ANTIMICROBIAL ACTIVITIES AND INTERACTION EFFECTS OF VIETNAMESE *LITSEA CUBEBA* (LOUR.) PERS ESSENTIAL OIL AND ITS ENDOPHYTIC ACTINOBACTERIA

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ABSTRACT

The aim of this study was to evaluate the antimicrobial activity of *Litsea cubeba* (Lour.) Pers (*L. cubeba*) essential oil and its endophytic actinobacterial crude extracts (EACE) against pathogenic bacteria in individual and in combination. Using the broth micro-dilution assay, the results indicated that both *L. cubeba* essential oil and its EACE showed inhibition effect against *Aeromonas hydrophila* ATCC 35654, *Escherichia coli* ATCC 25922, *Staphylococcus aureus ATCC* 25923, *Bacillus cereus* ATCC 13061, and drug resistant strains such as: Methicillin-resistant *Staphylococcus epidermidis* (MRSE) ATCC 35984 and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591. The minimal inhibitory concentration (MIC) of *L. cubeba* essential oil and its AECE were from 3.13 to 6.25 µl ml^{-1} and 50.0 to 333.3 µl ml^{-1} respectively. From the indices of fractional inhibitory concentration (FIC) of the combination of *L. cubeba* essential oil and its EACE, the synergistic effects were found against *E. coli, S. aureus, B. cereus* and MRSE. The combination treatment of *L. cubeba* essential oil and its EACE enhance the inhibition effect against food-borne bacteria 16 times comparing with individual treatment.

Keywords: antimicrobial activity, *Litsea cubeba*, essential oil, endophytic actinobacteria, FIC, synergistic effect.

1. INTRODUCTION

Since their discovery, antibiotics have provided the main basis for the treatment of infectious diseases caused by a variety of microorganisms in medical practices. However,

overuse and/or abuse of antibiotics have become the main factor in the emergence and dissemination of multidrug-resistant strains of bacteria. The antibiotic resistance in pathogenic bacteria is a significant clinical problem and a serious public health concern. Thus, the identification of new antimicrobial agents is a top research and development priority among scientists and pharmaceutical companies [1]. Medicinal plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, phenols and quinones. These compounds have been considered due to their inhibitory and bactericidal properties to kill pathogenic microorganisms. A growing interest has arisen towards herbal therapy for reducing the use of antibiotics, in food and aquaculture production in recent years [2]. The scientific value of the medical plants from the bioactive component, they are the environment for endophytic actinobacteria which are capable of antibiotic biosynthesis. Therefore, the recruitment of high biological active actinomycetes on the traditional medicinal plants is an important approach to the microbiology and pharmacy worldwide.

The complex (multi-component) nature of crude plant extracts may reduce the spontaneous occurrence of bacterial resistance since multiple simultaneous mutations may be required to overcome the antimicrobial actions of all active plant components. To enhance the antimicrobial activities, the essential oils (EOs) have been used in combination with other antibacterial agents and a variety of treatments. Indeed, the combined application of *Origanum vulgare* and *Rosmarinus officinalis* EOs have shown a synergic interaction against *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophilla* [3] .

The antibiotic activities of *Litsea cubeba (L. cubeba)* EOs in the treatment of infection diseases were proved worldwide suggested us to investigate the correlation between the antimicrobial products of plant metabolism such as EO with endophytic actinobacteria isolated from this medicinal plants. However, to the best of our knowledge, there are very few of study on the combination antibacterial effects of this species EO and its actinobacterial crude extract (EACE) against food-borne and pathogenic bacteria.

Thus, the objective of this study was to investigate the individual and combination antimicrobial effect of *L. cubeba* EO and its EACE against pathogenic bacteria such as: *Aeromonas hydrophila, Escherichia coli*, *Bacillus cereus, Staphylococcus aureus,* Methicillin-resistant *Staphylococcus epidermidis* (MRSE) and Methicillin-resistant *Staphylococcus aureus* (MRSA).

2. MATERIALS AND METHODS

2.1. Materials

Roots, steams, fruits and leaves of plant samples were harvested in PhuTho province in the North of Vietnam (21°24' N, 105°4' E). Plants were confirmed as *L. cubeba* (Lour.) Pers. at Department of Plant, Faculty of Agronomy, Vietnam National University of Agriculture (VNUA), where a voucher specimen was deposited. 200 g of the fresh cut fruits *L. cubeba* was subjected to balloon of 2 liters of water and then hydrodistillated for 4 h using a Clevenger-type apparatus to extract essential oil. They were dried over anhydrous sodium sulfate and stored at 4 °C in the dark glass bottle until used. Its endophytic actinobacterial crude extract (EACE) MPT28 was obtained from Institute of Biotechnology, Vietnam Academy of Science and Technology (VAST).

The tested strains obtained from Americane Type Culture Collection including two Gram-negatives reference strains: *A. hydrophila* ATCC 35654, *E. coli* ATCC 25922 and two Gram-

positives reference strains: *S. aureus* ATCC 25923, *B. cereus* ATCC 13061, two antibiotic resistant strains: MRSE ATCC 35984 and MRSA ATCC 33591 were used in this study. All strain was grown on Muller Hilton Agar (MHA, Merck) plates for 24 h at 30 ^{o}C for *A. hydrophila* and at 37 ^{o}C for the other strains.

2.2. Preparation of endophytic actinobacterial crude extracts (EACE)

The strain was isolated from different part of *L. cubeba* plant. Among isolates strains, the strain MPT28 was selected for further assays due to its antimicrobial activity. Seed culture for inoculation of isolate MPT28 was prepared in YIM38 medium (200 rpm, 28 °C, 4 days). Then, inoculant was transferred to the YIM61 medium [4] as the antibiotic producing medium (200 rpm, 28 °C, 5 days). After incubation, 100 ml of actinomycete MPT28 broth was centrifuged at 7000 rpm for 5 min; the supernatant was obtained and used to identify the antimicrobial activity and other tests.

2.3. Antimicrobial activity test

The MICs (Minimum Inhibitory Concentration) of *L. cubeba* fruit EO and its EACE MPT28 were determined by microbroth dilution assay in 96-well microplates [5]. *L. cubeba* EO was dissolved in distillated water containing 0.5 % Tween 80 to achieve a final concentration ranging from 50 µl ml^{-1} to 0.195 µl ml^{-1}. Each well contained 20 µl of test sample and 180 µl of bacterial suspension containing 10^6 CFU ml^{-1} in Muller Hilton Broth (MHB, Merck). After incubation for 24 h at 30 ^{o}C for *A. hydrophila* and at 37 ^{o}C for the other strains, the optical density (OD) was measured at 600nm using Elisa reader (Bio-rad Model 680, Japan). The MIC was determined as the lowest concentration showing no growth. The Minimum Bactericidal Concentration (MBC) was determined by spreading 100 µl of the cultures on MHA plates and then incubated for 24 h at 30^{o}C for and *A. hydrophila* and at 37 ^{o}C for the other strains. The MBC was identified as the lowest concentration showing no bacterial growth on agar plates. The assays were carried out in triplicate. A positive control containing the bacterial culture without the EO/EACE extract and a negative control containing the MHB, Tween and test sample (EO or EACE) were performed under the same conditions.

2.4. The combination effects of *L. cubeba* essential oil and its actinobacterial crude extract against pathogenic bacteria

The checkerboard method was performed using 96-well microtitre plates to obtain the Fractional Inhibitory Concentration (FIC) index of combinations in the MHB [6]. Plates consisted of columns containing 20 µl of *L. cubeba* EO (component A) diluted two-fold along the x axis as well as rows with the same amount of EACE (component B) diluted two-fold along the y axis. Subsequently, 160 µl of each tested bacteria suspension containing 10^6 CFU ml^{-1} were added to all wells. The concentrations of *L. cubeba* and EACE were prepared corresponding to 2, 1,1/2, 1/4, 1/8 and1/16 of the MIC values, respectively. Plates were then incubated at 30 ^{o}C for *A. hydrophila*, and at 37 ^{o}C for the other strains for 24 h. The FIC indices were calculated as $FIC_A + FIC_B$, where $FIC_A = (MIC_A \text{ combination}/MIC_A \text{ alone})$ and $FIC_B = (MIC_B \text{ combination}/MIC_B \text{ alone})$. The results were interpreted as synergy ($FIC < 0.5$), addition ($0.5 \leq FIC \leq 1$), indifference ($1 < FIC \leq 4$) or antagonism ($FIC > 4$). Experiments were performed in triplicate [6].

3. RESULTS AND DISCUSSION

25 endophytic actinomycetes were isolated from *L. cubeba* plant by Institute of Biotechnology, VAST. Among them, the isolate MPT28 was selected for further study on MIC and MBC due to its antimicrobial activity.

Bizuye et al [2] isolated 30 strains of endophytic actinomycetes from soil samples in north western Ethiopia. In particularly, there were 8 strains exhibiting inhibitory activity against at least one test microorganism (26.7 %). Several researchs in all over the world proved the inhibition of the endophytic actinomycetes to pathogenic microorganisms, especially those were isolated from medicinal plants. In general, endophytic actinomycetes isolated from medicinal plant (especially types contains oil or antimicrobial substances) have the antimicrobial resistance rate higher than those of originated from soil. The results showed the diversity in the ability to inhibit microbial pathogens of the endophytic actinomycetes isolated from medicinal plant *L. cubeba*.

3.1. Antibacterial activity of *L. cubeba* fruits EO and its actinobacterial crude MPT28 extracts

Both of *L. cubeba* EO and EACE showed the antimicrobial effect against all tested pathogenic strain (Table 1). The MIC values against all tested bacterial strains ranging from 3.13 to 6.25 µl ml^{-1} for EO and from 50 to 350 µl ml^{-1} for EACE. In the case of *L. cubeba* EACE, the most sensitive strains were *S. aureus* ATCC 25923, *B. cereus* ATCC 13061 and *A. hydrophila* ATCC 35654 whereas the less sensitive strains were MRSE ATCC 35984 and MRSA ATCC 33591. The EO showed higher activites against the pathogenic bacteria comparing to EACE's. The higher activity of *L. cubeba* fruits EO is correlated to the large content of citral (neral and geranial), whose antimicrobial effectiveness was reported in the literature [7]. Considering the ratio of MBCs and MICs, it appeared that the EO exerted a bacteriostatic effect (MBC/MIC>4) against *A. hydrophila* ATCC 35654, *B. cereus* ATCC 13061, MRSE ATCC 35984 and MRSA ATCC 33591 and a bactericidal effect (MBC/MIC < 4) against *E. coli* ATCC 25911 and *S. aureus* ATCC 25923.

Table 1. Antimicrobial activity of *L. cubeba* EO and its AECE MPT28, against the tested bacterial strains.

Strain species	*L. cubeba*		EACE	
	MIC (µl ml^{-1})	MBC (µl ml^{-1})	MIC (µl ml^{-1})	MBC (µl ml^{-1})
S. aureus ATCC 25923	3.13^{a}	8.33^{b}	50.0^{a}	50.0^{a}
B. cereus ATCC 13061	3.13^{a}	$>50^{c}$	66.7^{ab}	200.0^{c}
MRSE ATCC 35984	4.17^{ab}	$>50^{c}$	166.7^{c}	166.7^{bc}
MRSA ATCC 33591	4.17^{ab}	$>50^{c}$	333.3^{d}	-
A. hydrophila ATCC 35654	3.13^{a}	16.67^{a}	100.0^{b}	166.7^{bc}
E. coli ATCC 25922	6.25^{b}	12.5^{a}	100.0^{b}	150.0^{b}

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, (-): not determined
Data are expressed as means of triplicate. Values followed by different letters within a column are significantly different by Fisher's test ($p < 0.05$)

Plant EOs are generally more active against Gram-positive bacteria than Gram-negative bacteria [8]. Some authors suggested that the outer membrane surrounding the cell wall of

Gram-negative bacteria may restrict diffusion of hydrophobic compounds through its lipopolysaccharide covering [9]. In our work, Gram-positive strains, *S. aureus* ATCC 25923, *B. cereus* ATCC 13061, MRSA ATCC 33591 and MRSE ATCC 35984 were more sensitive to the action of the EOs than the Gram-negative *E. coli* ATCC 25922 except *A. hydrophila* ATCC 35654 (Table 1). Our results was in agreement with the study of Wan et al. [10] . *A. hydrophila* showed in fact to be one of the most sensitive species. Saikia et al. [7] observed a broader range of MICs (1.25 to 10 mgml^{-1}) against different bacterial strains of the EO from *L. cubeba* fruits harvested in Assam-India. Wang and Liu [11] described a low range of MIC values comprised between 0.1 and 0.7 mg ml^{-1} of the fruit EO of *L. cubeba* containing citral (63.75 %) from China against other bacterial strains (*B. subtilis, E. faecalis, P. aeruginosa*)..

To the best of our knowledge, few studies have reported the effect of cultural broth *L. cubeba* actinobacterial crude extract against bacterial pathogens. For instance, dragon's blood extract has a range of MIC/IC$_{50}$ from 4.88 to 9.77 (µg ml^{-1}) against same bacterial strain MRSE, MRSA, *E. coli* [12]. In this study, the cultured broth of MPT28 from *L. cubeba* also showed the antimicrobial effect against pathogenic bacteria.

3.2 The combination effects of *L. cubeba* EO and its EACE MPT28

FIC values frequently used to define or to describe the interactions between antibiotic or antibiotic and EOs. The combination effects of the antimicrobial activity of *L. cubeba* EO and EACE were shown in Table 2.

Table 2. FIC values and the combinations effects of *L. cubeba* EO and its EACE MPT28 against tested bacterial strains.

Strains	Agent	MIC (µl ml^{-1})		FIC	Sum FIC[b]	Interaction
		Individual	Combination			
S. aureus	*L. cubeba* EO	3.13	0.46	0.15	0.29	Synergistic
ATCC 25923	EACE	50.00	7.29	0.15		
B. cereus	*L. cubeba* EO	3.13	0.91	0.29	0.38	Synergistic
ATCC 13061	EACE	66.70	5.56	0.08		
MRSE	*L. cubeba* EO	4.17	0.26	0.06	0.23	Synergistic
ATCC 35984	EACE	166.70	27.78	0.17		
MRSA	*L. cubeba* EO	4.17	1.74	0.42	0.92	Additive
ATCC 33591	EACE	333.30	166.65	0.50		
A. hydrophila	*L. cubeba* EO	3.13	2.35	0.75	1.02	Indifferent
ATCC 35654	EACE	100.00	27.08	0.27		
E. coli ATCC	*L. cubeba* EO	6.25	0.39	0.06	0.35	Synergistic
25922	EACE	100.00	29.17	0.29		

Data are expressed as means of triplicate.
Results are interpreted as synergy (FIC < 0.5), addition(0.5 ≤ FIC ≤ 1), indifference (1 < FIC ≤ 4) or antagonism (FIC > 4).

The results indicated that most of combination show a synergistic effect (FIC < 0.5) against tested strains except for *A. hydrophila* ATCC 35654 (Indifferent, FIC = 1.02) and MRSA ATCC

33591 (Additive, FIC = 0.92) (Table 2). The synergistic effects of *L. cubeba*/EACE was strongest against MRSE ATCC 35984 (FIC = 0.23) in which the concentration of *L. cubeba* and EACE decreased 16 and 6 times in combination comparing with individual treatment, respectively. No antagonism was observed for any of the combinations evaluated.

Some studies have concluded that whole EOs have a greater antibacterial activity than the major components [5]. Burt [8] suggested that the minor components present in the EOs' extracts are more critical to the activity than EO main components mixed, and may have synergistic effects or a potentiating influence. The interaction (synergy, indifference, antagonism or addition) between two compounds depends on the concentrations of the single component and the overall susceptibility of the target microorganism. This may explain variation of interaction observed between combinations and strains [13]. Although, further studies need to be carried out, the synergistic effects of *L. cubeba* EO and its EACE also suggested the ideal that medical plant metabolites might also come from its endophytic actinobacteria.

4. CONCLUSION

The present study demonstrated that both of *L. cubeba* EO and its EACE had an antimicrobial effect with MICs ranged from 3.13 to 6.25 µl ml^{-1} and 50 to 333.3 µl ml^{-1}, respectively. In addition, their combination showed synergistic effect against tested strains except for *A. hydrophila* and MRSA. The best synergistic effect was obtained with combinations against MRSE. Furthermore, due to its wide distribution in East Asia, this species has been proposed to be tested as an industrial crop model [14]. Our results obtained could be a potential and promising application for sustainable therapy in food and aquaculture production.

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TÓM TẮT

NGHIÊN CỨU HOẠT TÍNH KHÁNG KHUẨN VÀ TƯƠNG TÁC CỦA TINH DẦU MÀNG TANG *LITSEA CUBEBA* (LOUR.) PERS VÀ XẠ KHUẨN NỘI CỘNG SINH TRÊN CÂY MÀNG TANG

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Mục đích của nghiên cứu này để đánh giá khả năng kháng khuẩn của tinh dầu màng tang *Litsea cubeba* (Lour.) Pers (*L. cubeba*), dịch nuôi xạ khuẩn phân lập từ Màng tang (EACE) với các chủng vi khuẩn gây bệnh khi sử dụng riêng rẽ và kết hợp. Bằng phương pháp pha loãng liên tục, kết quả nghiên cứu cho thấy cả tinh dầu Màng tang và EACE có tác dụng ức chế các chủng vi khuẩn chỉ thị như *Aeromonas hydrophila* ATCC 35654, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 13061, và vi khuẩn kháng thuốc Methicillin-resistant *Staphylococcus epidermidis* (MRSE - *S. epidermidis* kháng Methicilin) ATCC 35984 và Methicillin-resistant *Staphylococcus aureus* (MRSA – *S. aureus* kháng Methicilin) ATCC 33591. Nồng độ ức chế tối thiểu (MIC) của tinh dầu màng tang và EACE tương ứng là 3,13 - 6,25 µl ml^{-1} và 50,0 - 333,3 µl ml^{-1}. Dựa trên giá trị nồng độ ức chế riêng phần (FIC), sự kết hợp của *L. cubeba* và EACE có tác dụng hiệp đồng với *E. coli*, *S. aureus*, *B. cereus* và MRSE. Sự kết hợp của tinh dầu màng tang và EACE tăng cường hiệu lực chống lại vi khuẩn gây bệnh thực phẩm gấp 16 lần so với khi sử dụng riêng rẽ.

Từ khóa: xạ khuẩn, kháng khuẩn, endophytic actinobacteria, *Litseacubeba*, FIC, tác dụng hiệp đồng.