

THE POTENTIAL CO-TREATMENT EFFECTS OF THREE PLANT EXTRACTS AND THREE ANTIBIOTICS ON MULTIDRUG-RESISTANT BACTERIA.

Wisatre Kongcharoensuntorn^a*, Chintana Chirathaworn^b, Thitiporn Dechdougchan^a, Wipaphorn Jaikua^a,
Sungwan Hangla^a, Benjamas Sang Fai^a, Patra Rattanasinchiboon^a, Kaikwan Jaimeang^a

a Department of Biology, Faculty of Science, Burapha University, Chonburi 20131, Thailand.

b Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

ABSTRACT

The study was aimed to investigate the co-treatment effects of three Thai medicinal plants and three antibiotics on multidrug-resistant bacteria. The results indicated that the usage of medicinal plant extracts and antibiotics could inhibit the growth of multidrug-resistant bacteria: *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* better than the usage of plant extracts alone. The co-treatments of three antibiotics with *Dracaena loureiri* Gagnep, *Mansonia gagei* Drumm, and *Myristica fragrans* Houtt exhibited inhibitory effects on *A. baumannii* and *P. aeruginosa*. The minimum inhibitory concentration (MIC) values of dual treatments were lower than those values of individual treatments 16-32 times against *A. baumannii*, and 4-16 times against *P. aeruginosa*, respectively. In contrast, there was an antagonistic effect among the combination of *loureiri* and ampicillin against MRSA. It was detected by flow cytometry assay that *D. loureiri*, *M. fragrans*, and *M. gagei* enhanced bacterial permeability. The crude extracts of these plants could enhance the uptake of ethidium bromide into cells of multidrug-resistant *A. baumannii* and MRSA. The lowest concentration of *D. loureiri*, *M. fragrans* and *M. gagei* that could enhance the permeability of *A. baumannii* was 0.1, 0.5, and 5 mg/ml, respectively. The co-treatment of herb extracts by using 0.1 mg/ml each of *D. loureiri* and *M. fragrans* could increase the uptake of ethidium bromide into cells of MRSA.

Keywords: Co-treatments; plants, *Dracaena loureiri*, *Mansonia gagei*, *Myristicocsaе fragarus*; antibiotics; multidrug-resistant bacteria; flow cytometry.

* Corresponding author: Email address: wisatrek@yahoo.com

INTRODUCTION

Antibiotic resistance is one of the serious problems and impacts on the treatment of infectious diseases. Antibiotic resistance in bacteria is caused by innate genetics and physiology that are transmitted through species, and exchanged genetic material horizontally across species and genera. This combination of genetic strategy has resulted in accumulation of multidrug-resistant phenotypes in many species of bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Antibiotics have many modes of actions to inhibit protein synthesis by inhibiting ribosome functions, inhibit DNA replication and repair the binding to DNA Gyrase complexed with DNA, inhibit cell wall synthesis by inhibitor such as β -lactams (Wright and Sutherland, 2007).

Plants are a source of antibiotic agents used in the treatment of diseases. In Thailand, some plant species have traditionally been used in the treatment of several diseases, i.e., malaria, fungal and bacterial infections (Taylor and Attaur, 1994). Most remedy recipes of Thai medicinal plants (*Dracaena loureiri* Gagnep, *Mansonia gagei* Drumm, and *Myristica fragrans* Houtt.) have been applied for medicinal treatment, dietary supplements, and as gradients of some beverages (Bauer, 1985; Taylor and Attaur, 1994; Harborne and Baxter, 1995). It contains many bioactive compounds that can be a great significance in therapeutic treatment such as camphene, coumarins, euglenol, methoxyeuglenol, piene, sabinene, safrol, elemicin, euglenol, isoelemicin, isoflavans, retroridhydrochalcones, and lignan compounds (Meksuriyen and Cordell, 1988; Park et al., 2004; Capasso et al., 2000; Sonavane et al., 2002; Tiew et al., 2002; Morita et al., 2003). However, the mechanisms of these compounds that contribute to antibacterial drug resistance are not well understood. Indeed, some evidences have shown that sesquiterpenoids, i.e., nerolidol and farnesol, can enhance membrane permeability of membrane lipids, interfere permeability barriers of cell membrane and promote interaction with the interior of the lipid bilayer of cell membrane (Cornwell and Barry, 1993; Cornwell et al., 1996; Brehm and Johnson, 2003).

Several recent studies have commonly reported the comparative and combination studies on the antibacterial activity of phytochemicals and antibiotics against multidrug-resistant bacteria. It was found that the mixtures of herbs and antibiotics inhibited the growth of *A. baumannii*, MRSA, and *P. aeruginosa* (Tsuchiya et al., 1996; Muroi et al., 2004; Park et al., 2004; Kitahara et al., 2005). The evidence of infectious diseases caused by multidrug-resistant bacteria urged us to search for new antibacterial substances from many sources of plants, and new strategies in using a combination of herb treatment with antibiotics. It has been documented that some cellular targets of bacterial resistance to antimicrobial drugs are consisting of activating antibiotic efflux from the cell interior and prevention of entry of compound into the cell, altering permeability of the cell membrane to reduce drug uptake, inactivating antimicrobial agent such as hydrolyzing their β -lactam ring, and changing bacteria's cellular targets by mutation of target sites (Nikaido, 1994; Walsh, 2000; Wright and Sutherland, 2007).

One specific hypothesis to combat multidrug-resistant bacteria is to increase the uptake of antibacterial agents into bacterial cells by interfering with cellular permeability barriers of cell membrane and cell wall structures of microorganisms. We hypothesized that herb extracts improve the entrance of antibiotics to their target sites through bacterial membrane to bypass bacterial resistance. Our hypothesis was supported by the evidence of uptake of ethidium bromide into bacterial cells either with or without herb extracts. Because of acting as intercalating properties between the bases of DNA, ethidium bromide, it can be used as a probe to monitor the accumulative substances that pass through the membrane barrier and go to intercellular organelles and nucleus of the cells. Moreover, ethidium bromide gives fluorescent signals when cell damages or compromise occurred; thus, ethidium bromide can be used to examine the permeability of plant extract into bacterial cells (Brehm and Johnson, 2003; Midoux et al., 1995). To support the hypothesis, we observed an increasing of fluorescent signals in multidrug-resistant *A. baumannii* and MRSA in

parallel with an increasing of the concentrations of herb extracts.

In this study, we investigated the potential benefits of combination approach using antibiotics and Thai plant extracts for treatment of multidrug-resistant bacteria. We also investigated potential effects of some Thai plant extracts to mediate permeability of cell membrane of multidrug-resistant bacteria. The procedure was designed to detect an uptake of ethidium bromide by enhancing with a ranged concentration of herb extracts in living multidrug-resistant bacteria.

MATERIALS AND METHODS

Preparation of plant extracts

Parts of *Dracaena loureiri* Gagnep, *Mansonia gagei* Drumm, and *Myristica fragrans* Hoult were ground until a fine powder was obtained. The powder products were dried using a constant weight in desiccant at room temperature. In order to gain the most polyphenols from the extraction, we followed the method described by Cheng et al. (2003). The fine powder of herbs (100 g) was extracted using three volumes (300 ml) of 80% methanol for 48 hours at room temperature and subsequently kept at 4°C for 24 hours. The extracts were filtered through Whatman filter-paper No. 4 and then evaporated by rotary evaporator at 50°C. The extract was dissolved in dimethyl sulfoxide (DMSO) and filtered through 0.2 µm Millipore filter.

The stock solution of plant extracts consisting of 40 mg of crude extracts / 10% of DMSO was prepared as two-fold serial dilutions to cover a range from 10 mg/ml to 0.625 mg/ml and stored in the dark at 4°C.

Bacterial strains

The multidrug-resistant bacteria: *A. baumannii*, methicillin resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* were provided by Chulalongkorn University Hospital, Bangkok, Thailand. All multidrug-resistant strains are resistant to the specified antibiotics, i.e., *A. baumannii* is resistant to gentamicin, piperacillin/tezobactam, ceftriaxone,

ceftazidime, imipenem, meropenem, and cefepime. MRSA is resistant to oxacillin, clindamycin, erythromycin, cotrimoxazole, sulbactam/ampicillin, gentamicin, and cefoxitin. *P. aeruginosa* is resistant to gentamicin, netilmicin, amikacin, sulperazon, piperacillin/tazobactam, ceftriaxone, ceftazidime, imipenem, meropenem, and cefepime.

Agar diffusion susceptibility test

Antimicrobial activities of three herbs and to antibiotics alone were tested by agar diffusion susceptibility test (Barry and Thornberry, 1999). Bacterial suspensions were diluted to match the 0.5 MacFarland standard scales (approximately 1.5×10^8 CFU/ml). Then, 1 ml of adjusted bacterial suspensions was mixed with 19 ml of Mueller Hinton Agar (MHA) to make solid plates. The 0.6 cm diameter of punched hold was loaded with 40 µl of different amounts of plant extracts, or antibiotics (Ampicillin, Tetracycline and Chloramphenicol) by two-fold serial dilution of concentrations, and then incubated at 37°C for 24 hours. Triplicate tests were made for each experiment. The diameter in cm of inhibition zones around each of the holes was measured and recorded at the end of the incubation time.

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) of three plant extracts, of antibiotics alone, and of the combinations of plant extracts with antibiotics were determined by agar diffusion susceptibility tested as described before. Multidrug-resistant bacteria were inoculated with 1.5×10^8 CFU/ml onto Mueller Hinton agar plates (Merck, Germany) and were incubated at 37°C for 24 hours. MICs were defined as the lowest concentrations of plant extracts and antibiotics at which no visible growth were recorded.

Checkerboard dilution assay

The 1 ml of adjusted bacterial suspensions (1.5×10^8 CFU/ml) in each test sample was mixed with 19 ml of Mueller Hinton Agar (MHA) to make solid plates. The 0.6 cm diameter of punched hold was loaded with 20 µl of two-fold serial concentrations of herb extracts and 20 µl of two-fold serial

concentrations of antibiotics (ampicillin, tetracycline and chloramphenicol) started from 40 mg/ml. After 24 hours of incubation at 37°C, the synergistic MICs were recorded and compared to MICs of herb extracts and antibiotics alone. The efficiency of synergistic effects was indicated by titers as defined by the ratio of MICs of medicinal plant extracts, divided by MICs of co-treatment.

Flow cytometry assay

The ability of plant extracts to enhance permeability of multidrug-resistant bacterial cells was conducted by flow cytometry test of ethidium bromide uptake by *A. baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA). Ethidium bromide (Sigma) can intercalate double-strand DNA and becomes highly fluorescent when damaged cells or compromised cells are treated with this probe, and used to detect membrane integrity (Brehm and Johnson, 2003; Midoux et al., 1995). An overnight culture of non-multidrug-resistant (negative control) and multidrug-resistant strains (positive control) of *A. baumannii* and MRSA were grown at 37°C in Mueller Hinton broth (Merck, Germany). Cultures of positive and negative control organisms were then diluted to obtain the concentration of 10^7 CFU/ml in phosphate buffer saline (5.83 g of NaH_2PO_4 and 6.74 g of Na_2HPO_4 per liter of distilled water; pH 7.0). Fifteen μM of ethidium bromide was added to a positive control culture, and incubated at 55°C for 6 minutes. The cell that received 15 μM of ethidium bromide and 10% dimethyl sulfoxide (DMSO) was used for negative control test. The potential effects of plant extracts to mediate permeability of cell membrane of multidrug-resistant bacteria were determined by treating the cells with various concentrations (0.01, 0.05, 0.1, 0.5, 1, 2.5, and 5 mg/ml) from three plant extracts (in 10% DMSO) and 15 μM ethidium bromide, and incubated for 40 minutes at 25°C. Cells were subsequently diluted 1:10 in the same buffer and examined using FACScan flow cytometer (BD Bioscience, San Jose).

Statistical analysis

Combination treatment data were analyzed by completely randomized block design of ANOVA (SPSS version 12.0 program, InStat, GraphPad Software, Inc., San Diego, CA). Differences between means of the flow cytometry experiments and control groups were analyzed by paired t-test.

RESULTS

Evaluation of antimicrobial activity of herb extracts and antibiotics

The data indicated the potential antimicrobial activity of herb extracts and antibiotics against three multidrug-resistant bacteria, *A. baumannii*, MRSA, and *P. aeruginosa*, as shown by agar diffusion susceptibility test. The results are summarized in Tables 1 and 2, respectively. The concentration of herb extracts used in the test was ranging from 0.32-40 mg/ml. Three herb extracts showed variable degrees of antimicrobial activity to multidrug-resistant bacteria (1.25-20 mg/ml of MICs). Herb extracts of *D. loureiri*, *M. gagei*, and *M. fragrans* inhibited growths of MRSA and *P. aeruginosa*, but did not exhibit antibacterial activity against *A. baumannii*. Among three antibiotics, chloramphenicol and tetracycline demonstrated strong antibacterial effect on MRSA and *P. aeruginosa* (0.32-10 mg/ml of MICs), whereas ampicillin did not show any effect against *A. baumannii*.

Table 1. Antimicrobial activity of three antibiotics on multidrug-resistant bacteria isolated from clinical specimens as determined by disc diffusion method.

Multidrug-resistant bacteria	Antibiotics	Minimum inhibition concentration (mg/l)
<i>A. baumannii</i>	Ampicillin	0
	Chloramphenicol	1.25
	Tetracycline	0.31
<i>P. aeruginosa</i>	Ampicillin	0
	Chloramphenicol	20
	Tetracycline	20
Methicillin-resistant <i>S. aureus</i>	Ampicillin	10
	Chloramphenicol	1.25
	Tetracycline	10

Table 2. Antimicrobial activity of herb extracts on multidrug-resistant bacteria isolated from clinical specimens as determined by disc diffusion method.

Multidrug-resistant bacteria	Plant species	Minimum inhibition concentration (mg/l)
<i>A. baumannii</i>	<i>D. loureiri</i>	0
	<i>M. gagei</i>	0
	<i>M. fragrans</i>	0
<i>P. aeruginosa</i>	<i>D. loureiri</i>	20
	<i>M. gagei</i>	20
	<i>M. fragrans</i>	20
Methicillin-resistant <i>S. aureus</i>	<i>D. loureiri</i>	10
	<i>M. gagei</i>	1.25
	<i>M. fragrans</i>	20

Synergistic effects of herb extract and antibiotics on multidrug-resistant bacteria

The co-treatment effect of three medicinal plants and antibiotics inhibited significantly all multidrug-resistant bacteria better than the treatment of herbs or antibiotic alone as shown in Tables 3-5. The combination of three antibiotics with *D. loureiri*, *M. gagei*, and *M. fragrans* exhibited inhibitory effect

to multidrug-resistant strain of *A. baumannii* with 8-32 times of the MIC titer lower than that of their single treatment of herbs. Moreover, it was found that the synergistic effect of three herbs and antibiotics against *P. aeruginosa* reduced the MICs of single treatment of herbs to 4-16 times (Table 5). The treatment of the combination among chloramphenicol with three herbs, and ampicillin with three herbs

could lower the titer of MICs to 4-16 times. However, the combination of *M. fragrans* and tetracycline was not effective. In contrast, the use of herbs and antibiotics against MRSA showed antagonist effects, such as treatment of *M. fragrans* with ampicillin.

Moreover, this combination of using herbs with antibiotics showed the most effective antimicrobial activity when the doses of low concentration of antibiotics (less than 2.5 mg/ml in estimation) were used.

Table 3. Synergistic effects of herb extract and antibiotics on multidrug-resistant bacteria, *A. baumannii*, as determined by disc diffusion method.

Plant species	Antibiotics used in co-treatment	MIC alone (mg/l)	Ratio of MIC of herb extract per MIC of co-treatment
<i>D. loureiri</i>	Ampicillin	1.25	32
	Chloramphenicol	1.25	32
	Tetracycline	1.25	32
<i>M. gagei</i>	Ampicillin	1.25	32
	Chloramphenicol	1.25	32
	Tetracycline	1.25	32
<i>M. fragrans</i>	Ampicillin	1.25	32
	Chloramphenicol	1.25	32
	Tetracycline	5	8

Table 4. Synergistic effects of herb extract and antibiotics on multidrug-resistant bacteria, methicillin-resistant *S. aureus*, as determined by disc diffusion method.

Plant species	Antibiotics used in co-treatment	MIC alone (mg/l)	Ratio of MIC of herb extract per MIC of co-treatment
<i>D. loureiri</i>	Ampicillin	20	1
	Chloramphenicol	1.25	16
	Tetracycline	20	1
<i>M. gagei</i>	Ampicillin	10	1.25
	Chloramphenicol	10	125
	Tetracycline	10	1.25
<i>M. fragrans</i>	Ampicillin	40	0.5
	Chloramphenicol	1.25	16
	Tetracycline	20	1

Table 5. Synergistic effects of herb extract and antibiotics on multidrug-resistant bacteria, *P. aeruginosa*, as determined by disc diffusion method.

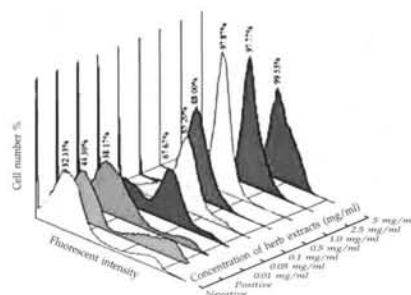
Plant species	Antibiotics used in co-treatment	MIC alone (mg/l)	Ratio of MIC of herb extract per MIC of co-treatment
<i>Dracaena loureiri</i>	Ampicillin	1.25	16
	Chloramphenicol	1.25	16
	Tetracycline	20	1
<i>Mansonia gagei</i>	Ampicillin	5	4
	Chloramphenicol	1.25	16
	Tetracycline	1.25	16
<i>Myristica fragrans</i>	Ampicillin	5	4
	Chloramphenicol	1.25	16
	Tetracycline	0	0

M. fragrans and *M. gagei* extracts enhance bacterial permeability of membrane by flow cytometry

The results indicated that *M. fragrans* and *M. gagei* could effectively enhance an accumulation of ethidium bromide in multidrug-resistant bacterial cells. The results of the flow cytometry experiments demonstrated that a dose response accumulation of ethidium bromide was induced by increasing the concentration of three herb extracts in bacterial cells of both *A. baumannii* and MRSA cells (Figures 6 and 7). In the treatment of *D. loureiri*, *M. fragrans* and *M. gagei* extracts, the cells of *A. baumannii* and MRSA were more fluorescent and more homogeneously stained than those of the bacterial cells treated with DMSO, and the treatment of ethidium bromide alone. After being exposed for more than 40 minutes, the herb extracts mediated staining of ethidium bromide and showed brighter and more uniformly signals when treated bacterial cells with herb extracts of more than 0.01 mg/ml. The lowest concentration of *D. loureiri*, *M. fragrans* and *M. gagei* which made *A. baumannii* cells show uniformly fluorescent were 0.1, 0.05 and 5 mg/ml, respectively (Figure 6, A-C). Increasing the dose of treatment to 0.01 mg/ml of *D. loureiri* or *M. fragrans* effectively permeabilized MRSA cells to

ethidium bromide (Figure 7, A -B). The results showed that the accumulation of ethidium bromide mediated by *D. loureiri*, *M. fragrans* and *M. gagei* had an impact on structure and functions of bacterial membrane in both gram-negative and gram-positive bacteria.

Figure 6. An enhancement of an accumulation of ethidium bromide in multidrug-resistant bacterial cells, *A. baumannii*, following the treatment of herb extracts. Results of cells treated with extract from *Dracaena loureiri* are shown in Figure A, cells treated with extract from *Myristica fragrans* are shown in Figure B and cells treated with extract from *Mansonia gagei* are shown in Figure C.



A

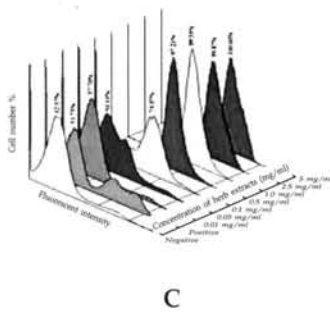
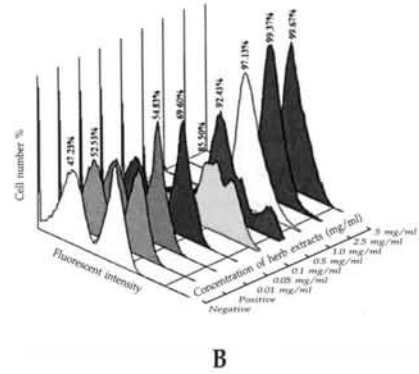
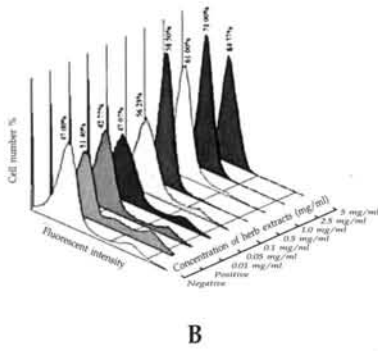
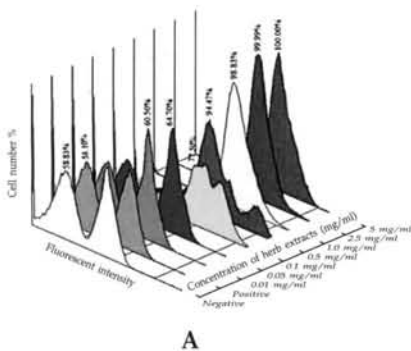


Figure 6. An enhancement of an accumulation of ethidium bromide in multidrug-resistant bacterial cells, methicillin-resistant *S. aureus*, following the treatment of herb extracts. Results of cells treated with extract from *Dracaena loureiri* are shown in Figure A, cells treated with extract from *Myristica fragrans* are shown in Figure B



DISCUSSION

The utilities of plant extracts as medicinal agents have been widely used as antimicrobial and anti-inflammatory effects against multidrug-resistant bacteria (Nascimento et al., 2000; Ahmad and Beg, 2001). Our results have shown that our tested plants can inhibit against multidrug-resistant *P. aeruginosa* and MRSA as was tested by other strains (Nascimento et al., 2000; Kumar et al., 2006). The different modes of actions of plant extract on bacterial activity are depended on different degree of antibacterial potency which may be caused by method of extraction, parts, and stage of plant sample. (Ahmad and Beg, 2001).

Our data have shown that the combinations of herb extracts with ampicillin and tetracycline strongly inhibit growth of multidrug-resistant bacteria, especially that of *A. baumannii* and *P. aeruginosa*, and are less effective on MRSA. This is due to an increase of permeability of outer membranes barrier of gram-negative bacteria (not being found in gram-positive bacteria) by interacting with cell membrane and/or lipopolysaccharide layer and around ampicillin and tetracycline easier to gain access to cytoplasmic target (Hancock, 1984; Park et al., 2004). Moreover, we found an antagonistic treatment, such as combination of *M. fragrans* and *M. gagei* with ampicillin or tetracycline, in the treatment of MRSA. Some bioactive compounds in crude extracts may interfere the activity of antibiotics. Also, we found some plant extracts, of which being separately used,

inhibit growth of antibiotic resistant bacteria, such as the treatment of tetracycline with *D. loureiri* inhibiting growth of *P. aeruginosa* and MRSA. Our results have shown that synergistic effects of herb extracts and antibiotics are useful for the treatment of multidrug-resistant bacteria, as those studied by Kak et al. (2000), Hu et al. (2001), and Sato et al. (2004). Antibacterial synergism of antibiotics and herbs and synthetic peptides has been reported. The combination of antibiotic peptides with chloramphenicol inhibited the growth of *S. aureus* and *P. aeruginosa* (Park et al., 2004). The combination of isoflavanone (bidwillon B) and mupirocin inhibited the growth of MRSA because mupirocin inhibited radio-labeled thymidine, uridine, glucose, and isoleucine incorporation into MRSA (Sato et al., 2004). The consequence of combination therapies could develop inhibitors of resistant enzymes or enhance accessibility of antibiotics to their site of action. The outcome of combination usage can rescue antimicrobial activity of the antibiotics that could not be used to kill multidrug-resistant bacteria.

Anacardic acids in combination with methicillin increased antimicrobial activity against MRSA when increasing the number of double bonds in the alkyl chain of anacardic acids. The antibiotic activity of anacardic acids was greater as the lengths of alkyl chain was longer up to maximum 10-12 carbon atoms, and their targets of actions were at lipophilic membrane of bacteria. Thus, penetrations of anacardic acids lead to bring methicillin pass to cytoplasmic targets of MRSA (Muroi et al., 2004).

We observed an increase of fluorescent signals in multidrug-resistant *A. baumannii* and MRSA when increasing the concentration of plant extracts. Thus, herb extracts can interfere with the permeability of membrane transportation, or interrupt with cell wall of multidrug-resistant bacteria, and allow ethidium bromide easier to access to cytoplasmic organelles and nucleus.

For application usage, combination of plant extracts and antibiotics can be a new choice for treatment of infectious diseases caused by resistant microorganism. This combination of usage can replace the use of respective antibiotics when it is no longer effective by itself. Our results suggest that the extracts of

D. loureiri, *M. gagei*, and *M. fragrans* may be used as enhancers of nonspecific permeability to antibiotics and antibacterial drugs.

In conclusion, our results suggest the co-treatments of three antibiotics with *D. loureiri*, *Mansonia gagei*, and *Myristica fragrans* have inhibitory effects to multidrug-resistant strains of *A. baumannii* and *P. aeruginosa*, but did not inhibit the growth of MRSA. Also, all three herb extracts may increase the uptake of ethidium bromide into the multidrug-resistant bacteria. The study of synergistic effects of herb extracts and antibiotics may be helpful for the therapy of some diseases caused by multidrug-resistant bacteria.

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