCs, values for *E. coli* were exceeding 100 µg/mL to 6.25 µg/mL (kariyat and carambola), and to 12.5 µg/mL (guava and noni), whereas for Salmonella sp. values were exceeding 150 µg/mL to 4.6875

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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