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Original Article

Synergistic activity of lawsone methyl ether in combination with some antibiotics and artocarpin against methicillin-resistant *Staphylococcus aureus*, *Candida albicans*, and *Trychophyton rubrum*

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ABSTRACT

Objective: One appealing strategy to overcome and prevent resistant problem is the use of combined two or more antibacterial substances. Lawsone methyl ether (LME) is the naphthoquinone found in the leaves of *Impatiens balsamina*. The objective of this study is to determine the interaction of LME with some antibiotics (ampicillin, tetracycline, norfloxacin, and clotrimazole) and a natural compound, artocarpin against methicillin-resistant *Staphylococcus aureus* (MRSA), *Candida albicans*, and *Trychophyton rubrum*.

Methods: A broth microdilution method was used to determine the minimum inhibition concentration (MIC). Synergistic effects were evaluated at their own MIC using the checkerboard method and time-kill assay.

Results: LME showed moderate antibacterial activity against MRSA with MIC value of 15.6 μg/mL, and exhibited strong antifungal activities against *T. rubrum* and *C. albicans* with MIC values of 7.8 and 3.9 μg/mL, respectively. The interaction of LME with the natural compound artocarpin against MRSA produced a synergy with fractional inhibitory concentration index (FICI) value of 0.31, while the combination of LME and clotrimazole exhibited synergy against *C. albicans* and *T. rubrum* with FICI values of 0.38 and 0.24, respectively. The time-kill assays confirmed that the compounds in combination enhanced their antimicrobial activities against the resistant microorganisms with different degrees.

Conclusion: LME in combination with clotrimazole exhibited synergy effect against *C. albicans* and *T. rubrum*. In combination with artocarpin, it showed synergy effect against MRSA.

1. Introduction

Infectious diseases caused by microorganisms, such as bacteria, fungi, and parasites are still a major health problem worldwide. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of major nosocomial pathogen that can cause an increasing of infection particularly in hospital and resulting in high morbidity and mortality (Qin et al., 2013). This bacterial strain is resistant toward many antibiotics, especially β-lactam antibiotics due to its ability to transfer of resistance genes including *mecA* gene (Mun et al., 2013). In addition, the commensal microbe, including *Candida albicans* may also cause systemic infection. The incidence of *C. albicans* infection was significantly increased, especially in immunocompromised patients (Sardi, Scorzoni, Bernadi, Fusco-Almeida & Mendes Giannini, 2013). Furthermore, superficial fungal infection has also resulted in increasing public health problem. *Trichophyton rubrum* infection is the most frequent fungal infection and causes skin infection. Dermatophytosis infected with *T. rubrum* can also lead to severe symptoms for patients, who have the high risk of infection (Soares et al., 2014). Although antibiotics have been used to eradicate the infections, however the irrational use of the regimens has led to the increasing incidence of antibiotics resistant. One appealing strategy to overcome this problem is the use of combination of two or more antimicrobial substances in order to enhance their activity (Wagner & Ulrich-Merzenich, 2009).

Plant-derived compounds are potential sources of antibacterial agents that can be used in combination with commercial antibiotics to achieve the synergy. Many classes of natural compound possessed antimicrobial activity, including naphthoquinones (Baker, Tatum & Nemec, 1990). Lawsone methyl ether (LME) (Fig. 1)

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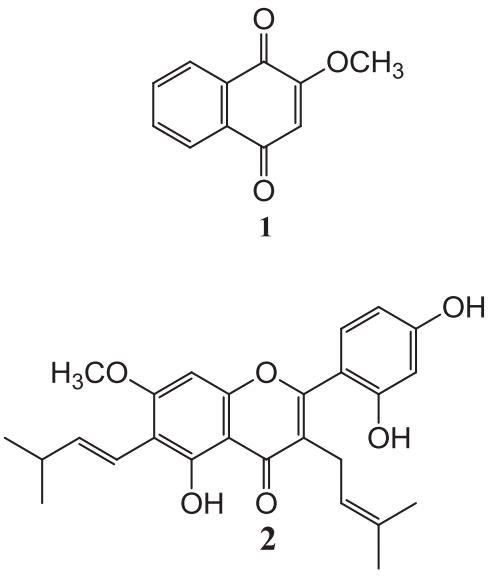

Fig. 1. Chemical structures of lawsone methyl ether (**1**) and artocarpin (**2**).

is one of such naphthoquinone compound that found in *Impatiens balsamina* L. It has been reported that LME displayed potent antifungal and antibacterial activities (Sakunphueak & Panichayupakranant, 2012; Yang et al., 2001). This compound also exhibited antitumor activity against HepG2, a hepatocellular carcinoma cell line (Ding, Jiang, Chen, Lv & Zhu, 2008). But, there is no data available concerning the activity of this compound in combination with antimicrobial substances. Therefore, this study was undertaken to investigate the interaction of lawsone methyl ether with commercial antimicrobials as well as natural compound, i.e. artocarpin (Fig. 1) against MRSA, *C. albicans* and *T. rubrum* in order to obtain the important insight of its synergistic effect.

2. Materials and methods

2.1. Chemicals

Lawsone methyl ether (LME) was prepared by methylation of lawsone in acid conditions as previously described by Panichayupakaranant and Raenmongkol (2002). Artocarpin was purified from the crude ethyl acetate extract of *Artocarpus heterophyllus* heartwoods using a method previously described by Septama and Panichayupakaranant (2015). The antibiotics, ampicillin, tetracycline, norfloxacin, and clotrimazole were from Sigma (Sigma-Aldrich, UK). Brain heart infusion (BHI), Sabouraud dextrose broth (SDB) and agar were from the Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA).

2.2. Microorganisms

Methicillin-resistant *Staphylococcus aureus* (DMST 20,654), *Candida albicans* (TISTR 5779), and *Trichophyton rubrum* were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

2.3. Determination of minimum inhibitory concentrations

The microdilution assay (NCCLS, 2008) with a slight modification was used to determine the MICs. Two-fold dilutions of each sample in BHI (MRSA) or SDB (*C. albicans* and *T. rubrum*) were prepared in a sterile 96-well plate. The cell suspensions were prepared in 0.85% NaCl, and the turbidity of the suspension was adjusted to the 0.5 McFarland standards (equivalent to 1×10^8 CFU/mL). This suspension was diluted with normal saline to contain (1×10^6) CFU/mL, and it was then added into each well. The final cell concentration was (5×10^5) CFU/mL. Meanwhile, a modified broth dilution through millipore filter was used to determine the MIC against *T. rubrum* (Koc, Silici, Ayangil, Ferahbas & Cankaya, 2005). Briefly, spore suspension was prepared in 0.85% NaCl. The optical density of suspension was read at 530 nm, and adjusted 0.15–0.17 to contain approximately (1×10^6) spores/mL. Afterward, the suspension was added to well to reach the final concentration (5×10^5) spores/mL. The plates were incubated at 37 °C for 24 h for MRSA and *C. albicans*, 5 d incubation at 26 °C for *T. rubrum*. The MIC was considered to be the lowest concentration of the sample that produced suppression of visible growth.

2.4. Checkerboard assay

The checkerboard method was used to determine the interaction of samples against tested microorganisms as described by Basri and Khairon (2012) with slight modification. This assay was conducted with LME in combination with antibiotics as well as artocarpin in 96-well plates. Briefly, LME was diluted by two-fold dilution along the *x*-axis of the plates, while two-fold dilution of antibiotics and artocarpin were prepared along *y*-axis. Subsequently, each well was inoculated with tested microorganisms (1×10^6 CFU/mL). The plates were then incubated at 37 °C for 24 h. The MIC was considered as the lowest concentration of the compounds alone, or in combination, required to inhibit the growth of microorganisms. The fractional inhibitory concentration (FIC) was defined as the MIC of LME and antibiotics/artocarpin in combination divided by the MIC of LME and antibiotics/artocarpin alone. The interaction between compounds was determined by calculating the fractional inhibitory concentration index (FICI), equal to the sum of FIC of LME and FIC of antibiotics/artocarpin. The results were considered as a synergy (FICI $\leq$ 0.5), additive (0.5 $<$ FICI $\leq$ 1), indifferent (1 $<$ FICI $\leq$ 4) or antagonistic (FICI $>$ 4) effects (Odds, 2003).

2.5. Time-kill assay

Time-kill assay was conducted for single compound as well as in combination in order to confirm any synergistic effect on the dynamic microbial kills as previously described (Basri & Sandra, 2016). The microbial suspensions contained (1×10^6) CFU/mL was added to broth containing the mixture of samples to reach the final cell concentration of (5×10^5) CFU/mL, which was incubated at 37 °C. Afterward, aliquots (50 µL) of the cultures were removed at eight time intervals of incubation (0, 1, 2, 4, 6, 8, 12, and 24 h), and ten-fold serial dilutions were prepared in normal saline. Then, 20 µL of each dilution was cultured on BHI agar for MRSA and on SD agar for *C. albicans* and *T. rubrum*. The numbers of viable colonies were then calculated after 24 h incubation. DMSO was used as negative control. The limit of quantification was 100 CFU/mL (Hamoud, Zimmermann & Reichling, 2014).

3. Results and discussion

Antimicrobial susceptibility tests of LME, artocarpin and some antibiotics against MRSA, *C. albicans*, and *T. rubrum* was performed using the broth microdilution method. LME andantibiotics, including norfloxacin, ampicillin and tetracycline exhibited moderate antibacterial activity against MRSA, with MIC values of 15.6 – 31.2 µg/mL (Table 1), while artocarpin showed weak antibacterial activity with MIC value of 62.5 µg/mL. For anti-candida activity, LME and clotrimazole displayed strong inhibitory activities, with MIC values of 7.8 and 0.9 µg/mL, respectively. Arto-

Table 1
Antibacterial activity of combination of LME and antibiotics/artocarpin against MRSA.

Administrations	MIC*a* /(μg · mL^{-1})	MIC*c* /(μg · mL^{-1})	FIC	FICI	Interactions
LME-Norfloxacin				1.12	Indifferent
LME	15.6	15.6	1		
Norfloxacin	31.2	3.9	0.12		
LME-Ampicillin				0.62	Addition
LME	15.6	1.9	0.12		
Ampicillin	31.2	15.6	0.5		
LME-Tetracycline				1.12	Indifferent
LME	15.6	15.6	1		
Tetracycline	15.6	1.9	0.12		
LME-Artocarpin				0.31	Synergistic
LME	15.6	3.9	0.25		
Artocarpin	62.5	3.9	0.06		

MIC*a* (MIC of sample alone), MIC*c* (MIC of samples in combination).
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index).

carpin showed moderate activity against *C. albicans* with MIC value of 31.2 μg/mL (Table 2). LME and clotrimazole also exhibited strong anti-dermathophyte activities against *T. rubrum* with MIC values of 3.9 and 0.5 μg/mL, respectively. However, a weak anti-dermathophyte activity was shown by artocarpin with 62.5 μg/mL MIC (Table 3).

Further study was performed to determine the interaction of LME with antibiotics as well as LME with the natural flavonoid, artocarpin using the checkerboard method. The interactions were interpreted using their FICI values. The combination of LME with some antibiotics did not show any synergistic activity against MRSA, LME (1.9 μg/mL) only gave an additive effect with ampicillin (15.6 μg/mL). Interestingly, LME exhibited a synergy on the antibacterial activity of artocarpin against MRSA with FICI value of 0.31 (Table 1) by decreasing 16-fold concentration of artocarpin. To confirm the synergistic effect of LME with artocarpin against MRSA, a time-kill assay was performed. It has been known that time-kill assay provides more accurate results to determine the synergy effect. The obtained result from time-killing method was more favored compared to checkerboard titration method. The combination of LME (3.9 μg/mL) and artocarpin (3.9 μg/mL) was able to reduce the number of colony counts of MRSA after 8 h incubation. Fig. 2A showed that single dose of LME (15.6 μg/mL) and artocarpin (62.5 μg/mL) used alone also reduced the bacterial colony counts after 22 h and 4 h incubation, respectively at the higher concentrations.

Moreover, LME (1.9 μg/mL) in combination with clotrimazole (0.1 μg/mL) displayed a synergistic effect against *C. albicans* with FICI value of 0.38. But, it only showed additive effect with artocarpin (FICI value of 0.75) (Table 2). The time-kill assays confirmed the synergistic of LME with clotrimazole, as shown in Fig. 2B. The combination of 1.9 μg/mL of LME enhanced the anti-candida activity of 0.1 μg/mL clotrimazole by completely inhibiting the microbial growth after 6 h incubation. While, the single use of LME (7.8 μg/mL) and clotrimazole (0.9 μg/mL) were able to kill the microbial after 24 h incubation. In addition, LME (0.5 μg/mL) also enhanced the anti-dermatophyte activity of clotrimazole (0.1 μg/mL) against *T. rubrum* with a synergy effect (FICI value of 0.24). LME combined with artocarpin only produced an addition effect with FICI value of 0.75 (Table 3). The time-kill assays also confirmed their synergistic effect (Fig. 2C). The combination of 0.5 μg/mL of LME with 0.1 μg/mL clotrimazole showed killing effect after 12 h incubation.

The results also showed that the required dose of single compounds to inhibit the microbial growth was higher than the dose in their combination. It has been known that the use of high dose of antibiotics in clinical may lead to the antibiotic resistant problems (Zhao & Drlica, 2001). Therefore, the alternative approach

Table 2
Antibacterial activity of the combination of LME and clotrimazole/artocarpin against *C. albicans*.

Administrations	MIC*a* /(μg · mL^{-1})	MIC*c* /(μg · mL^{-1})	FIC	FICI	Interactions
LME-Clotrimazole				0.38	Synergistic
LME	7.8	1.9	0.25		
Clotrimazole	0.9	0.1	0.13		
LME-Artocarpin				0.75	Addition
LME	7.8	3.9	0.5		
Artocarpin	31.2	7.8	0.25		

MIC*a* (MIC of sample alone), MIC*c* (MIC of samples in combination).
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index).

Table 3
Antibacterial activity of the combination of LME and clotrimazole/artocarpin against *T. rubrum*.

Administrations	MIC*a* /(μg · mL^{-1})	MIC*c* /(μg · mL^{-1})	FIC	FICI	Interactions
LME-Clotrimazole				0.24	Synergistic
LME	3.9	0.5	0.12		
Clotrimazole	0.5	0.1	0.12		
LME-Artocarpin				0.75	Addition
LME	3.9	1.9	0.5		
Artocarpin	62.5	15.6	0.25		

MIC*a* (MIC of sample alone), MIC*c* (MIC of samples in combination).
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index).

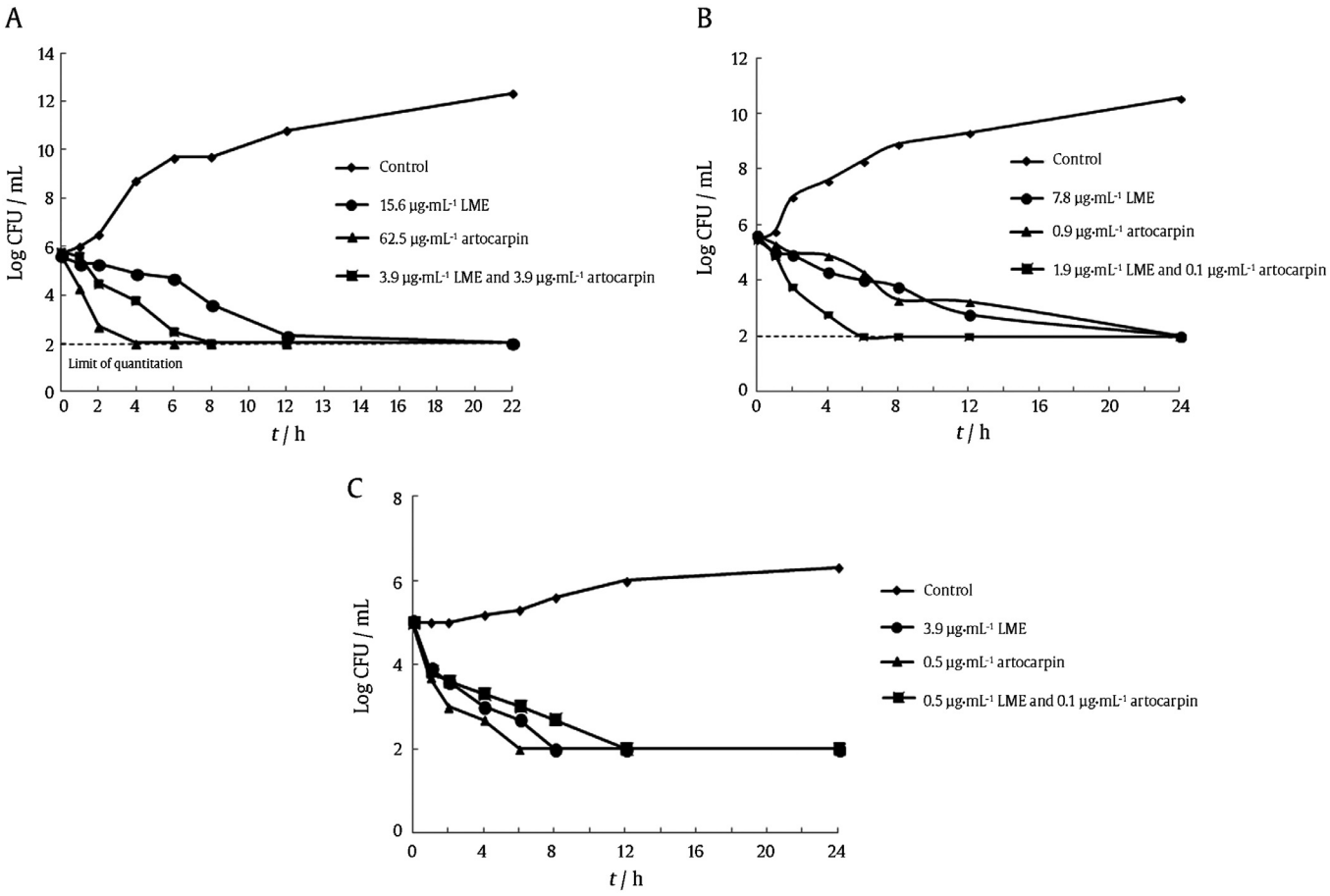

Fig. 2. Time-kill curves of LME, artocarpin, and their combination against MRSA.

using combination of antibacterial compounds in order to reduce the dose of antibiotics might be beneficial to overcome the resistant problems associated with pathogenic infection.

The use of some antimicrobial compounds in combination may increase their biological activity. It can be due to several ways, for instance different compounds probably occupy at the same site of action by agonistic or synergistic way. On the other hand, the different compounds may also regulate the different targets of action in order to achieve the same response, which in turn enhancing their biological properties (Yang et al., 2014). The mechanism underlying synergistic action of LME with artocarpin against MRSA may be due to through the different targets of actions. It has been known that the interaction of flavonoid compounds with lipophilic site of membrane will reduce the membrane fluidity (Cushnie & Lamb, 2011). Therefore, it may allow LME to give its antimicrobial action by altering the mitochondria respiration of microbes (Vanichayachart et al., 1992). In the case of combination LME with clotrimazole against *C. albicans* and *T. rubrum*, the possible mechanism of synergistic effect is the different compounds may regulate the different target in various pathways inside the cells. It has been known that the mechanism of action of clotrimazole is inhibiting synthesis of protein and RNA of microbes (Iwata, Yamaguchi & Hiratani, 1973). It will corporate with mechanism of action of LME in a synergistic way. The synergistic effect of LME with antibiotics as well as natural compound may provide an important insight on the ability of LME in enhancing the antimicrobial activity of clotrimazole against *C. albicans* and *T. rubrum*.

4. Conclusions

In conclusion, the results revealed the potential of LME as an adjuvant of antibiotics by reducing their therapeutic doses. This finding is also the first report regarding the synergistic activity of LME in combination with artocarpin. It may provide the prospect for the use of this combination to overcome some resistant problems. Nevertheless, further experiments are required to elucidate their mechanism of action that underlies this synergistic activity.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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