

## INTERACTION EFFECTS OF *LITSEA CUBEBA* ESSENTIAL OIL AND ANTIBIOTICS ON ANTIBACTERIAL ACTIVITY AGAINST PATHOGENIC BACTERIA IN AQUACULTURE

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### ABSTRACT

In this study, the interaction effects of *Litsea cubeba* essential oil (EO) and two antibiotics (ABs) on the antibacterial activity against pathogenic bacteria in aquaculture were investigated. Two reference strains (*Escherichia coli* ATCC 25922 (*E. coli* ATCC 25922) and *Vibrio parahaemolyticus* ATCC 17802 (*V. parahaemolyticus* ATCC 17802)) and six locally isolated aquatic pathogenic strains (*E. coli* 9C48, *E. coli* 11C123, *Vibrio* 2S4, *Vibrio* 2N38, *V. parahaemolyticus* ND201 and *V. parahaemolyticus* TB81) were used. The inhibitory effects of individual antimicrobial agent (*L. cubeba* EO, nalidixic acid, and oxytetracycline) were tested against eight strains by using broth microdilution assay in 96-well microplates. A higher inhibitory effect of *L. cubeba* EO was observed against isolated aquatic pathogenic (MIC = 1.15 - 2.30 mg/mL) than that in the reference strains (MIC = 5.53 mg/mL). The combination effects of *L. cubeba* EO and ABs often used in the treatment of bacteria effects in aquaculture (nalidixic acid and oxytetracycline) were evaluated by the checkerboard method. Fractional Inhibitory Concentration (FIC) values were determined to characterize the interaction among combinations. Out of 16 AB-EO combinations tested, 11 of them showed a synergistic effect ( $FIC \leq 0.5$ ), 3 of them showed an additional effect ( $0.5 < FIC \leq 1.0$ ) and 2 of them showed indifferent effect ( $1 < FIC \leq 4$ ), no antagonistic effect was observed. The antimicrobial synergy of EO and AB could enhance efficacy, reduce toxicity, decrease adverse side effects, and lower the dose of ABs used in aquaculture.

**Keywords:** *Litsea cubeba*, essential oil, antibacterial activity, synergistic effect.

### 1. INTRODUCTION

According to the worldwide extension of aquaculture activity, new emerged diseases and the occurrence of other diseases increased year by year, in which, bacteria are a major cause of diseases [1]. *Vibrio* spp. and *E. coli* are the pathogenic bacteria which may be found in aquatic animals [2]. Since antibiotics (ABs) have the capacity to kill or inhibit the growth of bacteria by

having specific mechanisms toward the pathogens targeted, producers have to make use of massive amounts of ABs in order to control mortality and avoid huge economic losses. However, the use of a wide variety of ABs (e.g., oxytetracycline, nalidixic acid, amoxicillin, sulfonamides) [3] in large amounts, including non-biodegradable ABs useful in human medicine, ensures that they remain in the aquatic environment, exerting their selective pressure for long periods of time. This process has resulted in the emergence of AB-resistant bacteria in aquaculture environments, in the increase of AB resistance in fish pathogens, in the transfer of these resistance determinants to bacteria of land animals and to human pathogens, and in alterations of the bacterial flora both in sediments and in the water column. The use of large amounts of ABs that have to be mixed with fish food also creates problems for industrial health and increases the opportunities for the presence of residual ABs in fish meat and fish products [4]. Thus the reduction in ABs consumption in aquaculture is clearly important to minimize this problem. Plant secondary metabolites, e.g. essential oils (EOs) are known to display antimicrobial properties that could be relevant alternatives to ABs.

EOs are products of the secondary metabolites formed by aromatic plants. May Chang *Litsea cubeba* (so-called “Mãng tang” in Vietnamese), a plant of *Litsea* genera that has been known for a long time as a popular remedy, is a potential therapeutic plant. *L. cubeba* has been used in traditional medicine to treat headache, fatigue, muscle pain, depression, sores and furuncles [5]. Its extracts have been reported for their antibacterial, antifungal, antioxidant and anticancer activities [5]. However, in many case, natural antimicrobial compounds from plant possess relatively weak activity, compared to synthetic ABs.

The combination of antibacterial agents is based on the Principle that the formulation may enhance efficacy, reduce toxicity, decrease adverse negative effects, lower dose of antibacterial agents [6]. Various antimicrobial interactions have been reported for EOs and ABs when tested in binary combinations [7 - 10]. For instance, lemongrass (*Cymbopogon citratus*) EO and its main component, citral, combined with streptomycin and kanamycin exhibited synergistic or additive effect against *Salmonella typhimurium* [9]. Oregano (*Origanum vulgare*) EO in combination with doxycycline, florfenicol or sarafloxacin possessed synergistic effects against *E. coli* isolated from chickens [10]. However, to the best of our knowledge, the combination of antibacterial activities between *L. cubeba* EO and ABs against reference and isolate strains for aquaculture have not been reported yet.

The aim of this work was to investigate the antibacterial effects of *L. cubeba* EO and nalidixic acid and oxytetracycline individual and in combination against two reference strains (*E. coli* ATCC 25922, and *V. parahaemolyticus* ATCC 17802) and 6 isolated aquatic pathogenic strains (*E. coli* 9C48, *E. coli* 11C123, *V. parahaemolyticus* ND201, *V. parahaemolyticus* TB81, *Vibrio* 2S4 and *Vibrio* 2N38).

## 2. MATERIALS AND METHODS

### 2.1. Materials

*L. cubeba* EO purchased from Aromasia company (> 98 % pure) (Vietnam), and nalidixic acid and oxytetracycline (Sigma Aldrich, France) were tested towards eight strains including: two reference strains: *E. coli* ATCC 25922, *V. parahaemolyticus* ATCC 17802; two *Vibrio* spp. isolated strains *V. parahaemolyticus* ND201 and *V. parahaemolyticus* TB81 isolated from the whiteleg shrimp *Litopenaeus vannamei* Acute Hepatopancreatic Necrosis Syndrome (AHPNS) hatchery of local farming in Nam Dinh and Thai Binh (National Veterinary Diagnostic Center –

Hanoi); four isolated strains kindly provided by the Pathology Laboratory of the Department of Aquatic Animal Pathology, Faculty of Fisheries, Nong Lam University including *Vibrio* 2S4, *Vibrio* 2N38, and *E. coli* 9C48, *E. coli* 11C123 isolated from cockle and catfish, respectively.

## 2.2. Determination of minimum inhibition concentration (MIC)

The minimum inhibition concentration (MIC) of the antibacterial agents (EO and AB) was determined using microbroth dilution assay in 96-well microplates. The EO was dissolved in distilled water containing 0.5 % Tween 80 to achieve a final concentration ranging from 0.195  $\mu\text{L}/\text{mL}$  to 50  $\mu\text{L}/\text{mL}$ . Bacterial cultures were prepared in Mueller Hilton Broth (MHB, Merck) or MHB plus NaCl 2 % for vibrios. An overnight culture was prepared and adjusted to obtain a suspension of  $10^7$  CFU/mL by measuring the Optical Density (OD) at 600 nm. Each well contained 20  $\mu\text{L}$  of test sample, 20  $\mu\text{L}$  of bacterial suspension and 160  $\mu\text{L}$  MHB. Thus, the final bacterial concentration for the assay was then  $10^6$  CFU/mL. After incubation for 24 h at 28 °C for vibrios and at 37 °C for *E. coli*, the OD was measured at 600 nm using Elisa reader (Bio-rad Model 680, Japan). The MIC was determined as the lowest concentration showing no growth [5]. The assays were carried out in triplicate. A positive control containing the bacterial culture without the EO and a negative control containing the MHB, Tween and EOs were performed in the same conditions [5].

## 2.3. Checkerboard method: interaction effect on antibacterial activity of *L. cubeba* and AB

The checkerboard method was performed using 96-well microplates to obtain the fractional inhibitory concentration (FIC) index [8]. Six serial (2, 1, 1/2, 1/4, 1/8 and 1/16 MIC), two-fold dilutions of *L. cubeba* EO (A) and antibiotics (B) were prepared as in the MIC tests. 10  $\mu\text{L}$  of EO dilution were added to the plate in a vertical orientation and 10  $\mu\text{L}$  of AB dilution were added in a horizontal orientation. Then, 20  $\mu\text{L}$  of the suspension bacterial overnight culture containing  $10^7$  CFU/mL of the strain were added to all wells. 160  $\mu\text{L}$  of media MHB were filled to obtain total of 200  $\mu\text{L}$ . Plates were then incubated at 37 °C for *E. coli* and 28°C for vibrios for 24 h.

The FIC index was calculated as  $\sum FIC = FIC_A + FIC_B$

where:

$$FIC_A = \frac{MIC_{A\text{ combination}}}{MIC_{A\text{ alone}}};$$

$$FIC_B = \frac{MIC_{B\text{ combination}}}{MIC_{B\text{ alone}}}$$

The results were interpreted as synergistic ( $FIC \leq 0.5$ ), additional ( $0.5 < FIC \leq 1$ ), indifferent ( $1 < FIC \leq 4$ ) or antagonistic ( $FIC > 4$ ) [8]. Experiments were performed in triplicate.

## 3. RESULTS AND DISCUSSION

The antimicrobial activity individual and in combination of EO and AB (MIC and FIC values) against *Vibrio* spp. and *E. coli* were presented in Table 1 and Table 2, respectively.

Table 1. FIC values and the combinations effects of *L. cubeba* fruit EO and antibiotic against *Vibrio* spp.

Strains	Antimicrobial agents	MIC (mg/mL)		FIC	Sum FIC	Interaction
		Alone	Combination			
<i>V. parahaemolyticus</i> ATCC 17802	<i>L. cubeba</i>	5.53	0.69	0.13	0.23	Synergy
	Nalidixic acid	2.67. 10 <sup>-3</sup>	0.27. 10 <sup>-3</sup>	0.10		
	<i>L. cubeba</i>	5.53	0.61	0.11	0.28	Synergy
	Oxytetracycline	2.67. 10 <sup>-3</sup>	0.45. 10 <sup>-3</sup>	0.17		
<i>Vibrio</i> 2S4	<i>L. cubeba</i>	1.15	0.77	0.67	1.17	Indifference
	Nalidixic acid	64.0. 10 <sup>-3</sup>	32.0. 10 <sup>-3</sup>	0.50		
	<i>L. cubeba</i>	1.15	0.96	0.83	1.33	Indifference
	Oxytetracycline	16.0. 10 <sup>-3</sup>	8.0. 10 <sup>-3</sup>	0.50		
<i>Vibrio</i> 2N38	<i>L. cubeba</i>	2.30	0.97	0.42	0.92	Addition
	Nalidixic acid	53.3. 10 <sup>-3</sup>	26.7. 10 <sup>-3</sup>	0.50		
	<i>L. cubeba</i>	2.30	0.76	0.33	0.83	Addition
	Oxytetracycline	8.0. 10 <sup>-3</sup>	4.0. 10 <sup>-3</sup>	0.50		
<i>V. parahaemolyticus</i> ND201	<i>L. cubeba</i>	1.67	0.22	0.13	0.30	Synergy
	Nalidixic acid	8.0. 10 <sup>-3</sup>	1.4. 10 <sup>-3</sup>	0.17		
	<i>L. cubeba</i>	1.67	0.32	0.19	0.44	Synergy
	Oxytetracycline	16.7. 10 <sup>-3</sup>	4.2. 10 <sup>-3</sup>	0.25		
<i>V. parahaemolyticus</i> TB81	<i>L. cubeba</i>	1.75	0.37	0.21	0.38	Synergy
	Nalidixic acid	9.3. 10 <sup>-3</sup>	1.6. 10 <sup>-3</sup>	0.17		
	<i>L. cubeba</i>	1.75	0.19	0.11	0.32	Synergy
	Oxytetracycline	16.0. 10 <sup>-3</sup>	3.4. 10 <sup>-3</sup>	0.21		

The MIC values of *L. cubeba* EO against all tested strain ranging from 1.15 - 5.53 mg/mL. The same antibacterial activities (MIC = 1.25 - 10 mg/mL) were reported for *L. cubeba* EO rich in citral (83.9 %) from India [11]. Recent observations by transmission electron microscopy showed holes and gaps in the cell wall of *E. coli* and that the bacteria were killed or destroyed within 2 h when treated with 0.125 % v/v of *L. cubeba* [12].

In addition, *L. cubeba* EO had higher inhibitory effect against isolated strains than reference strains. Indeed, the MIC values against isolated strains lower 2.4 - 4.8 times (corresponding to 1.15 - 2.30 mg/mL) compared to those (5.53 mg/mL) against references strains. Generally, a higher amount of AB was required when treating bacteria resistant to AB. However, the effect of EOs to isolated bacteria was variable. A higher MIC values (MIC = 0.015 - 0.062 µg/mL) of clove (*Syzygium aromaticum*) and citronella (*Cymbopogon nardus*) EO

against 32 strains including *Vibrio* spp., *Edwardsiella* spp., *Aeromonas* spp., *E. coli*, isolated from aquatic

Table 2. FIC values and the combinations effects of *L. cubeba* fruit EO and antibiotic against *E. coli*.

Strains	Antimicrobial agents	MIC (mg/mL)		FIC	Sum FIC	Interaction
		Alone	Combination			
<i>E. coli</i> ATCC 25922	<i>L. cubeba</i>	5.53	0.44	0.08	0.33	Synergy
	Nalidixic acid	4.0. 10 <sup>-3</sup>	1.0. 10 <sup>-3</sup>	0.25		
	<i>L. cubeba</i>	5.53	1.38	0.25	0.40	Synergy
	Oxytetracycline	1.67. 10 <sup>-3</sup>	0.25. 10 <sup>-3</sup>	0.15		
<i>E. coli</i> 9C48	<i>L. cubeba</i>	1.38	0.08	0.06	0.14	Synergy
	Nalidixic acid	16.0. 10 <sup>-3</sup>	1.3. 10 <sup>-3</sup>	0.08		
	<i>L. cubeba</i>	1.38	0.08	0.06	0.14	Synergy
	Oxytetracycline	32.0. 10 <sup>-3</sup>	2.6. 10 <sup>-3</sup>	0.08		
<i>E. coli</i> 11C123	<i>L. cubeba</i>	2.30	0.58	0.25	0.50	Synergy
	Nalidixic acid	21.3. 10 <sup>-3</sup>	5.3. 10 <sup>-3</sup>	0.25		
	<i>L. cubeba</i>	2.30	0.97	0.42	0.92	Addition
	Oxytetracycline	21.3. 10 <sup>-3</sup>	10.7. 10 <sup>-3</sup>	0.50		

animals than those against reference strains (MIC = 0.015 µg/mL) were reported [13]. Otherwise, the better inhibitory effects were observed when compared the MIC values of *Cinnamomum cassia*, *C. verum*, *Origanum compactum*, *Thymus capitatus* and *T. vulgaris thymoliferum* against *Listeria monocytogenes*, *Salmonella Typhimurium* and *E. coli* isolated strains from meat than references strains [14].

In order to reduce the consumption of ABs as well as economic losses due to the use large quantities of EOs, the *L. cubeba* fruit EO were used in combination with ABs. The FIC indices ranged from 0.23 - 1.17 and from 0.14 - 0.92 for the combined antimicrobial substances against *Vibrio* spp. and *E. coli*, respectively. Overall, no antagonistic effect was observed for any of the combination evaluated. A majority of combination provided a synergistic effect against tested microorganisms. In fact, out of 16 AB-EO pairs tested, 11 of them showed synergistic effect (FIC ≤ 0.5), 3 of them showed addition effect (0.5 < FIC ≤ 1.0) and 2 of them showed indifferent effect (1 < FIC ≤ 4). The best combination was found for *L. cubeba* and nalidixic acid, oxytetracycline against *E. coli* 11C123, in which FIC values were 0.14 and showed a total synergistic effect (MIC reduce 12 - 17 fold). A significant 4 to 10 times and 4 to 13 times reduction in the MIC of the ABs and *L. cubeba*, were observed in rest combinations with FIC index ranging from 0.23 to 0.50, respectively.

Several studies have described various interaction effects for ABs and EOs. Tea tree (*M. alternifolia*) EO combinations with gentamicin or tobramycin had a synergistic effect against *E. coli* and *S. aureus* [9]. The author suggested that aminoglycosides ABs inhibit protein synthesis and tea tree EO damages the cytoplasmic membrane of bacteria; possibly this combination possessed multi-target synergy [9]. The decrease of MIC values when used in

combination could be explained by the presence of various antimicrobial components. Since many EOs or EO compounds generally affected the cell membranes of bacteria and most ABs have specific targets in protein or DNA synthesis, it seems likely that synergy in most cases may be due to multi-target effects. The multi-targets include receptor site modification, enzymatic degradation, reduced accumulation of drug within the bacterial cell, decreased membrane permeability, and efflux pumps [6]. In addition, some mechanisms pathways of EO/ABs synergism were reported, such as the inhibition of common biochemical pathways, the inhibition of protective enzymes, and the combination of membrane active agents. These mechanisms lead to enhance the diffusion of other antimicrobials [15].

#### 4. CONCLUSION

In conclusion, our study showed the antibacterial effect of *L. cubeba* EO against isolated strains. Lower antibacterial activity (higher MIC values) of *L. cubeba* EO against reference strains (5.53 mg/mL) was found than isolated strains (1.15 - 2.30 mg/mL). Most of combinations showed synergistic effect and the best synergistic effect were obtained for *L. cubeba* and nalidixic acid or oxytetracycline combination against isolate *E. coli* 9C48. The reduction in the effective dose of antimicrobial agents could be useful for treatments in the clinical setting in order to decrease the adverse effects of ABs as well as EOs in the future therapy. The results obtained could be a potential and promising application for sustainable therapy in aquaculture.

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## TÓM TẮT

### NGHIÊN CỨU TƯƠNG TÁC KHÁNG KHUẨN CỦA TINH DẦU MÀNG TANG *LITSEA CUBEBA* VÀ KHÁNG SINH TRONG THUỶ SẢN

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Nghiên cứu này đánh giá tương tác kháng khuẩn giữa tinh dầu màng tang (*Litsea cubeba*) và một số kháng sinh đối với 2 chủng vi khuẩn kiểm định và 6 chủng vi khuẩn phân lập từ thủy sản bao gồm *Escherichia coli* ATCC 25922 và *Vibrio parahaemolyticus* ATCC 17802; và *E. coli* 9C48, *E. coli* 11C123, *Vibrio* 2S4, *Vibrio* 2N38, *V. parahaemolyticus* ND201 và *V.*

*parahaemolyticus* TB81. Khả năng kháng khuẩn của tinh dầu, axit nalidixic và oxytetracycline được xác định bằng phương pháp pha loãng liên tục sử dụng phen 96 giếng. Tác dụng kháng khuẩn của tinh dầu màng tang đối với vi khuẩn phân lập (MIC = 1,15 - 2,30 mg/mL) tốt hơn đối với vi khuẩn kiểm định (MIC = 5,53 mg/mL). Hiệu ứng tương tác giữa tinh dầu màng tang và axit nalidixic hoặc oxytetracycline được xác định bằng phương pháp bàn cờ thông qua việc xác định giá trị nồng độ ức chế riêng phần (FIC). Trong 16 tương tác tinh dầu - kháng sinh, có 11 tương tác hiệp đồng (FIC  $\leq$  0,5), 3 tương tác cộng hợp (0,5 < FIC  $\leq$  1,0) và 2 tương tác không khác biệt (1,0 < FIC  $\leq$  4,0). Tương tác hiệp đồng giữa tinh dầu và kháng sinh có thể làm tăng hiệu quả, giảm độc tính, giảm tác dụng phụ, giảm nồng độ sử dụng của kháng sinh được sử dụng trong nuôi trồng thủy sản.

*Từ khóa:* *Litsea cubeba*, tinh dầu, hoạt tính kháng khuẩn, tác dụng hiệp đồng.