MINISTRY OF EDUCATION AND TRAINING
HANOI UNIVERSITY OF SCIENCE AND TECHNOLOGY

NGUYEN HAI VAN

ANTIBACTERIAL ACTIVITY AND MECHANISM OF ACTION OF MAY CHANG *(LITSEA CUBEBA)* ESSENTIAL OIL AGAINST PATHOGENIC BACTERIA AND ITS POTENTIAL APPLICATION IN AQUACULTURE

DISSERTATION
for the degree of

DOCTOR OF PHILOSOPHY IN FOOD TECHNOLOGY

Hanoi - 2018

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Major: Food Technology
Code No.: 9540101

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SUPERVISORS:
1. Assoc. Prof. Chu Ky Son
2. Dr. Samira Sarter

DECLARATION

I hereby declare that this thesis is an original work and that it has not been previously presented at my university or any other university for any degree.

I also declare that, to the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made.

On behalf of the supervisors Date

CHU Ky Son NGUYEN Hai Van

ACKNOWLEDGEMENTS

I would like thank my co-supervisor Dr. Samira SARTER and Assoc. Prof. CHU-KY Son who have supported and guided me from the initial to the final level to develop an understanding of the subject. I have been extremely lucky to have advisors who cared so much about my work, and who responded to my questions and queries so promptly. This thesis would not have been possible without their encouragement and support. Thank you for all you have taught me and the support you gave me during the preparation of my thesis.

I am deeply grateful to all members of the jury for agreeing to read the manuscript and to participate in the defense of this thesis.

I wish to express my gratitude to Dr. Jean-Christophe MEILE who kindly taught me knowledge and laboratory techniques in antibacterial mechanisms. I am also very grateful to Dr. Marc LEBRUN who helped me to learn techniques to analyze composition in his laboratory. I also like to thank all the members of staff at CIRAD, UMR Qualisud who helped me in my supervisor's absence.

I would like to thank Dr. Domenico CARUSO from URM ISEM (IRD), Dr. NGUYEN Ngoc Tuan and Ms. TRINH Thi Trang from Vietnam National Uinverist of Agriculture for their helps and teaching me about providing for the research, especially in aquaculture sector. In particular, I wish to thanks Dr. Domenico CARUSO for helping me in the implementation of immunological protocols.

I thank Dr. NGUYEN Thi Hong Van from Vietnam Academy of Science and Technology who taught me the extraction technique of essential oils.

I extend my sincere thanks to all members of the Center for Research and Development in Biotechnology, the Department of Food Technology and the School of Biotechnology and Food Technology, and all those who contributed directly or indirectly to my thesis.

I could not have achieved this goal without the help and support of so many people. Many thanks to Dr. NGUYEN Hoang Nam Kha, NGUYEN Huu Cuong and Marc LARTAUD for their kind help with my research. I am grateful to Hien, Hai, Hoa, Nhi, Nam, Chanh and Nhan for all their help and assistance with my experiments. I have been blessed with a

friendly and cheerful group of fellow students.

I would like to thank all my friends, Phuong Anh, Phuong, Lan, Nghia, Van, Tam… for their encouragement. Thank is not enough for Amandine for her support in my first time in France.

I would especially like to thank all members of my family for the love, support and constant encouragement I have gotten over the years. You are the salt of the earth, and I undoubtedly could not have done this without you.

To anyone that may I have forgotten. I apologize. Thank you as well.

This project was made possible thanks to the grant provided by the CIRAD – France, as well as the French Ministry of Foreign Office for providing part of funds to the project ESTAFS (Ethnobotany for Sustainable and Therapy in Aquaculture and Food Safety) project in the frame of the Bio-Asia program.

ABSTRACT

The threat of bacterial resistance to antibiotics has created an urgent need to develop new antimicrobials. In this context, a growing interest has arisen towards herbal therapy. For the screening test, the antibacterial activities and chemical compositions of nine commercial essential oils obtained from Aromasia company (clove basil *Ocimum gratissimum*, cajeput *Meulaleuca leucadendron*, cinnamon *Cinnamomum cassia*, Indian prickly ash *Zanthoxylum rhesta*, sweet wormwood *Artemisia annua*, basil *Ocimum basilicum*, Mexican tea *Chenepodium ambrosioides*, corn mint *Mentha arvensis*, may chang *Litsea cubeba*) were tested. Due of high antibacterial acitivities, widely distributed and limit of studies in Vietnam, May chang *L. cubeba* was selected for further research.

L. cubeba leaf EOs collected from North Vietnam were characterized by their high content in either 1,8-cineole or linalool. Linalool-type EOs were more effective than 1,8-cineole-type. EO leaf samples, LC19 (1,8-cineole-type) and BV27 (linalool-type), showed strong bactericidal effect against *Escherichia coli*. EOs caused changes in cell morphology, loss of integrity and permeability of the cell membrane, as well as DNA loss. However, LC19 showed antimicrobial effects against *E. coli* differed with BV27. LC19 mostly affected to the cell membrane, leding to a significant cell filamentation rate and altered cell width, whereas BV27 damaged cell membrane integrity leading to cell permeabilization and altered the nucleoid morphology.

L. cubeba fruit EO, as similar with oxytetracycline, exhibited higher survival rates and lower bacterial concentrations of the whiteleg shrimp than the control (EO and antibiotic-free). However, the application of *L. cubeba* EO in aquaculture has been limited. The common carp was fed with 0 (control), 2, 4 and 8% (w/w) *L. cubeba* leaf powder supplementation diets. Weight gain, specific growth rate and feed conversion ratio were improved together with the increasing of *L. cubeba* leaf powder in a dose-related manner. *L. cubeba* leaf powder increased nonspecific immunity (lysozyme, haemolytic and bactericidal activities of plasma) of carps. After infection with *A. hydrophila*, the survival percent of fish fed with *L. cubeba* leaf powder were significantly higher than that of control. Therefore, our results could be of great potential for the discovery of plant candidates for the sustainable therapy in aquaculture including fish and shrimp to improve food quality and safety.

Keywords: *Litsea cubeba*, essential oils, antibacterial mechanism, chemical diversity, pathogenic bacteria, aquaculture, sustainable therapy, Vietnam

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LIST OF ABBREVIATION

ACP:	Alternative Complement Pathway
AHPND:	Acute Hepatopancreas Necrosis Disease
AHPNS:	Acute Hepatopancreas Necrosis Syndrome
ANOVA:	Analysis of variance
ATCC:	American Type Culture Collection
CCP:	Classical Complement Pathway
DAPI:	4′,6-dia-mino-2-phenylindole
EO:	Essential oil
FAO:	Food and Agriculture Organization
FCE:	Food Conversion Efficiency
FCR:	Feed Conversion Ratio
FIC:	Fractional Inhibitory Concentration
GC:	Gas Chromatography
GC/MS:	Gas Chromatography – Mass Spectrometry
g/t:	Gram per metric ton
h:	hour
HP:	Hepatopancreas
i.p:	intraperitoneally
KI:	Kovats Index
LCP:	Lectin Complement Pathway
LD50:	Lethal Dose 50
LPS:	Lipopolysaccharides
MBC:	Minimum Bactericidal Concentration
MHA:	Mueller Hinton Agar
MHB:	Mueller Hinton Broth
MIC:	Minimum Inhibitory Concentration
NA:	Nutrient Agar
OD:	Optical Density
OM:	Outer Membrane
OTC:	Oxytetracycline
PI:	Propidium Iodide

RBC:	Red Blood Cell
SEM:	Scanning Electron Microscopy
SGR:	Specific Growth Rate
TEM:	Transmission Electron Microscopy
VASEP:	Vietnam Association of Seafood Exporters and Processors
WBC:	White Blood Cell
WG:	Weight Gain
WHO:	World Health Organization

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INTRODUCTION

Aquaculture is the fastest growing food sector globally and is established itself as a high protein resource to fulfill the human food demand since the natural resources exhibits over exploitation. Vietnam has been geographically endowed with ideal conditions (3260 km coastline, 3000 islands and 2860 rivers) for the thriving fishery sector. Consequently, this sector plays an important role in the national economy with the high growth of aquatic production over the year. According to Food and Agriculture Organization (FAO) of the United Nations, Vietnam has become the fifth top producer of aquaculture products [64].

According to the worldwide extension of aquaculture activity, new emerging diseases and the occurrence of other diseases increased year by year. Recently, the outbreak diseases caused by bacterial pathogens such as *Aeromonas hydrophila* and *Vibrio parahaemolyticus* spread around the world, and led to massive economic losses. Antibiotics have been widely used in aquaculture to promote growth, to increase feed efficiency and to prevent infections. However, the overuse of antibiotics is considered to be one of the major reasons for the development of bacterial resistance to antibiotic. In addition, the resistance genes can spread through horizontal genetic transfer between zoonotic and commensal bacteria of different niches along the food chain [179]. With the widespread of resistance among zoonotic bacteria that may be pathogenic to humans, new strategies are needed to control these organisms in food producing systems and reduce the use of antibiotic. In this regard, there is a growing interest in investigating natural antimicrobials such as plant-based products and these could be a potential alternative to antibiotic used in aquaculture.

Essential oils are produced as secondary metabolites by many plants and can be distilled from all part of plants such as flowers (jasmine, lavender), leaves (thyme, "may chang"-Chinese pepper, basil), bark (cinnamon), fruits (anis, star anise, "may chang") [75]. EOs containing bioactive compounds have been known for the biological activities, remarkably antimicrobial activity against pathogenic bacteria and which depend on their chemical composition [21, 31, 119]. The antibacterial mechanism of EOs is not specific [31]. The hydrophobic nature of EOs may facilitate their penetration into the cell via interaction with cell membranes [31]. In fact, EOs may have several effects including the degradation of the cell wall, damaging the cytoplasmic membrane, cytoplasm coagulation, damaging the membrane proteins, increased permeability leading to leakage of the cell contents [188], reducing the proton motive force [164], reducing the nuclear DNA content [46], reducing

the intracellular ATP pool via decreased ATP synthesis [166] and reducing the membrane potential via increased membrane permeability [181].

Several studies on application of EOs, plant extract or plants in aquaculture have shown to enhance growth promotion, immunostimulation as well as antibacterial effects [43, 76, 136]. However, to the best of our knowledge, the investigation on this field in Vietnam is still limited in Vietnam.

Therefore, we conducted the thesis entitled **"Antibacterial activity and mechanism of May Chang (*Litsea cubeba*) essential oil against pathogenic bacteria and its potential application in aquaculture"**.

OBJECTIVES OF THESIS

The objectives of the study are as follows:

- To screen a potential EO in Vietnam having antibacterial activites against pathogenic bacteria in food and aquaculture.
- To investigate the mechanism of action of EOs against pathogenic bacteria.
- To apply the results obtained in aquaculture.

CONTENTS OF THESIS

- Investigation of the antibacterial activities of some EOs from Vietnam against pathogenic bacteria such as *Escherichia coli*, *Aeromonas* spp., *Vibrio* spp., …
- Investigation of the chemical compositions diversity and antibacterial activities of one EO having the best antibacterial activity (among tested EOs content 1) in Vietnam
- Investigation the mechanism of action of the EO in term of cell viability, membrane integrity, membrane permeabilization, cell size and DNA of *E. coli*
- Application in aquaculture in Vietnam such as whiteleg shrimp (*Litopenaeus vannamei*) and common carp (*Cyprinus carpio*)

THEORETICAL AND PRATICAL SIGNIFICANCE OF THESIS

- **Theoretical significance**

The results of this study

- Contributions significance to the scientific data on the diversity of chemical composition of May chang (*Litsea cubeba*)
- Explanation the antibacterial mechanism of *L. cubeba* essential oils against *Escherichia coli*, a representative of gram-negative bacteria

- **Pratical significance**

This study is the basic research for the application of May chang *Litsea cubeba*-derived having antibacterial activities with the potential of exploitation and application in aquaculture to reduce the use of antibiotics for sustainable agricultural development.

NOVELTY OF THESIS

- This is the first report reporting the antibacterial mechanism of May chang *Litsea cubeba* essential oils against bacterial model *E. coli.*
- This is the first report evaluating the effect of May chang *Litsea cubeba* on common carp and whiteleg shrimp.

CHAPTER 1. LITERATURE REVIEW

1.1. Essential oils: plant-based alternatives to antibiotics

1.1.1. Definitions and biological activities of essential oils

Essential oils (EOs) are the liquid secondary metabolites which are synthesized by various organs of aromatic plants such as buds, flowers, leaves, stems, branches or seeds and characterized by strong odors and usually clear (uncolored) appearances [21].

Nowadays, the properties of EOs have been known better and thanks to these properties, their usage areas have been extensively enlarged. EOs or their components are being used commercially in the production of cosmetics (fragrances and aftershaves), food additives and in aromatherapy of agriculture or medicine [21].

EOs roles in the plants are mainly protecting the plants from pathogens and predators by their antibacterial and antifungal activities due to the presence of the terpenoids and phenolic compounds in EOs. The functional properties of EOs mainly depend on their chemical compositions [31]. Tea tree (*Melaleuca alternifolia*), cinnamon leaf oil (*Cinnamomum zeylanicum* Blume.), 1,8-cineole, linalool, citral are some examples of EOs and components that are reported to have anti-inflammatory effects [31]. Paula-Freire et al. investigated and proved the antinociceptive effect of *Ocimum gratissimum* EO [124]. Hwang et al. (2005) have investigated the antioxidant effect of *Litsea cubeba* [82]. The most abundant data collected about EOs may be about their antimicrobial activity. The antibacterial effect of EOs has been verified by several studies [140, 153]. The EOs of cinnamon (*Cinnamomum cassia*), oregano (*Origanum vulgare* L.), mint (*Mentha piperita*), basil (*Ocimum basilicum*) are showing better antibacterial effects compared to other EOs like bitter orange (*Citrus aurantium*), sage (*Salvia officinalis* L.) and many others [153].

Steam or water distillation technique is the most frequently used method for the production of EOs [31].

1.1.2. Chemical composition of essential oils

The chemical analysis of EOs is generally performed using gas chromatography (GC) (qualitative analysis) and gas chromatography-mass spectrometry (GC/MS) (quantitative analysis). The identification of the main components is carried out by the comparison of both the GC retention times and the MS data against those of the reference

standards, Kovats retention indices (KI) and comparison with previous literature. The compounds found in EOs are from a variety of chemical classes, predominantly terpenes, and phenylpropanoids and other compounds in smaller proportions. They are all hydrocarbons and their oxygenated derivatives, and also contain nitrogen or sulfur [32].

1.1.2.1. Terpenes

Terpenes are the largest group of natural compounds, with over 30,000 known structures. Terpenes are polymers of isoprene (C_5H_8) joined together (Fig. 1.1). A terpene containing oxygen is called a terpenoid.

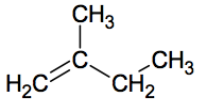


Figure 1.1: Isoprene unit ([32])

Terpenes are classified by the number of isoprene (Table 1.1). EOs are mainly composed of monoterpens and sesquiterpenens and their oxygenated derivatives. The high molecular compounds (diterpenes, triterpenes) were rare found in EOs.

Table 1.1: Classification terpenes by the number of isoprene units ([32])

n	Number of carbon	Terpenes	Example
2	10	Monoterpenes ($C_{10}H_{16}$)	Geraniol
3	15	Sesquiterpenes ($C_{15}H_{24}$)	Farneol
4	20	Diterpenes ($C_{20}H_{32}$)	Ginkgolide
6	30	Triterpenes ($C_{30}H_{48}$)	Squalene
8	40	Tetraterpenes ($C_{40}H_{64}$)	β-carotene
>8	>40	Polyterpenes $(C_5H_8)_n$	Rubber

a. Monoterpenes

Monoterpenes are formed from two C_5 isoprene units which made a skeleton with the molecular formula $C_{10}H_{16}$ (Table 1.1). Monoterpenes may be cyclic (ring-forming) or acyclic (linear), and their derivatives include alcohols, esters, phenols, ketones, lactones, aldehydes and oxides (Table 1.2 and Fig. 1.2) [32].

Table 1.2: Classification monoterpenes by functional groups ([32])

Functional groups	**Examples**
Carbure	myrcene, ocimene, terpinenes, p-cimene, δ-3-carene, sabinene,...
Alcohols	geraniol, linalol, citronellol, lavandulol, menthol, α-terpineol, …
Aldehydes	geranial, neral, citronellal, …

Ketone	menthones, carvone, pulegone, piperitone, camphor, thuyone, …
Esters	linalyl acetate, citronellyl acetate, menthyl, α-terpinyl acetate, …
Ethers	1,8-cineole, menthofurane, …
Peroxydes	ascaridole, …
Phenols	thymol, carvacrol, …

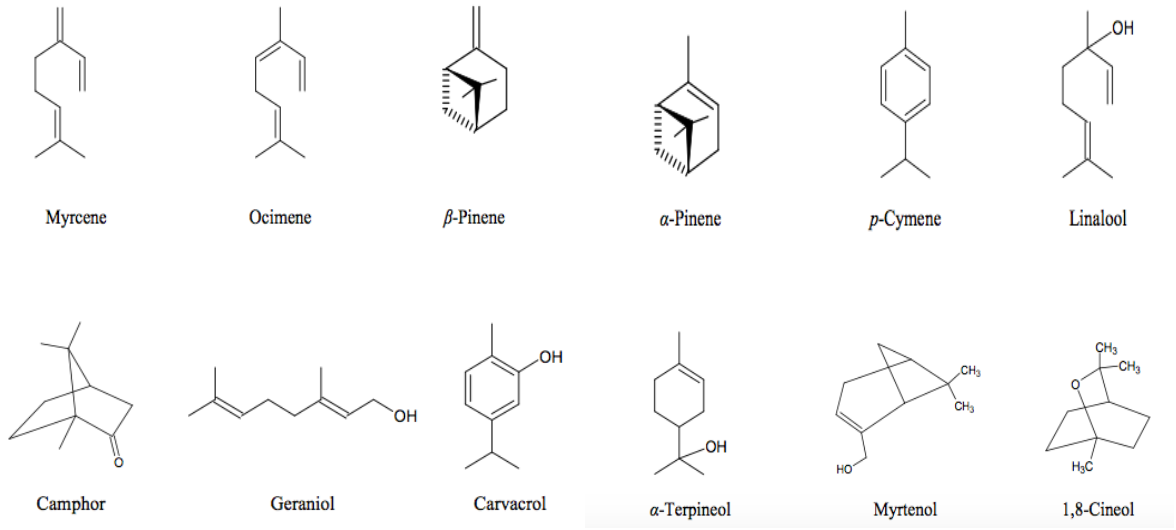


Figure 1.2: Structure of some monoterpenes of essential oils ([32])

b. Sesquiterpenes

Following monoterpenes, sesquiterpenes are the second most frequently presented in EOs. Their molecular formula is $C_{15}H_{24}$ which formed from three isoprene units combined (Table 1.1). Sesquiterpenes may be linear, branched or cyclic (Fig. 1.3).

Alcohols: bisabol, cedrol, β-nerolidol, farnesol, carotol, β-santalol, patchoulol…

Ketones: germacrone, nootkatone, cis-longipinan-2,7-dione, β-vetinone, turmerones…

Epoxide: caryophyllene oxide, humulene epoxides, …

Carbures: cadinenes, β-caryophyllene, curcumenes, farnesenes, zingebrenene…

Examples of plants containing these compounds are angelica, bergamot, caraway, celery, citronella, coriander, eucalyptus, geranium, juniper, lavandin, lavander, lemon, lemongrass, mandarin, mint, orange, peppermint, pine, rosemary, sage, thyme [3].

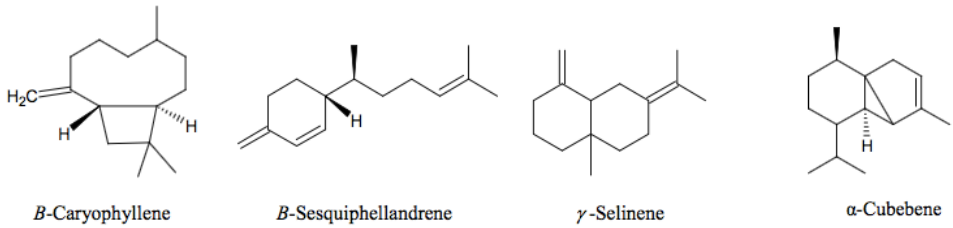
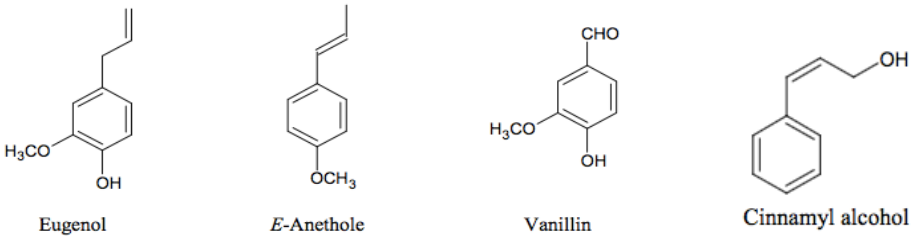


Figure 1.3: Structure of some sesquiterpenes of essential oils ([32])

1.1.2.2. Phenylpropanoids

Phenylpropanoids have a C_6C_3 skeleton composed of a six carbon aromatic ring (also known as benzene ring) with a three-carbon side chain. Only approximately 50 phenylpropanoids have been described. Phenylpropanoids are far less common than terpenoids. However, some of the EOs in which phenylpropanoids occur contain significant proportions of them, such as the eugenol in clove EO, present 70 to 90% of the EO or cinnamon *C. cassia* EO containing 90% cinnamladehyde [117].



Eugenol *E*-Anethole Vanillin Cinnamyl alcohol

Figure 1.4: Structure of some phenylpropanoid of essential oils ([32])

1.1.2.3. Sulfur and nitrogen compounds of essential oils

More rarely, a few compounds found in EOs contain one or more sulfur or nitrogen molecules. The presence of sulfur in particular confers an often strong, characteristic odour [32]. Sulfur- and nitrogen-containing compounds occur mainly as glucosinolates or isothiocyanate derivatives. EOs from plants in the Alliaceae family are also particularly well known for sulfur-containing compounds; these include plants such as *Allium cepa* L. (onion), *Allium porrum* L. (leek) and *Allium sativum* L. (garlic), in which the sulfur compounds are responsible for the characteristic aroma and taste [31].

EOs are mixtures of 20 to 60 individual compounds and sometimes they may contain up to approximately 100 components. EOs are usually composed of one, two or three major components with quite high percentage (20% to 70%), where the remaining part presented in trace amounts (Table 1.3). Generally, the biological properties of the EOs is dependent on their chemical composition and the amount of the single components. However, the composition of EOs depends on spices, geographic, seasonal and climate, extraction techniques [31]. These factor could also affect on the yield of EO [31]. Thus, these variations are significant influences the biological activities of EOs.

1.1.3. Antibacterial activity of essential oils

EOs have been studied extensively for their antimicrobial properties among other

biological properties. EOs as well as their compounds have been reported to have antimicrobial activity against a wide range of spoilage and pathogenic bacteria. EOs are usually mixtures of several components in which phenolic groups were reported the most effective, followed by cinnamic aldehyde, aldehydes, ketones, alcohols, ethers, and hydrocarbons [88, 153]. Generally, both Gram-positive and Gram-negative bacteria have demonstrated susceptibility to EOs and their components. The methods used are usually disc diffusion method or broth-dilution method. To assess the activities of EOs, the plant spices, EO compositions and microorganism are important factors.

1.1.3.1. In vitro tests of antibacterial activity of essential oils

EOs form Apiaceae family can be obtained from both seeds and leaf materials and therefore, the composition and antibacterial activity of these EOs may be different. For example, coriander *Coriandrum sativum* (Apiaceae family) seed EO rich in linalool and had MICs ranged from 0.006 to 1% against *Staphylococcus aureus*, *E. coli* and *Candida albicans*, whereas coriander leaf EO contains predominantly decanal, decen-1-ol and *n*-decanol, possessed a higher MIC values ranged from 10.8 to 21.7% for a same broad of pathogenic bacteria [75]. In addition, clove basil *O. gratissiumum* EO inhibited both Gram-positive bacteria (*S. aureus* and *Bacillus* spp.) at the concentration of 93.7-150 mg/mL and Gram-negative bacteria (*E. coli, P. aeruginosa, S*. Typhimurium, *Klebsiella pneumoniae, Proteus mirabilis*) at the concentration of 107-750 mg/mL [127]. *Cinnamomum verum, Cinnamomum cassia* bark EOs (Lauraceae family) were dominated by cinnamaldehyde where leaf EOs contained high level of eugenol. Cinnamon bark EOs possessed a strong antibacterial activity which low MIC values ranged from 0.012 to 0.05% against *E. coli, C. jejuni, S. aureus, S. enteritidis, S.* Typhimurium, *L. innocua* and *L. monocytogenes*; whereas MIC for cinnamon leaf EOs were 0.31 to 1.25% for a same broad of pathogens. These data suggest that bark EOs possessed a higher antimicrobial activity than leaf EOs [75, 117, 164].

Table 1.3: Lists of the essential oils used in this study

***Botanical name* (Family)**	**Common name**	**Part used**	**Origins**	**Oil yield (% w/w)**	**Main compounds**	**Properties**	**Ref**
Ocimum gratissimum (Lamiaceae)	Clove basil	Leaf	India	0.2-0.6	eugenol 84.1%	Used in folk medicine to treat: upper respiratory tract infections, diarrhea, headache, ophthalmic, skin diseases, pneumonia, cough, fever, conjunctivitis	[134]
Melaleuca leucadendron (Myrtaceae)	Cajeput	Leaf	Indonesia, Egypt, Pakistan	0.61-1.59	1,8-cineole 64.3%; terpinolene 29.2%, α-terpinen 22.6%	Use for the treatment of colic, cholera, headaches, toothache and various skin diseases	[65, 151]
Cinnamomum cassia (Lauraceae)	Cinnamon	Bark	China, Vietnam	2.70-3.11	trans-cinnamaldehyde (90.08%)	Used to treat diseases: carminative, stomachic, astringent, stimulant and antiseptic	[117, 164]
Zanthoxylum rhetsa (Rutaceae)	Cape yellowwood	Seed	India	1.5-1.8	linalool 71%, limonene 8.2%	Used in medicine for the treatment cholera, antiseptic, disinfectant, asthma, toothache, rheumatism, anti-diabetes, antispasmodic, diuretic, anti-inflammatory	[139]
		Fruit	India	1.8-2.22	sabinene 29.5%, terpinen-4-ol 22.7%		
Artemisia annua (Asteraceae)	Sweet wormwood	Leaf	Bosnia, Vietnam, India, Iran	0.30-0.54	camphor 44%, germacrene D 16%, artemisia ketone 22.3%	Used in infusion as poison antidote, activates the blood circulation, antimalaric and anthelmintic	[27, 37]
		Root	India	0.25	cis-arteannuic alcohol 25.9%, (E)-β-farnesene		

					6.7%, β-maaliene 6.3%		
Litsea cubeba (Lauraceae)	May chang, Chinese pepper	Fruit	China, Vietnam	2 -8	citral 45.7-83.8%	Used in medicine for headache, fatigue, muscle pain and depression; and fresh leaves were mashed and used for skin problems, such as sore and furuncles	[25, 140]
		Leaf	China, India, Vietnam	0.9-1.3	citronellal 78.2%; 1,8-cineole 51.7%/linalool 91.1%/sabinene 54.6%		
Ocimum basilicum (Lamiaceae)	Basil/ Sweet basil	Leaf	India, Vietnam	0.2-0.5	methyl chavicol 87%; methyl chavicol 61.5%/linalool 28.6%	Used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunctions	[134]
Chenopodium ambrosioides (Chenopodiaceae)	Mexican tea Wormseed	Leaf	Nigeria, India	0.17-0.3	α-terpinene 44.7-63.1%, p-cymene 21.3-26.4%, ascaridole 3.9-17.9%	Used in medicine to treat anthelmintic, wounds, respiratory, inflammatory, painful, bronchitis, tuberculosis, rheumatism, snake bites	[13, 142]
		Leaf	Yemen, Vietnam, Brazil	0.52-0.71	ascaridole 41.8-61.4%, isoascaridole 18.1-18.6%, p-cymene 12.7-16.2%		
		Fruit	Philipine, Vietnam	0.5-2.5	limonene32.5%, trans-pinocarveol 27%, ascaridole 41.8-61.4%,		
Mentha arvensis (Lamiaceae)	Corn mint/ Menthol mint	Leaf	Vietnam, India	0.3-1.2	menthol 56.4%, menthone 10.9%	Used as nasal decongestant, carminative, gastric and skin diseases	[28, 173]

In addition, cajeput *Melaleuca leucadendron* EOs contained eugenol methy ether showed higher antimicrobial activity (MIC=4-8 μg/mL) than cajeput EOs rich in 1,8-cineole (MIC=1067 and 1191 μg/mL) [15, 151]. Eucalyptus *E. globulus* EO contained high levels of 1,8-cineole and the MIC values of this EO were 1.25 to 8.75% against some strain pathogenic such as *Streptococcus, Staphylococcus, E. coli*. MIC values for clove *Syzygium aromaticum* EO, which contained predominantly eugenol, ranged from 0.013 to 0.62% for a range of pathogens including *S. aureus, E. coli* and *C. albicans* [75].

Limonene were found as major component of *Citrus* spices (Rutaceae family). High MIC values (1 to >2%) were reported for citrus EO including orange *C. sinensis*, bergamot *C. aurantium*, lemon *C. limon* and graphefruit *C. paradisi* against *S. aureus, E. coli, K. pneumoniae* and *E. faecalis.* However, a low MIC (0.062%) were also found for orange EO against *V. cholerae* and *A. hydrophila* [75]. Wormseed *C. ambrosioides* EO (Chenopodiaceae) showed an important inhibiting activity on *S. aureus* than *E. coli*. Indeed, the MIC values ranged from 1.56-1.71 mg/mL against *S. aureus*, whereas those against ranged from 6.69-6.86 mg/mL [13]. The same MIC values of 10 mg/mL was found of sweet wormwood *A. annua* (Asteraceae) against both Gram-negative (*E. coli*) and Gram-positive bacteria (*S. aureus, B. cereus, B. subtilis*) were reported [27].

Table 1.4: MICs of essential oils tested in vitro against pathogenic bacteria

Family	**Plants**	**Bacteria**	**MIC (%v/v)**	**Ref**
Apiaceae	Coriander seed *Coriandrum sativum*	*S. aureus, E. coli*	0.006 - 1	[75]
	Coriander leaf *Coriandrum sativum*	*S. aureus, E. coli*	10.8 - 21.7	[75]
Lamiaceae	Peppermint *Mentha piperita*	*S. aureus, E. coli*	0.125- 2	[75]
	Corn mint *Mentha arvensis*	*B. cereus, S.* Typhimurium, *P. aeruginosa, E. coli*	0.078 – 5	[173].
	Basil *O. basilicum*	*S. aureus, Bacillus* spp., *E. coli, P. aeruginosa, S.* Typhimurium, *K. pneumoniae, P. mirabilis*	0.001 - >2	[127]
	Rosemary *Rosmarinus officinalis*	*E. coli, S. aureus, B. cereus*	0.012 - 2	[75]
	Lavender *Lavandula* spp.	*Staphylococcus* spp., *Enterobacter* spp., *E. coli*	0.25 - 8.75	[75]
Myrtaceae	Tea *Melaleuca alternifolia*	*Staphylococcus* spp., *Enterococcus* spp., *Bacillus* spp., *Klebsiella* spp., *Salmonella* spp.	0.06 - 1.87	[15, 151]

Poaceae	Citronella *Cymbopogon nardus*	*E. coli*, *S.* Typhimurium, *S. aureus*, *L. monocytogenes*	0.025- 0.2%	[75]

1.1.3.2. Litsea cubeba

The genus *Litsea* (Lauraceae family) is composed of 622 species, distributed mainly in tropical and subtropical areas such as Australia, New Zealand, South America, southern China, Japan, Taiwan and South-East Asia [39]. *Litsea cubeba* (also called May Chang or "Màng tang" in Vietnamese) has been more popular than other species in the genus due to its distribution [39].

Figure 1.5: Plant of Litsea cubeba

L. cubeba is a small to medium-sized tree (5-8 m high) and its fruit is a berry-like spherical drupe. It usually generates flower buds in winter and flowers in the spring of the following year. The fruits of 4-5 mm in diameter are green when immature and turn black at maturity in autumn. *L. cubeba* tends to grow on barren mountains and wastelands, ranging in elevation from 300 to 1,800 m, as well as in shrubby areas and sparse woods of the forest fringe [39]. Since ancient times, *L. cubeba* fruit has been used in traditional medicine to treat headache, muscle pain, stomach distension, asthma, diarrhea, turbid urine [25, 39, 91]. *L. cubeba* leaf has been used to promote blood circulation, treat mamitis as well as used for for hemostasis, sores, furuncle, insect and snake bites sores and furuncles [25, 39, 91]. It is widely used in cosmetics, soap, perfume, skin cleaner, and acne medicine [39].

L. cubeba plants contain biologically active chemicals such as ligans, amides, steroids, fatty acids, butanoilides and butenolactones, alkaloids, flavonoids, terpenoids, and volatile oil components [176]. The EOs of *L. cubeba* can be extracted from different parts of plant, including fruit, leaf, flower, flower bud, stem, wood and roots with significant

diversity in composition and yield [176]. *L. cubeba* contains most of its EOs in the fruit (2-8%) and smaller amounts in leaves (0.9-1.3%) and wood (0.5-2.5%). *L. cubeba* EO is a flowing, pale yellow liquid, with an intensely lemonlike, spicy aroma. Wang et al. (2010) analyzed the chemical compositions from different parts of *L. cubeba* EO. The authors demonstrated that β-phellandrene (18.7%), terpinene-4-ol (12.1%), limonene (9.8%) were the main components of stem EO. In the root EO, neral B (21.5%), citronellal (8.6%), and linalool (7.5%) were the main constituents. In the alabastrum EO, the contents of β-phellandrene, cineole, α-pinene, and β-pinene were 33.7, 11.2, 9.0, and 8.3%, respectively. The main components of flower EO were β-terpinene (33.2%), cineole (13.7%), α-pinene (7.5%), and β-pinene (7.3%). Citral was found as the main compound of fruits *L. cubeba* EO (60-90%) and it was reported in other research [26, 155, 176]. Whereas, the major compounds found in leaves were either 1,8-cineole, linalool, sabinene or neral depend on the origin of EO [26, 155, 176].

The *L. cubeba* EO is widely used as a flavor enhancer in foods, cosmetics and cigarettes; as raw material for the manufacture of citral, vitamins A, E and K, ionone, methylionone, and perfumes. Its extracts have been reported for their antibacterial [78], antifungal [140], antioxidant [82] and anticancer [78] activities. MIC values *L. cubeba* fruit EO against *E. coli, S. aureus*, *S.* Typhimurium, *Bacillus* spp., *Listeria* spp.… ranging from 0.01-1%. However, *L. cubeba* leaf EOs possessed a lower antimicrobial activity (MIC = 0.02-2%) against the same broad of bacteria [140, 176].

The application of *L. cubeba* EO in food system, mainly concentrate to *L. cubeba* fruit EO, were reported. Indeed, *L. cubeba* fruit EO could be used as natural additives to preserve foods [157, 161]. Treatment with *L. cubeba* fruit EO maintained the colour and remained the phenolic compounds and antioxidant activity of fresh-cut pears during 14 days of storage at 2°C [161]. *L. cubeba* fruit EO vapor with the laser treatment could inhibited completely the growth of natural molds on the brown rice snack bars for at least 25 days, compared to 3 days of control (without EO vapor and laser treatment) [157]. However, in the food systems, the concentration of EO required to inhibit the growth of organism was much higher from 2-100-fold compared to *in vitro* experiments [101]. The MIC values of *L. cubeba* fruit EO against *Vibrio spp.* in oysters was 3000 μg/g compared with MIC=375 μg/g in broth system [101].

To the best of our knowledge, the applications of *L. cubeba* in the aquaculture

system have never been reported.

1.1.4. Synergistic effects of essential oils on the antibacterial activity

The therapeutic value of synergistic interactions has been known since antiquity. Recently, the application of combination therapy has gained a wider acceptance, especially in the treatment of infectious diseases [170]. Indeed, the interaction between antimicrobials in a combination can have four different outcomes, synergistic, additive, indifferent or antagonistic [170]. Various antimicrobial interaction have been reported for EOs or their constituents and antibiotic when tested in binary combinations [73, 83, 146]. For example, combination of oregano (*O. vulgare*), thyme (*T. vulgaris*), basil (*O. basilicum*), marjoram (*O. majorana*), rosemary (*R. officinalis*) and lemon balm (*Melissa officinalis*) were observed against a broad of pathogenic bacteria. For instance, the existing studies have focused on the antimicrobial activity of the following: basil/oregano and thyme/oregano EO mixtures against *E. coli* [72]; thyme/oregano EO mixture against *S. aureus* and *S.* Typhimurium [156]; thyme/cinnamon EO mixtures against *S. aureus* [90]; thyme/peppermint, and thyme/lemon balm EO mixtures against *E. coli* [90]; thyme/lavender, thyme/peppermint, and thyme/rosemary EO mixtures against *S. aureus*, *B. cereus*, *P. aeruginosa*, and *E. coli* [63]. Among these combinations, only thyme/oregano [156], thyme/cinnamon [90]; and thyme/peppermint EO mixtures [63] displayed a synergistic effect. Other combinations have shown indifferent, additive or antagonistic effects [146]. Mixtures of cinnamaldehyde with carvacrol or thymol yielded in most cases synergistic effects against *E. coli* and *S.* Typhinurium. 1,8-cineole in combination with aromadendrene and limonene were found to have additive and synergistic effects, respectively. Other combinations including α-pinene with limonene or linalool also showed additive and synergistic effects [22].

A combination of antagonistic, synergistic and additive interactions was observed between ciprofloxacin and tea-tree *M. alternifolia*, thyme *T. vulgaris*, peppermint *M. piperita* and rosemary *R. officinalis* EOs against *K. pneumonia* and *S. aureus*. Promising synergy was observed between rosemary EO and ciprofloxacin. Combination of lemon grass (*C. citratus*) with kanamycin and streptomycin also showed synergy effect against *S.* Typhimurium. Synergism has been observed between oregano EO and levofloxacin, florfenicol and doxycycline against *E. coli* [22, 150]. Non-antibiotic agents may also synergistical act with EOs, including organic acids, sodium chloride, nisin, bacteriocin

…[22].

The concept of antimicrobial synergy is based on the principle that, in combination, the formulation may enhance efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose and reduce the advance of antimicrobial resistance [170]. Therefore, the synergistic interaction could be potential application of EOs and antibiotics in food and aquaculture system.

1.1.5. Antibacterial mechanism of essential oils

The most appropriate method for determining the bactericidal effect as well as a for obtaining information about the dynamic interaction between the EOs and the bacterial strain is the time-kill test. A time-dependent and a concentration-dependent antibacterial effect is also investigated by the time-kill test. Li et al. (2014) reported that the kinetic curves of *L. cubeba* fruit EO at 0.0625% (v/v) was able to prolong the lag phase growth of *E. coli* cells to approximate 12 h while the cells were completely killed at 0.125% (v/v) within 2 h [100]. Destruction of the *E. coli* outer and inner membrane might be due to the penetration of the *L. cubeba* fruit EO with the observation of many holes and gaps on the damaged cells, which led to killing them eventually [100].

The antibacterial mechanism varies with the type of EO or the bacterial strain used. It is well known that in comparison to Gram-negative bacteria, Gram-positive bacteria are more susceptible to EOs [79]. This can be attributed to the fact that Gram-negative bacteria have an outer membrane which is rigid, rich in lipopolysaccharide (LPS) and more complex, thereby limiting the diffusion of hydrophobic compounds through it, while this extra complex membrane is absent in Gram-positive bacteria which instead are surrounded by a thick peptidoglycan wall not dense enough to resist small antimicrobial molecules, facilitating the access to the cell membrane [83]. Moreover, Gram-positive bacteria may ease the infiltration of hydrophobic compounds of EOs due to the lipophilic ends of lipoteichoic acid present in cell membrane (Fig 1.6) [116].

Because of the wide variety of molecules present in the natural extracts, the antimicrobial activity of the EOs cannot be attributed to a single mechanism. Instead, different biochemical and structural mechanisms are involved at multiple sites within the cell and on the cell surface. Generally, due to the hydrophobicity properties, EOs usually lead to the disruption of bacterial structures, destabilization of the double phospholipid layer, degradation of the cell wall, damaging the cytoplasmic membrane, cytoplasm

coagulation, damaging the membrane proteins, increased permeability leading to leakage of the cell contents [95], reducing the proton motive force [164], reducing the intracellular ATP, reducing the membrane potential via increased membrane permeability. These major physiological changes can ultimately result in cell lysis and death [113].

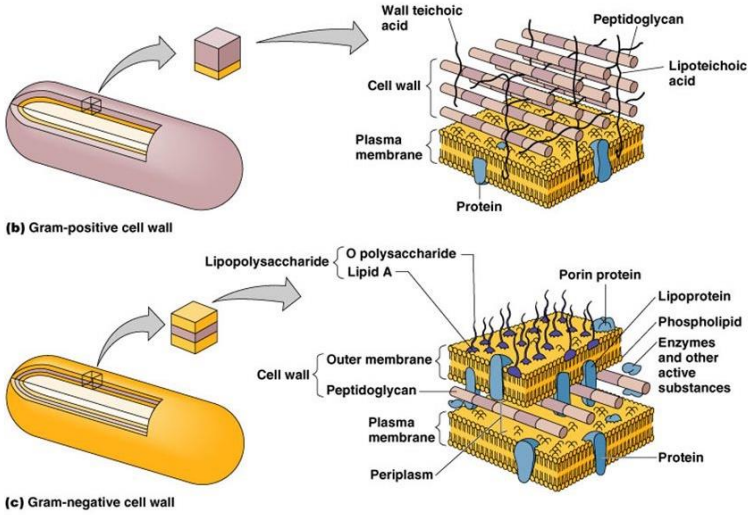


Figure 1.6: Schematic of cell wall of Gram-positive and Gram-negative bacteria ([83])

EOs, particularly EOs that are rich in phenolics, are able to the surface of the cell and thereafter penetrate to the phospholipid bilayer of the cell membrane. The structural of cell membrane integrity is disturbed by the accumulation of EOs, which can harmfully influence the cell metabolism causing cell death. This phenomenon indicates that the cell wall and cell membrane is the first target of EOs [113], therefore, many of studies focused on the change of the cell membrane [29, 30, 49, 118].

The cell wall integrity is critical for the bacteria survival because it is an important element for the essential biological activities occurs within the cells. The membrane acts an effectual barrier between the external environment and the cytoplasm; the import and export of the materials (metabolites and ions) indispensable for all activities happening in the microorganism cell occur through the cell membrane. When EOs are existing in environment surrounding microbial, the bacteria could react by modifying the fatty acids and membrane proteins synthesis to alter the fluidity of the membrane [20].

For instance, *Laurus nobilis* and *Satureja calanmitha* EO, which is rich in 1,8-cineole, caused the loss of membrane integrity and membrane fluidity of *L. innocua* LRGIA 1 and *E. coli* CECT 471, confirmed by using LIVE/DEAD Baclight Kit and by measuring the fluorescence anisotropy of DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1,(4-(trimethyl-amino)-phenyl]-6-phenylhexa-1,3,5-triene) [41]. The LIVE/DEAD

BacLight kit was used to estimate the proportion of cells with an intact cytoplasmic membrane. This kit is generally associated with cells lost their membrane integrity. In addition, PI was also used to evaluate the effect on membrane permeability [24].

Membrane potential is used by the cell to perform actions necessary for life, such as synthesis of enzymes, nucleic acids, polysaccharides, and other cell components, for cell maintenance and repair of damage, for motility, and for numerous other processes [116], and decrease in this membrane potential is indicative of damage to the cell membrane. Recently, Trinh et al. (2015) employed 3,3'-dipropythiacarbocyanide iodide (DiSC35) to confirmed the disruption of membrane potential of *L. innocua* by cinnamon *C. cassia* EO and cinnamaldehyde. Loss of membrane potential is adverse to cell survival, but could be a consequence of membrane disruption [164].

Ginkgo biloba leaf EO caused morphological alterations on the cell wall of *B. cereus* and *E. coli* leading to disruption and lysed cell formation. These morphological alterations in bacterial cells might be occurred due to the effect of *G. biloba* EO on membrane integrity, thereby resulting in the lysis of bacterial cell wall followed by the loss of intracellular dense material of treated cells [20]. Cinnamaldehyde and limonene disrupted the external envelope of *S.* Typhimurium and *Pseudomonas* spp. Indeed, *S.* Typhimurium cell membrane showed altering/disrupted and swelling after exposure to EO. The authors suggested that the components accumulated in the membrane, causing a loss of membrane integrity and dissipation of the proton motive force. In particular, *E. coli* cells had holes or white spots on the cell wall. In addition, the effects of sublethal concentrations of carvacrol and 1,8-cineole alone and in combination on the morphology, cell viability and membrane permeability of *P. fluorescens* were investigated [49]. The ultrastructural changes after 1 h of exposure included shrunken protoplasm, discontinuity of the outer and cytoplasmic membranes and leakage of the intracellular material. A decrease in the number of SYTO-9 cells (intact cells) with a concomitant increase in the number of PI-positive cells (dead cells) were also observed. The author suggested that the morphological changes were indicative of increased membrane permeability and the loss of bacterial envelope integrity, which ultimately lead to cell death [49]. Cell death may have been the result of the extensive loss of cell contents, the exit of critical molecules and ions, or the initiation of autolytic processes [188]. Therefore, release of intracellular components is also a good indicator of membrane integrity.

Further, action of EO on the the integrity of cell membrane changes the membrane permeability which leads to loss of vital intracellular contents like proteins, reducing sugars, ATP and DNA, while inhibiting the energy (ATP) generation and related enzymes leading to the destruction of cell and leakage of electrolytes [46, 95]. Efflux of small ions is not necessarily indicative of complete loss of membrane function, and can be observed in viable cells where growth is inhibited because the cell uses energy for repair or survival rather than cell proliferation [29]. Effects on the cell membrane that lead to cell death is more accurately predicted by detecting the efflux of larger molecules like ATP or carboxyfluorescein diacetate (cFDA) after esterase reaction [181], or by influx of large polar organic DNA-binding stains like DAPI [46] and PI [29].

4′,6-dia-mino-2-phenylindole (DAPI), which gives blue fluorescence to cells, is a fluorescent dye that could penetrate into the bacteria cells and integrate with DNA. The reduction of DNA content of *E. coli* and *S. aureus* cells treated with *Salvia sclarea* EO were observed by the fluorescence spectrophotometer measurements. This may be due to the interaction between *S. sclarea* EO and the bacterial cell membrane [46]. *C. citratus*, *O. gratissimum*, or *T. vulgaris* EO caused an increase in carboxyfluorescein released by *L. innocua* cells [114] and thus a measure of the disruption of the cell membrane. In addition, carvacrol and thymol were leakage of carboxyfluorescein from within *E. coli* cells [181]. *E. coli* and *P. aeruginosa* were much more susceptible, in term of PI uptake, than *S. aureus* when cells exposed to tea tree and cinnamon EO, respectively [29, 45].

Another strategy for determining the mode of action of EOs against bacteria was performed on the basis of the cell constituents release determined by the measurement of the absorbance at 260 nm and 280 nm of the supernatant of treated strains. For instance, Spanish oregano *Corydothymus capitatus*, cinnamon *C. cassia*, and savory *Satureja montana* EOs were able to cause a significant increase amount of 260 nm absorbing material of *E. coli* O157:H7 and *L. monocytogenes* [118]. de Sousa et al. (2013) investigated the effects of oregano *O. vulgare* (rich in carvacrol) and rosemary *R. officinalis* (rich in 1,8-cineole) EOs on *P. fluorescens* [50]. They found that cell material was released immediately after exposure with either EO singly or in combination. Electron microscopy of exposed cells revealed alteration in the cell wall structure, rupture of the plasma membrane, shrinking of the cells, condensation of the cytoplasmic content, and leakage of the intracellular material after 2 and 3 h exposure time [50]. Confocal scanning laser microscopy revealed increased cell membrane permeability, resulting in cell death

after exposure times of only 15 and 30 min [50].

Zengin et al. (2014) confirmed that 1,8-cineole, linalool and α-terpineol caused permeability alteration of the outer membrane, alteration of cell membrane function and leakage of intracellular materials. Indeed, after exposure to linalool, 1,8-cineole, α-terpineol and α-pinene alone and in combination, the cell constituents release increased visibly compared to the control group. In addition, SEM observations confirmed the damage to the structural integrity of the cells and considerable morphological alteration to *S. aureus, E. coli* O157:H7. Treatment with 1,8-cineole and linalool caused pores on the outer membrane of *E. coli* O157:H7 cells which enabled the cytoplasmic constituents to excrete and also caused collapsing of the cells [188].

The leakage of potassium into the extracellular space is considered an indicator for an increase in membrane permeability and ultimate loss of viability for the cell. Tea tree, oregano, cinnamon and ginkgo EO caused the potassium leakage of *E. coli, S. aureus, P. aeruginosa* and *B. cereus*. EO-treated cells showed disruption cell membranes and swelling of the cells which led to leakage of intracellular material [20, 29, 30, 45]. Other intracellular events may contribute to the intracellular ATP decrease; for example, inorganic phosphate may have been lost by passing through the compromised permeable membrane [118], or the proton motive force and changes in the balance of some essential ions, such as K+ and H+, may have been disrupted [118, 164]. These studies indicate that EOs and components are able to cause macromolecular permeability in a variety of bacteria.

There are several additional antimicrobial effects which are different than those already discussed. For example, *B. cereus* cell separation occurs when the cinnamaldehyde binds to FtsZ, a cell-division regulator, and disturbs Z-ring formation [77]. Cinnamon EO was reduced respiratory enzyme activity of *P. aeruginosa* [30]. Clove, eucalyptus and citrus EO inhibited the communication of cell-to-cell among bacteria (quorum sensing) [116].

Table 1.5 describes some potential mechanisms of action of the EOs and/or their components and shows the potential cell targets of their antimicrobial activity. However, each of these actions cannot be considered separate events but instead may be a consequence of the other activities.

1.2. Aquaculture in Vietnam

1.2.1. Overview of aquaculture in Vietnam

Aquaculture is growing rapidly in many regions of the world, and play an important food supply. About 90% of the global aquaculture is produced in Asia [160]. According the Food and Agriculture Organization (FAO) of the United Nations, Vietnam has become the fifth top producer of aquaculture products, including China, India, Bangladesh and Egypt [64]. Vietnam, with a coastline of over 3260 kilometers and more than 3000 islands and islets scattered offshore, plus up to 2860 rivers [55] and estuaries, has been geographically endowed with ideal conditions for the thriving fishery sector which currently exists. Consequently, the fishery sector plays an important role in the national economy, accounting for about 0.09% of Gross Domestic Product (GDP) in 2016 and even reached 12% of total export value in 2001 [2]. The aquatic production in Vietnam has maintained continuous growth in 20 years (1995 – 2015) with an average growth rate of 9.07%/year. The Mekong River Delta in the South and the Red River Delta in the North have been used for wild catch fishing as well as extensive fish farming.

Although there is a growing domestic market as incomes improve and local demand increases, a strong export market is the driving force behind the growth in aquaculture. Products are exported to 164 countries and regions around the world [5], the major markets being the United States of America (20%), EU (18%), Japan (15%), China (9.4%) and ASEAN (7.6%). In 2016, the total exports reached USD 7.053 billion, an increase of 7.4% compared to 2015, contributing more than 22% in export turnover of agriculture, fishery and forestry sector. Currently, shrimp is the largest export product (value of exported shrimp reached USD 3.1 billion) followed by pangasius, tuna, other fish, squid and octopus [5]. With a twenty-four-fold increase in fishery exports since the 1990's, Vietnam now ranks among top five seafood exporters in the world and the sector aquaculture has ranked fourth in the league of the key economic sectors of Vietnam (Fig 1.7) [5].

Table 1.5: Essential oils/components and their identified target sites and modes of action

Common name (Species name)	Major constituents of EOs	Bacterial species (MIC)	Mechanism	Ref
Cinnamon (*Cinnmomum cassia*)	Cinnamaldehyde (75.3%)	*B. cereus* (339 μg/mL) *E. coli* (2640 μg/mL) *L. monocytogenes* (2640 μg/mL) *S. aureus* (1320 μg/mL)	Release of cellular content; reduce of intracellular pH; alteration of membrane integrity and membrane permeabilization	[118, 164]
Coriander (*Coriandrum sativum*)	Linalool (25.9–64.4%)	*L. monocytogenes* (0.018–0.074% v/v)	Damage of cytoplasmic membrane; release of cellular content	[152]
Cinnamomum longepaniculatum	1,8-cineole (58.55%), α-terpineol (15.43%)	*E. coli* (3.125 μL/mL) *S. aureus* (6.25 μL/mL)	Rupture of cell wall and membrane, cytoplasmic coagulation	[99]
Litsea cubeba	Citral (36.17%), citral isomer (32.08%)	*E. coli* 0.125% (v/v)	Damage of cell membranes and flagella of cell	[100]
Linalool		*E. coli* (515–2145 μg/mL) *S. typhimurium* (1000 μg/mL) *B. cereus* (1073 μg/mL) *S. aureus* (1073–2145 μg/mL)	Release of intracellular material, permeabilization of membranes, damage or alteration of cell mebrane function	[10, 188]
1, 8-cineole		*E. coli* (3.125 μL/mL) *S. aureus* (6.25 μL/mL)	Leakage of intracellular material, permeabilization of membranes, alteration of cell membrane	[10, 188]
Eugenol, thymol, carvacrol		*E. coli* O157:H7	Alteration cell morphology and outer envelope, disrupting membrane and leakage of intracellular constituents	[54]

1.2.2. Cultured species

Vietnam aquaculture uses a wide range of species that provide significant potential for further aquaculture development. Among cultured species, common carp (*Cyprinus carpio*) and whiteleg shrimp (*Litopenaeus vannamei*) contribute significantly to food security and economic security by providing souces of animal protein, essential nutrients and income [18, 64, 109].

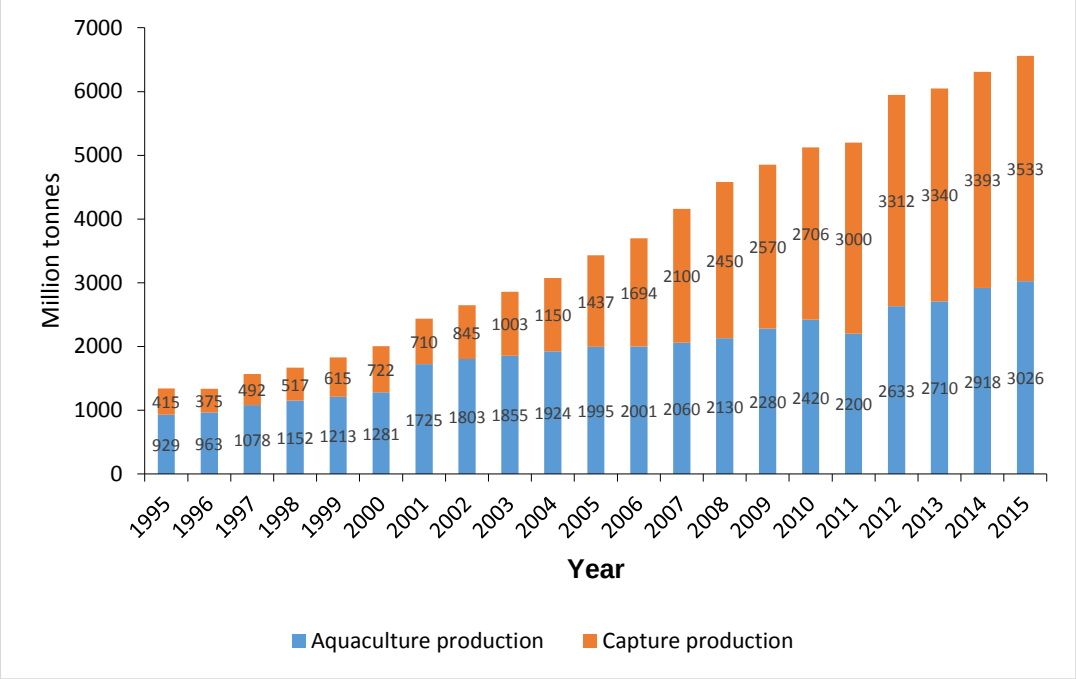


Figure 1.7: Vietnam capture fisheries and aquaculture production (1995 – 2015) [5]

1.2.2.1. Common carp (Cyprinus carpio)

Common carp (*C. carpio*) belongs to the the family Cyprinidae. It generally inhabits freshwater environments, especially ponds, lakes and rivers, and also rarely inhabits brackish-water environments [109]. It is widely distributed in almost all countries of the world but is very popular in Asia and some European countries.

Common carp is the third most widely cultivated and commercially important freshwater fish species in the world. In 2010, it ranked third (after grass carp and silver carp) in terms of worldwide finfish aquaculture production, contributing 9% of the world's total finfish aquaculture production, and Asia accounted for more than 90% of common carp's aquaculture production. Vietnam ranked third (after China and Indonesia) of the world’s aquaculture production of common carp (3, 216, 203 tons) in 2009 (Fig 1.8) [109].

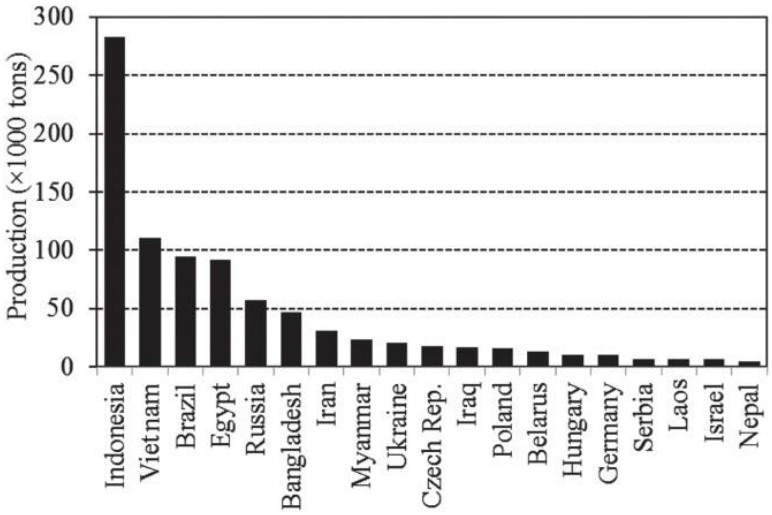


Figure 1.8: Major common carp-producing countries (except China) and their production in 2010 ([109])

1.2.2.2. Whiteleg shrimp (Litopenaeus vannamei)

Whiteleg shrimp (*L. vannamei*), is a variety of prawn of the eastern Pacific Ocean commonly caught or farmed for food. It is capable of tolerating a wide range of salinities. Whiteleg shrimp culture production has undergone a great expansion, from just 8,000 tons in 1980 to over three million tons currently [18, 64].

In 2001, *L. vannamei* was introduced into Vietnam. Vietnam shrimp production is especially development in Mekong River Delta with 67 000 ha of farming area in 2014, increase of 68% compared to 2013. Vietnam is one of the most important shrimp production in the world (including China, India, Indonesia and Bangladesh in Asia regions, Ecuador, Brazil, and Mexico in Americas regions) [64].

1.2.3. Bacterial diseases in aquaculture

Currently, outbreaks of parasitic, bacterial, fungal and viruses act as major pathogens that are affecting the aquaculture industry (Table 1.6).

Table 1.6: Main causes of outbreaks diseases in shrimps and fish farming

Aquatic animals	Pathogens	Name of pathogens	Ref
Shrimp	Virus	Taura syndrome virus, yellow head virus, infectious hypodermal and hematopoietic necrosis virus, white spot syndrome virus, infectious myonecrosis virus, Macrobrachium rosenbergii nodavirus	[89, 175]
	Fungi	*Fusarium incarnatum, Fusarium solani, Enterocytozoon hepatopenaei, Lagenidium* spp., *Sirolpidium* spp.,...	[89]
	Parasites	*Microsporidia* spp., *Haplospora* spp., *Epistylis* spp.,	[89]

		Zoothamnium spp., *Gregarina* spp., *Acineta* spp.	
	Bacteria	*Vibrio* spp: *V. harveyi, V. parahaemolyticus, …*	[89]
Fish	Virus	Koi herpesvirus, infectious haematopoietic necrosis virus, viral haemorrhagic septicaemia virus, epizootic haematopoietic necrosis virus, spring viraemia of carp virus, red sea bream iridoviral, viral nervous necrosis virus	[175]
	Parasites	*Henneguya* spp., *Myxobolus cerebralis*, *Ichthyophthirius multifiliis …*	[168]
	Fungi	*Saprolegnia* spp., *Branchiomyces* spp., *Achyla* spp., *Dermocystidium spp, Basidiobolus spp., Aspergillus* spp.	[168]
	Bacteria	*Aeromonas* spp., *Edwardsiella* spp.,*Flavobacterium* spp., *Francisella* spp., *Photobacterium* spp., *Piscirickettsia* spp., *Pseudomonas* spp., *Tenacibaculum* spp., *Vibrio* spp., *Yersinia* spp., *Lactococcus* spp., *Renibacterium* spp., *Streptococcus* spp.	[168]

A wide variety of pathogens have been associated with aquatic animals diseases [89, 175]. For example, *A. hydrophila* (haemorrhagic septicaemia; fin/tail rot) and *A. salmonicida* (furunculosis; ulcer disease), *Edwardsiella* spp. (edwardsiellosis) in fish, *Vibrio* spp. (vibriosis, early mortality syndrome) and virus (white spot syndrome, yellowhead disease) in shrimps have been reported to cause disease leading to high mortalities. Among this, *Aeromonas* and *Vibrio* genera were the most common diseases which have a relatively high antibiotic resistance [89, 168].

1.2.3.1. Aeromonas hydrophila

A. hydrophila (Aeromonadaceae) is Gram-negative, facultative anaerobic, non-spore forming, rod shaped bacteria and has a size of 0.3-1.0 x 1.0-3.5 μm. This strain which may cause zoonotic diseases, is an emerging aquatic pathogen widely distributed in the environment and has been reported in seafood, shellfish, meat, raw milk, raw vegetable and poultry [48].

The pathologies, in which liver and kidney are commonly the targets, including dermal ulceration, tail and/or fin rot, exophthalmia, erythrodermatitis, hemorrhagic septicaemia, red sore disease, red rot disease and scale protrusion disease [168] (Fig. 1.9). Affected fish with *A. hydrophila* show hemorrhage and ulceration on the body surface, eye abnormalities and accumulation of red- colored ascetic fluid [168].

A high rate of *A. hydrophila* isolated from water, food and clinical specimens was resistant (100%) and multi-resistant (48%) to antibiotic applied in clinical practice such as

chloramphenicol, tetracycline, erythromycin, nalidixic acid, streptomycin… It become difficult to treat diseases caused by *A. hydrophila* [48].

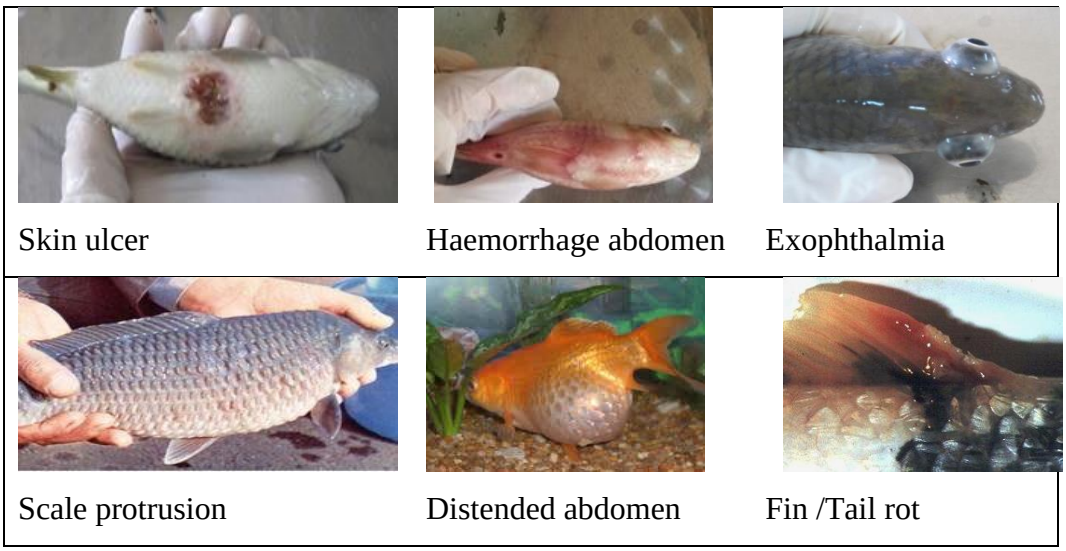
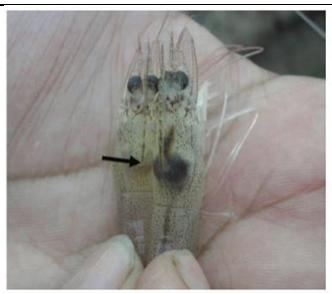


Figure 1.9: Common symptoms of Aeromonas hydrophila infected fish ([168])

1.2.3.2. Vibrio parahaemolyticus

Acute Hepatopancreatic Necrosis Disease AHPND (also call Acute Hepatopancreatic Necrosis Syndrome AHPNS or Early Mortality Syndrome EMS) is a new disease causing unusually heavy mortality (>70%) in cultured shrimps at approximately 30-45 days of culture. It was first reported in 2009 in China and has spread through Southeast Asia to Vietnam (2010), Malaysia (2011), and Thailand (2012), Mexico (2013) and Philippines (2014) [97]. Shrimp production within the AHPND-affected region dropped to 60% compared with 2012, and the disease has caused global losses to the shrimp farming industry estimated at more than 1 billion USD per year [97]. In 2015, the economic loss was estimated to 8,9 million USD in *L. vannamei* and 1,8 million USD in *P. monodon* productions by this outbreak in Mekong Delta, respectively.

In 2013, Tran et al. showed that the causative agent of AHPND was a specific strain of the common Gram-negative halophilic marine bacterium *Vibrio parahaemolyticus*, a common inhabitant of coastal and estuarine environments [163]. Through some unknown mechanism, this strain had become virulent, and, in infected shrimp, it induced AHPND's characteristic symptoms, i.e, a pale and atrophied hepatopancreas (HP) together with an empty stomach and midgut, slow growth, corkscrew swimming, loose shells (Fig 1.10) [97]. *V. harveyi* and *V. owensii* were also reported causing AHPND in shirmps [97].



L. vannamei: pale atrophied hepatopancreas; empty stomach and midgut (arrow)

P. monodon: normal hepatopancreas (arrow left); atrophied hepatopancreas (two arrows right)

L. vannamei: atrophied hepatopancreas

P. monodon: pale and trophied hepatopancreas; empty midgut

Figure 1.10: Common symptoms of infected shrimp with EMS/AHPND ([97])

In addition, thousands of foodborne pathogenic bacteria have been found to affect the health and safety of the world's populations of humans and seafood accounts for up to 10% of all outbreaks. The maining identified causative agents were *Salmonella*, *S. aureus*, *E. coli* O157:H7, *B. cereus*, *Clostridium perfringens* and *V. parahaemolyticus* … which are commonly found in many raw foods [10, 31].

1.2.4. Utilization of antibiotic in aquaculture

1.2.4.1. Situation of antibiotic utilization in aquaculture

According to worldwide extension of aquaculture activity, new occurred diseases and existence of other diseases increased year by year. At present, outbreaks of parasitic, fungal, and bacterial diseases play as major limiting factors for shrimp and fish farming, meaning that producers have to use of massive amounts of disinfectants, pesticides and antibiotics in order to control mortality and prevent huge economic losses. For instance, a wide variety of substances are currently used in aquaculture production, including disinfectants (e.g., hydrogen peroxide and malachite green), antibiotics (e.g., oxytetracyclines (OTC), oxolinic acid, amoxicillin, sulfadiazine, florfenicol, sulfonamides) [137], anthelmintic agents (e.g., avermectins) and vaccination. However, the disinfectants cause many side-effects such as expansion of resistance, being dangerous for animal health and environmental disadvantages. Additionally, commercial vaccines are too expensive for widespread and a single vaccine is effective against only one type of pathogens [51].

Antibiotics have been used mostly for therapeutic purposes in aquaculture. In addition, as bacteria are a major cause of diseases, the misuse of antibiotics in fish and shrimp farming is widespread and now it is becoming more and more regular.

Antibiotics, also known as antimicrobial agents, can be defined as compounds that have the capacity to kill or inhibit the growth of microorganisms (bacteria). They should be safe (non-toxic) to the host, permitting their use as chemotherapeutic agents for the treatment of bacterial infectious diseases. In aquaculture, antibiotics are used for prophylaxy, therapy and growth promotion. Regarding therapeutic levels, they are regularly administered for short periods of time via the oral route to groups of fish that share tanks or cages. In aquaculture, all drugs legally used must be approved by the government agency responsible for veterinary medicine, for example, Ministry of Agriculture and Rural Development in Vietnam. These supervisory agencies may set guidelines for antibiotic use, including allowable routes of delivery, dose forms, withdrawal times, tolerances, and use by species, including dose rates and limitations. For example, in Vietnam, the prohibited antibiotics in aquaculture are chloramphenicol, chloroform, chlorpromazine, dimetridazole, dapsone, dimetridazole, metronidazole, nitrofuran, ronidazole, ipronidazole, nitroimidazole, trichlorfon, trifluralin, cypermethrin, cypermethrin, enrofloxacin, ciprofloxacin, fluoroquinolones [1].

Intensive fish and shrimp farming has promoted some bacterial diseases, which has led to a rise in the use of antimicrobials [51]. Several authors reported that the amount of antibiotics and other compounds used in aquaculture varied significantly between countries. For instance, Defoirdt et al. (2011) estimated that approximately 500–600 metric tons of antibiotics were used in shrimp farm production in Thailand; the author also emphasized the large variation between countries, with antibiotic use ranging from 1 g/t of production in Norway to 700 g/t in Vietnam [51]. There are a large number of freshwater farms that use antibiotics in raising fish or shrimp in Vietnam [125]. A total of 10 different classes of antibiotics were used by the farms in which the four most commonly used were sulfamethoxazole (41.5%), OTC (30.9%), trimethoprim (30.8%) and sulfadiazine (17.0%). The farms used antibiotics at high density of fish or shrimp of water surface: 10.6 kg/t/m^2 versus 7.8 kg/t/m^2. Furthermore, 72.3% (68/94;) of farms surveyed used at least one antibiotic at any time in the production cycle and a considerable number of farms (23.4%; 22/94) used antibiotics up to harvest time [125].

Nevertheless, aquatic animals don’t efficiently metabolize antibiotics and will

excrete them mostly unused back into the water in feces. It has been projected that 75% of the antibiotics administered to fish are excreted into the environment. For example, OTC, one of the most frequently used antibiotics in fish farms, is poorly absorbed through the intestinal tract of fish. It has to be administered at a high dosage rate of 100-150 mg/ kg fish/ day for 10-15 days. This treatment subsequently causes the slow excretion of large volumes of this antibiotic, thus increasing the selective pressure which might lead to the selection of OTC-resistant bacteria in the gut [112].

1.2.4.2. Consequences of antibiotic overuse in aquaculture

Consequently, massive use of antibiotics in combination with high population densities, low water quality has resulted in the development of resistant bacteria strains, or the presence of residual antibiotics in the muscle of commercialized fish and thus has potential effects on human health and rise the reservoirs of antimicrobial-resistant bacteria in the environment [51]. Resistant bacteria to antibiotics can transfer the resistance determinants to other bacteria (even to bacteria of different genera) that have never been exposed to the antibiotic (known as horizontal gene transfer). At the same time, one microorganism acquiring resistance against an antibiotic seems to help it in becoming resistant against others; this capacity is known as co-selection [179].

A large number of *Vibrio* spp. (n=50) isolates from diseased and healthy prawn larval *Marcobrachium rosenbergii* were resistant to penicillin (98%), vancomycin (90%) and polymyxin B (64%). All of *Edwardsiella ictaluri* (n=13) isolates from diseased catfish *Pangasius hypopothalmus* were resistant to OTC, oxolinic acid and sulphonamid. Morover, among 123 cloramphenicol resistant isolates from water, sediment and apparently healthy fishes, 90% were resistant to tetracycline, 89% were resistant to trimethoprim/sulfadiazine, 76% resistant to ampicillin, 65% were resistant to nitrofuratoin and 33% were resistant to norfloxacin [126].

In addition, Sarter et al. (2007) tested the susceptibility to antibiotic of *Enterobacteriaceae, Pseudomonads* and *Vibrionaceae* isolated from catfish farms in Mekong Delta River in Vietnam [143]. The results indicated that 78.1% bacteria isolates (n=92) were resistant to at least 2 antibiotics showing 17 multiple antibiotic resistance profiles. The major profiles were ampicillin – OTC – trimethoprim – sulphamethoxazole – nalidixic acid; OTC – sulphamethoxazole – nalidixic acid; and ampicillin – chloramphenicol – nitrofurantoin – sulphamethoxazole – nalidixic acid.

In consequence, there was an increase in the number of shipments being rejected by importing countries due to antibiotic residues and other contaminants being detected during routine testing [5]. Vietnam Association of Seafood Exporters and Processors (VASEP) announced that Vietnamese seafood exporters have had shipments totaling 32,000 tons of various fisheries products rejected by importing countries during the past two years after these were found to contain banned antibiotic residues [5].

1.3. Potential of plant-based products in aquaculture

Seeing the risky of chemical drug usages on the environment and human health, using natural products (plant extracts or whole plants) in the culture of fish and shrimp [43, 76, 136] is one of the proposed alternatives to reduce antibiotic use. The plant-based products promise a sustainable and effective substitute for chemical treatments and an increasing number of studies highlighting their potential application in aquaculture have been published [76, 136]. Besides, their use could reduce costs of treatment and be more environmentally friendly and they are less likely to produce drug resistance in bacteria due to the high diversity of plant extract molecules [136]. A high potential of exploiting the herbal products in Vietnam has been reported. With 30 national parks and 60 natural conservation areas (2, 326, 388 ha), Vietnam possesses plant resources at a high level of diversity and abundance with approximately 4, 000 plant species are used. However, about 300 species have been exploited for using with total 20, 000 tons every year [81].

In aquaculture, plants coud be used as powder [60, 93, 148], EO [132], raw materials or solvent extract (methanol, ethanol, n-hexane) [84, 104] products (Table 1.7 and Table 1.8). They are commonly administrated through oral (enriching diet), injection or bath immersion.

Plant extracts have been reported to favour various activities like anti-stress, growth promotion, appetite stimulation, enhancement immunostimulation and anti pathogen properties in fish and shrimp aquaculture due to active components such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids or EOs [43].

1.3.1. Plants as a growth promoter

In aquaculture, numerous of additives are added to the diets to enhance the nutrient utilization, growth performance and survival of cultured fish such as probiotics, yeast, amino acids, vitamins, hormones, aromatic compounds, certain organic acids/salts and

plant extracts [69]. The effects of dietary plants extract supplementation on growth are well evaluated with several spices of fish and shrimp (Table 1.7).

Table 1.7: Effect of plant extract on growth promotion of fish

Plant (*latin name*)	**Spices**	**Type of plant-product**	**Type of adminis-tration**	**Length of treatment (days)**	**Ref**
Garlic (*A. sativum*)	Tilapia (*Tilapia zilii*)	Powder	Oral	75	[148]
Bermuda grass (*Cynodon dactylon)*, beal (*Aegle marmelos)*, winter cherry (*Withania somnifera)*, ginger (*Zingiber officinale)*	Tilapia (*Oreochromis mossambicus*)	Acetone extract	Oral	45	[84]
Moringa *(Moringa oleifera)*	Tilapia (*O. mossambicus*)	Powder (leaf)	Oral	45	[60]
Fenugreek (*Trigonella foenum graecum*)	Common carp (*C. carpio*)	Seed meal powder	Oral	56	[138]
Aloe vera *(Aloe vera)*	Common carp (*C. carpio*)	Ethanol extract	Oral	56	[104]
Garlic (*A. sativum*)	White leg shrimp (*L. vannamei)*	Garlic powder	Oral	60	[93]

Several plant extracts were reported to promote weight gain when they were administered to cultured fish and shrimp. Shalaby et al. (2006) showed that food intake, Specific Growth Rate (SGR) and Final Weight (FW) of Nile tilapia (*O. niloticus*) increased when garlic was incorporated in the diet [148]. Similar effects have been observed in tilapia *Oreochromis mossambicus* fed diets supplemented with acetone extract of *A. marmelos, C. dactylon, W. somnifera* or *Z. officinale* [84]. Moreover, diets enriched with garlic *A. sativum* powder 6%, have been reported to significantly decrease the feed conversion ratio (FCR) in whiteleg shrimp *L. vannamei* [148].

In another studies, Mahdavi et al. (2013) showed that dietary *A. vera* ethanol extract at 0.5 and 2.5% supplementation was efficient in growth performance (SGR, FCR and FCE) of common carp *C. carpio* [104]. According to Bahrami et al. (2014), ethanol extract of wood betony *Stachys lavandulifolia* at 2, 4 and 8% showed significantly increased the final weight, SGR and decreased FCR in a dose dependent manner in common carp [19]. The best growth parameters were archived in the groups of fish receiving the highest concentration (8%) of wood betony. Roohi et al. (2017) showed that this species fed with seed powder of Fenugreek (*Trigonella foenum graecum*) had higher

Weight Gain (WG) and SGR and higher FCR than the control fish, indicating that feeding with the herbal mixture improves the growth promoter [138].

However, the mechanisms contributing to the growth promoting effects of plant extract are yet to be fully clarified. Some authors suggest that a dietary supplement with plants could improve lipid metabolism (lower plasma triglyceride and high plasma HDL-CHO (high-density lipoprotein cholesterol) levels and modulate the activities of trypsin-like enzymes during digestive processes, resulting in an efficient protein deposition, improvement digestibility of nutrients and growth performance [136].

1.3.2. Plants as a immunostimulants of fish

The immune system is classified into innate and adaptive immunity system. As shown in Fig 1.11, fish defense mechanisms for protection against infections depend on physiological mechanisms of immunity [67]. Non-specific system is the first line of defense and their major component are macrophages, monocytes, granulocytes and humoral elements, including lysozymes or complement systems [136].

An immunostimulant is a chemical, drug, stressor or action that improves fish resistance to infectious diseases, not only stimulating the acquired immune response, but also enhancing innate, humoral and cellular defense mechanisms [67, 136]. Several substances have demonstrated effectiveness in increasing the immune response of fish including synthetic chemical (levamisole), bacterial derivatives (β-glucan), nutritional factors (vitamin A, D, E, C), hormones (interferon), animal derivatives (chitosan) and plant derivatives. Immunostimulants are considered safe and more environmental friendly than chemotherapeutics in addition to their wider efficacy [141].

The use of plant extracts as fish immunostimulants has been investigated in the last decade [76, 136]. Several studies showed that some immunological parameters such as lysozyme, complement, phagocytic, respiratory burst and plasma protein (globulin and albumin) activity have increased after administering plant products to some fish species [11, 108, 154] (Table 1.8).

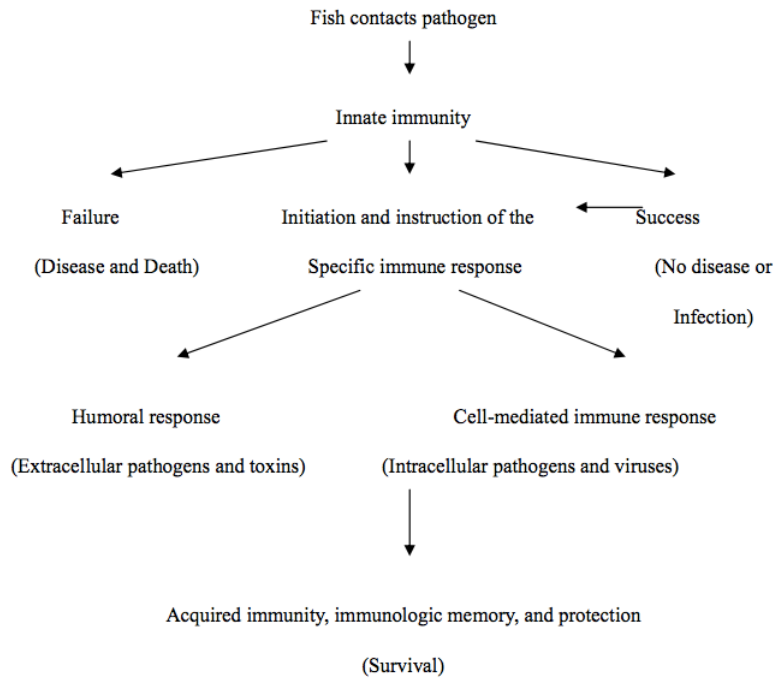


Figure 1.11: Schematic representation of the immune response of fish following contact with a pathogen ([67])

For example, Soltanian et al. (2016) showed that common carp *C. carpio* intraperitoneally (i.p.) injected with methanol extract of *Lawsonia inermis* (henna) displayed an increase of activity for which non-specific immune parameters were monitored (lysozyme, bactericidal, phagocytic and respiratory burst, total leucocyte count (TLC), lymphocyte, monocyte and neutrophil number). After that period, fish were challenged with *A. hydrophila* and treated fish had 28% more survivability than the control group [154]. Non-specific immune response in carp *C. carpio* was enhanced (lysozyme, alternative haemolytic complement (ACH50, bactericidal) with dietary supplementation of extracts of *Oliviera decumbens* and *Satureja khuzestanica* for 35 days [11]. Another study showed that carp *C. carpio* infected with *A. hydrophila* presented more viability (23-43%) when fed with mixture ethanol extract of *Inula helenium, Tussilago farfaea, Brassica nigra, Echinaceae purpurea* and *Chelidoniume majus* enriched diet compared to control group (viability of 31%). Serum bactericidal, lysozyme, serum protein, albumin, globulin, WBC, RBC, haemogloblin and respiratory burst activity increased, indicating that enhancement of immunological system leads to a better protection of common carp against *A. hydrophila* [108].

Table 1.8: Effect of plant extract on immunostimulant and antibacterial activities of fish ([11, 108, 154])

Plant (*latin name*)	**Spices**	**Type of plant - product**	**Type of adminis-tration**	**Length of treatment (days)**	**Bacterial**
Indian bael *(Aegle marmelos)*	*C. carpio*	Powder (leaf)	Oral	50	*A. hydrophila*
Garlic *(Allium sativum)*	*Labeo rohita*	Powder	Oral	60	*A. hydrophila*
Kalmegh *(Andrographis paniculata)*	*Oreochromis niloticus*	Powder	Oral	14	*S. agalactiae*
Cinnamon *(Cinnamomum verum)*	*O. niloticus*	EO	Oral	14	*S. iniae*
Coriander *(Coriandrum sativum)*	*Catla catla*	Powder leaf	Oral	14	*A. hydrophilla*
Curcumin *(Curcuma longa)*	*L. rohita*	Powder root	Oral	60	*A. hydrophilla*
Bermuda Brass *(Cynodon dactylon)*	*C. catla*	Ethanol	Oral	60	*A. hydrophilla*
Holy Basil *(Ocimum sanctum)*	*C. carpio*	Methanol	I.p		*A. hydrophilla*
	O. mossambicus	Water	I.p, Oral	1, 2, 4	*A. hydrophilla*
Rosemary *(Rosmarinus officinalis)*	*Oreochrmonis sp.*	Powder, Ethyacetate	Oral	15	*S. iniae*
Tropical Almond *(Terminalia catappa)*	*Beta splendens Regan*	Water	Bath		*A. hydrophilla*
Ginger *(Zingiber offininale)*	*L. calcarifer*	Powder	Oral	15	*V. harveyi*
	O. mykiss	Water, Powder	Oral	1, 4	*A. hydrophilla*

Lysozyme is a bactericidal enzyme that hydrolyses the β-1,4 glycosidic linkage between N-acetyl glucosamine and N-acetyl muramic acid of bacterial cell wall peptidoglycan, thereby causing bacteriolysis and preventing the growth of bacteria [138]. The medicinal herbs *Rheum officinale* [180], *C. zeylanicum, J. regia, M. piperita, O. basilicum* [6] and *A. marmelos* [129] enhanced lysozyme activity in common carps.

The alternative complement pathway is known to be one of the powerful non-specific defense mechanisms which protect fish from a wide range of bacteria [62]. The serum natural haemolytic complement activity was improved by oral administration of *A. sinensis* and *A. membranaceus* mixture in yellow croaker [87] and Jian carp [86], *Oliviera decumbens* and *Satureja khuzestanica* extracts in common carp *C. carpio* [11].

Moreover, the presence of antimicrobial agents in fish blood can be evaluated by serum bactericidal activity and this is an important tool to analyze the innate immune system [108]. The serum bactericidal activities of the Indian major carp [171] and the common carp [11] have increased after the administration of *Solanum trilobatum, Achyranthes aspera* and *A. vera* extracts, respectively [108].

Therefore, it has been widely established that certain herbs can improve the resistance of fish to bacterial diseases, as an overall consequence of their immunostimulatory effects. Diet supplementation with *R. officinale* [180], *A. membranaceus* [186], *A. marmelos* extract [129], *B. nigra, C. majus, E. purpurea, I. helenium, T. farfara* extracts [108], *C. zeylanicum, J. regia, M. piperita* and *O. basilicum* extracts [6] or *A. vera* extract [11, 12] improved the resistance of *C. carpio* to *A. hydrophila*. In addition, oral administration of *A. marmelos, C. dactylon, W. somnifera* or *Z. officinale* acetone extract significantly increased the resistance of tilapia *O. mossambicus* challenged with *Vibrio vulnificus* by enhancing lysozyme, phagocyte, leucocrit value and albumin, globulin, cholesterol, glucose and triglyceride levels [84].

1.3.3. Plants as a antibacterial agents

The ability of medicinal plants to inhibit activity of pathogenic bacteria in aquaculture such as *Vibrio* spp. and *Aeromonas* spp. has been well reported. However, it was referred to limited number of plant species.

The methanolic herbal extracts of *S. trilobatum, A. paniculata* and *P. corylifolia* enriched Artemia reduced the *Vibrio* concentration in *P. monodon* post-larvae tissues (hepatopancreas and muscle) [43]. Guava (*P. guajava*) eliminated luminous bacteria from black tiger shrimp (*P. monodon*) more effectively than with OTC [56]. Yeh et al. (2009) reported that whiteleg shrimp (*L. vannamei*) treated with hot-water extracts from twigs of stout camphor tree *Cinnamomum kanehirae* (Lauraceae family) showed a significant decrease in their sensitivity to *V. alginolyticus* [184]. An increasing of survival of the shrimp larvae (*P. monodon*) concomitant with a decrease in bacterial concentration were observed when *Cinnamosma fragans* EO was added directly to the water tank [132]. Similarly, two EOs of *C. fragrans* (B8: linalool-type and B143: 1,8-cineole-type) reduced the total heterotrophic aerobic bacteria and the *Vibrio* concentrations in the rearing water of *P. monodon* shrimp larvae [144]. Recently, infected shrimps *L. vannamei* were fed with feed mixed with oregano EOs (containing mainly carvacrol or thymol). The positive effect

of dietary oregano EOs on the growth of *Vibrio* bacteria in shrimps was observed. The microbial counts of *Vibrio* were significantly lower in tissues from shrimps whose food was supplemented with oregano EO [70].

In Vietnam, several medicinal plant have been used in aquaculture including *Allium sativum* L., *Phyllanthus urinaria* L., *Houttuynia cordata* Thumb, *Melia azedarach* L., *Portuiacaoler acea* L., *Eclipta alba* Hassk [35]. They have been administered as whole plant or parts (raw material) based on the farmer experiences. Some of mixture of medicinal plant products have been commercialized in Vietnam such as KN-04-12 (mixture powder of garlic, wedelia Chinese, false daisy), VTS1-C, VTS1-T to treat the infection of fish and shrimp [35]. However, the chemical composition as well as the biological effects of that products were not reported.

1.4. Aims of the thesis

Based on the literature and the limited knowledge on the EOs in Vietnam, our research focused on finding new potential plants for their antibacterial mechanism and to application in aquaculture. The general approaches in this thesis were divided into four areas:

a) Biological investigation
b) Chemical composition diversity
c) Antibacterial mechanism
d) Application in aquaculture

In the biological studies, experiments were conducted to: i) evaluate the antibacterial activity of EOs through determination of inhibition zone and minimum inhibitory concentration (MIC) values, ii) investigate the synergistic effects of combination of EOs between them or with antibiotic via Fractional Inhibitory Concentration (FIC) values.

The chemical composition diversity included: i) collection and extraction of plant materials, ii) determination of the chemical composition of EOs.

The antibacterial mechanism of EOs were carried out to explore: i) effects of EO on the viability of *E. coli* cells, ii) effects of EO on the integrity and permeabilization of cell membrane, iii) effect of EO on the morphology of *E. coli* cell (size), iv) effects of EO on DNA of *E. coli* cell.

The experiment in aquaculture included: In shrimp i) effect of EO on the bacterial concentration as well as survival rate of shrimp infected with *V. parahaemolyticus*. In fish ii) feeding experiment for carp with plant powder, iii) evaluation effect of plant powder on the growth promotion and the immunostimulation of carp e.g. serum lysozyme bactericidal and alternative complement activities, iv) effect of plant powder on the survival rate of carp challenged with *A. hydrophila*.

The aims of the study were as follows:

- To evaluate the antibacterial activities of nine EOs in Vietnam against 10 pathogenic bacteria via determination of the major component, inhibition zone and MIC values.
- To investigate the chemical compositions diversity and antibacterial activities of selected EO (*L. cubeba*) from some provinces North Vietnam.
- To determine the antibacterial mechanism of selected EO (*L. cubeba*) against *E. coli* as a model organism in term of cell viability, membrane integrity, membrane permeabilization, cell size and DNA
- To examine the effect of plants extract on the growth parameter; non-specific immunity, and protection against pathogenic bacteria such as *A. hydrophila* and *V. parahaemolyticus* in common carp *C. carpio* and whiteleg shrimp *L. vannamei,* respectively.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Essential oils and other antibacterial agents

2.1.1.1. Commercial EOs

For the screening test, nine EOs used in this study were purchased from Aromasia company in the North Vietnam including clove basil (*Ocimum gratissimum)*, cajeput (*Melaleuca leucadendron*), cinnamon (*Cinnamomum cassia*), Indian prickly ash (*Zanthoxylum rhetsa)*, sweet wormwood (*Artemisia annua)*, basil (*Ocimum basilicum*), Mexican tea (*Chenopodium ambrosioides*), corn mint (*Mentha arvensis*) (Fig 2.1) and fruit of May Chang or Chinese pepper (*Litsea cubeba*) (Fig 2.2). These EOs samples were selected based on literature survey and their use in traditional medicine. The EOs were obtained by hydrodistillation methods and the quality of the EOs was ascertained to be more than 98% pure.

Cinnamon *Cinnamomum cassia*

Corn mint *Mentha arvensis*

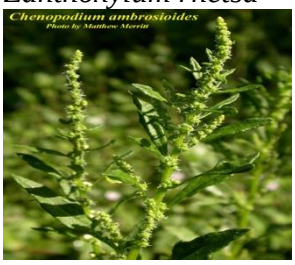
Cajeput *Melaleuca leucadendron*

Indian prickly ash *Zanthoxylum rhetsa*

Basil *Ocimum basilicum*

Clove basil *Ocimum gratissimum*

Sweet wormwood *Artemisia annua*



Mexican tea *Chenopodium ambrosioides*

Figure 2.1: Plants used in the current study

After screening the antibacterial activities of the nine EOs from Vietnam, *L. cubeba* fruit commercial EO was selected to further investigate the interaction effect with other antibacterial agents (EO and antibiotic) and shrimp experiments.

2.1.1.2. Extracted L. cubeba leaf EOs

Leaves of *L. cubeba* were harvested during the pre-flower phase, in seven provinces of North Vietnam for all assays regarding the chemotype characterization, the antibacterial activity and the mode of action. For each tree, one sample of leaves was manually collected and then identified at the Department of Plant, Faculty of Agronomy, Vietnam National University of Agriculture (VNUA), where a voucher specimen was deposited. The samples (n=25) and their location are listed in Table 3.4 (Annex Fig. 1).

25 samples of *L. cubeba* leaf EOs were extracted via hydrodistillation using a Clevenger-type apparatus [133]. These EOs were used for assays including chemotype characterization, antibacterial activity, antibacterial mechanism and fish experiments.

Figure 2.2: Plant of Litsea cubeba from Bavi

2.1.1.3. Antibiotics

The disc antibiotics including amikacin 30µg/disc (AK 30) and cefoxitin 30µg/disc (FOX 30) were purchased from Liofilchem. OTC and nalidixic acid were purchased from Sigma Aldrich.

2.1.2. Bacterial strains

The effects of EOs samples were tested against a panel of bacteria, including:

- Five Gram-negative bacteria: *E. coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* Typhimurium ATCC 14028, *Vibrio alginolyticus* ATCC 17749, *V. parahaemolyticus* ATCC 17802, *Aeromonas hydrophila* ATCC 35654.
- Five Gram-positive bacteria: *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC

29212, *Bacillus cereus* ATCC 13061, *Bacillus subtilis* ATCC 11774, *Listeria innocua* ATCC 33090.

- Bacterial isolates:
    - *V. parahaemolyticus* ND201 and *V. parahaemolyticus* TB81 isolated from *L. vannamei* (shrimp having Acute Hepatopancreatic Necrosis Syndrome) from a hatchery of local farming in Nam Dinh and Thai Binh (National Veterinary Diagnostic Center – Hanoi)
    - four strains *Vibrio* sp. 2S4, *Vibrio* sp. 2N38, and *E. coli* 9C48, *E. coli* 11C123 isolated from cockle and catfish and *A. hydrophila* isolated from diseased catfish *Pangasianodon hypophtalmus* kindly provided by the Pathology Laboratory of the Department of Aquatic Animal Pathology, Faculty of Fisheries, Nong Lam University, respectively
    - two isolated strains from diseased fish given by the collection of the National Veterinary Laboratory (Montpellier, France): *Edwarsiella tarda* AR 436 and *Streptococcus garvieae* AR 759

The bacterial cultures were maintained in thiosulfate citrate bile sucrose (TCBS) Agar (Merck) for vibrios and in Nutrient Agar (NA) for the others strains at 4ºC throughout this work and used as stocked cultures.

2.2. Methods

The antibacterial activities of the nine EOs were carried out by the disc diffusion and micro-dilution methods in order to determine the diameter of inhibition, MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration). After determining the main chemical composition and antimicrobial activity of nine EOs, *Litsea cubeba* was chosen for further research due of high antibacterial activities.

2.2.1. Extraction and yield of *L. cubeba* leaf EOs

Fresh leaves of *L. cubeba* (200 g/sample, n=25) were washed, cut into small pieces and subjected to hydrodistillation using a Clevenger-type apparatus to extract EO during 4 h, after which no more EO was obtained. After extraction, anhydrous sodium sulfate was added to remove residual water from the oil. The EOs were stored at 4°C in dark glass bottles until use (Annex Fig. 2) [133].

The yield of EOs obtained was calculated using the equation [44]:

$Y=V/B_m$ (2.1)

Where

V: the volume of the extracted EO (mL)

B_m: the initial fresh leaves (g)

2.2.2. Chemical analysis of EOs

GC/MS analyses were performed for nine commercial EOs and 25 extracted *L. cubeba* leaf EOs from Vietnam using a model 6890/5973N/Gerstel Multipurpose Sample MPS-2 (Agilent Technologies, Palo Alto, USA).

The column was a DB WAX (J&W, Scientific Folsom, CA, USA, 30m × 0.25 mm) [183]. Elution was performed following the temperature program: 3°C/min from 40°C to 170°C, then 10°C/min up to 240°C and held for 10 min. Helium was the carrier gas at a flow rate of 1 mL/min and 1 µL (100-fold dilution in n-hexane) was injected at 250°C with X20 split ratio. Mass units were monitored from 40 to 450 Dalton in EI mode at 70 eV. The semi quantification of compounds was estimated by integrating the peak area of 2-octanol as internal standard (Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 65.6 g/L. The identification of peaks was performed on MSDchem version E by comparing their mass spectra with those from the NIST 2011 (National Institute of Standard Technology) database and based on Kovats Index, calculated after injection of a series of alkanes C_7-C_{25} (Sigma-Aldrich, St. Louis, MO, USA) as reference standard (Annex Table 1), and comparison on both Pherobase (www.pherobase.com) and Flavornet (www.flavornet.org) databases.

Calculation of Kovats indices

Kovats retention indices can be obtained by calculating the temperature program linear retention indices of a chemical compound from the gas chromatogram and by logarithmic interpolation between bracketing alkanes. The Kovats index is given by the equation (using definition of Van den Dool and Kratz) [169]:

$$I = 100 \times (n + \frac{t_{r,un} - t_{r,n}}{t_{r,N} - t_{r,n}}) \quad (2.2)$$

Where:

I: Kovats retention index

n: the number of carbon atoms in the smaller n-alkane

N: the number of carbon atoms in the larger n-alkane

t_r: the retention time

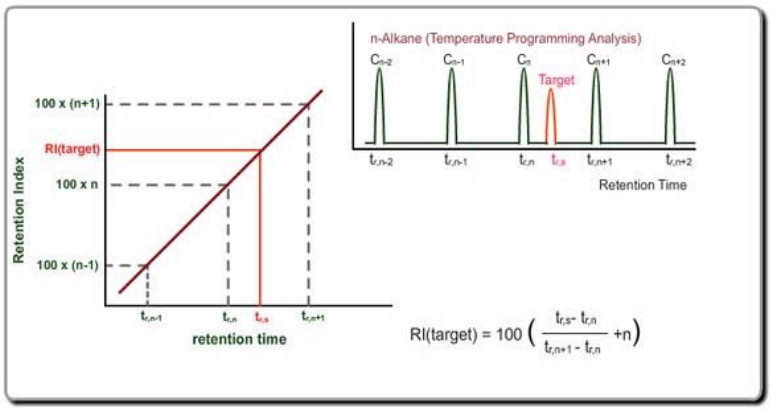


Figure 2.3: Graphical calculation of the Kovats Retention Index

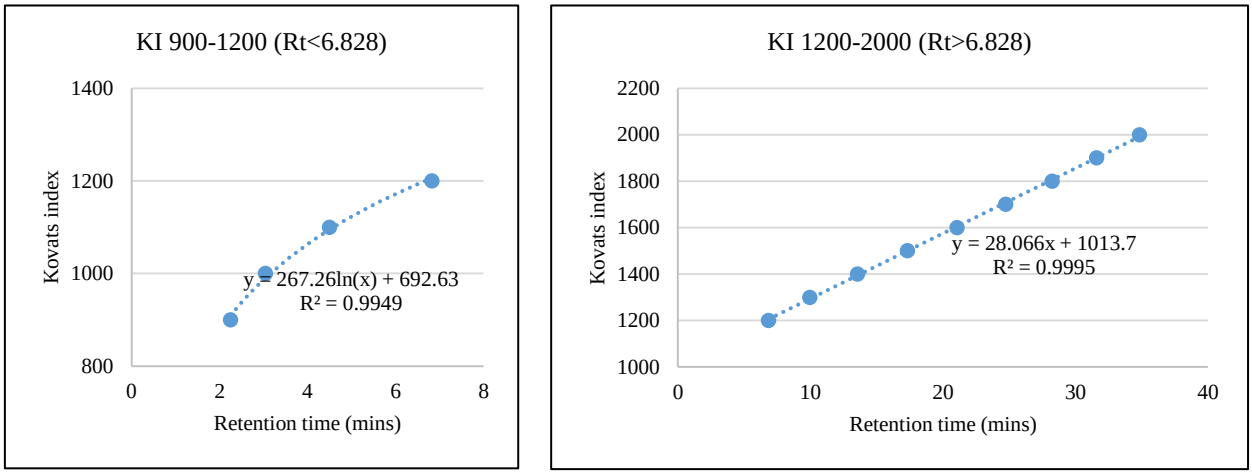


Figure 2.4: Retention index found in our study on the DB WAX column

2.2.3. Disc diffusion method

The screening of the nine commercial EOs from Vietnam was done by the disc diffusion method, which was used as a preliminary check to select efficient EOs.

From cultures of bacteria that were grown for 24 h, 5 colonies were solubilized in sterile physiological saline solution (0.9% NaCl). The optical density (OD) of the inoculum was measured at 600 nm (OD_{600} = 0.1) and then diluted to reach a final OD_{600} = 0.001 in the assay. 2 mL of test microorganisms containing 10^6 CFU/mL were inoculated on the surface of Mueller Hilton Agar (MHA, Biokar, France) plates and MHA supplemented with 2% NaCl for vibrios. After 5 min, the excess of inoculum was eliminated. Six sterile

discs with a diameter of 6 mm (BioMérieux, France) were placed onto each inoculated agar plate. Then 10 µL of pure EO were dropped onto each disc under sterile conditions. The plates were incubated 24 h at 30°C for vibrios and *A. hydrophila* and at 37°C for other strains. All experiments were performed in triplicate.

The antimicrobial activities of all solutions were detected as clear zones around the assay discs. This region is called the "zone of inhibition". Thus, the size of the zone of inhibition (including diameter of paper disc 6 mm) is a measure of the compound's effectiveness (Annex Fig. 3). The larger the clear zone around the assay disc, the more effective the solution is. The antibiotics, amikacin 30µg/disc (AK 30) and cefoxitin 30µg/disc (FOX 30) (Liofilchem) were used as positive control [178].

2.2.4. Microdilution method

The MIC and MBC of the nine commercial EOs against *S.* Typhimurium, *E. coli, A. hydrophila, V. parahaemolyticus, B. cereus* and *S. aureus* were determined using microbroth dilution assay in 96-well microplates.

In addition, at least one EO from each region were selected to investigate the inhibitory effect of leaf EOs. Indeed, eight *L. cubeba* leaf EOs from six regions including four 1,8-cineole type samples (HG01, YB12, LC17, LC19) and four linalool type samples (TD18, TD21, PT13, BV27) among 25 extracted *L. cubeba* leaf EOs were selected. The antibacterial activities of eight *L. cubeba* leaf EOs samples were determined against selected pathogenic strains and isolated strains including *B. subtilis, A. hydrophila, E. coli, S.* Typhimurium, *V. parahaemolyticus, V. furnissii*, *E. tarda* AR436 and *S. garvieae* AR759.

EOs was dissolved in distillated water containing 0.5% Tween 80 to achieve a final concentration ranging from 0.195 µL/mL to 50 µL/mL. Bacterial cultures were prepared in Mueller Hilton broth (MHB, Merck) or MHB plus NaCl 2% for vibrios. The concentration of an overnight culture was determined using previously established standard curves showing the OD at 600 nm versus the bacterial concentration (CFU/mL). The suspensions were then diluted in MHB to reach a final concentration of 10^7 CFU/mL. Each well contained 20 µL of test sample, 20 µL of bacterial suspension and 160 µL MHB. Thus, the final bacterial concentration for the assay was then 10^6 CFU/mL. After incubation for 24 h at 30°C for *Vibrio* spp. and *Aeromonas* spp. and at 37°C for *E. coli*, the OD was measured at 600 nm using Elisa reader. The MIC was determined as the lowest concentration

showing no growth [38].

The MBC was determined by spreading 100 µL of the cultures on Mueller Hinton Agar (MHA, Merck) plates and then incubated 24 h at 30ºC for *Vibrio* spp. and *Aeromonas* spp. and at 37ºC for other strains. The MBC was identified as the lowest concentration showing no bacterial growth on agar plates. The assays were carried out in triplicate (Annex Fig. 4). A positive control containing the bacterial culture without the EO extract and a negative control containing the MHB, Tween and EOs were performed in the same conditions [38].

2.2.5. Synergy studies of *L. cubeba* fruit EO and other antibacterial agents by checkerboard method

For further synergy study, MIC of *L. cubeba* fruit EO and antibiotics (nalidixic acid, OTC) against *V. parahaemolyticus*, *E. coli* and 6 strains isolated *V. parahaemolyticus* ND201 and *V. parahaemolyticus* TB81, *Vibrio* sp. 2S4, *Vibrio* sp. 2N38, *E. coli* 9C48, *E. coli* 11C123 were carried out as described above (section 2.2.4).

The checkerboard method was performed using 96-well microplates to obtain the fractional inhibitory concentration (FIC) index of fruit *L. cubeba* fruit EO (A) and antibiotics or other EOs (B). Plates consisted of columns containing 10 µL of *L. cubeba* diluted twofold in distillated water along the x axis as well as rows with the same amount of antibiotics/EOs diluted twofold in the same media along the y axis. Subsequently, 20 µL of the suspension bacterial overnight culture containing 10^7 CFU/mL of the indicator strain were added to all wells. 160µL of media MHB were filled to obtain total of 200 µL (Annex Table 2). Plates were then incubated at 37ºC for *E. coli* and 30ºC for vibrios for 24 h.

The FIC indices were calculated as $\sum FIC = FIC_A + FIC_B$ (2.3)

where:

$$FIC_A = \frac{MIC_A combination}{MIC_A alone} \quad (2.4)$$

$$FIC_B = \frac{MIC_B combination}{MIC_B alone} \quad (2.5)$$

The results were interpreted as synergy (FIC≤0.5), addition (0.5<FIC≤1), indifference (1<FIC≤4) or antagonism (FIC>4) (Table 2.1). Experiments were performed

in triplicate.

Various bacterial species were investigated to evaluate the combination effects of *L. cubeba* fruit EO and an antibiotic. Combinations of *L. cubeba* and OTC, nalidixic acid were tested against *V. parahaemolyticus* ATCC 17802, *E. coli* ATCC 25922 and six bacteria isolated *V. parahaemolyticus* ND201, *V. parahaemolyticus* TB81, *Vibrio* sp. 2S4, *Vibrio* sp. 2N38, *E. coli* 9C48 and *E. coli* 11C123. The results obtained will be used for treating antibiotic resistant bacteria in aquaculture.

Among nine EOs tested in our study, *C. cassia*, *C. ambrosioides* and *M. leucadendron* showed higher antibacterial activity than the others. Therefore, the combination of *L. cubeba* and three EOs were tested against *V. parahaemolyticus* ATCC 17802, *E. coli* ATCC 25922 to evaluate the potential of application in food and aquaculture system.

Concentrations used for the combinations were based on MIC values obtained in antibacterial activity assays section [73, 170].

Table 2.1: *Interaction between two antibacterial agents ([73, 170])*

FIC	*L. cubeba* EO (A)						Interaction
Others (B)	2 MIC	1 MIC	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC	
2 MIC	4.00	3.00	2.50	2.25	2.13	2.06	**Indifference 1< ΣFIC ≤4**
1 MIC	3.00	2.00	1.50	1.25	1.13	1.06	
1/2 MIC	2.50	1.50	1.00	0.75	0.63	0.56	**Addition ΣFIC > 0.5–1.0**
1/4 MIC	2.25	1.25	0.75	0.50	0.38	0.31	**Synergy ΣFIC ≤ 0.5**
1/8 MIC	2.13	1.13	0.63	0.38	0.25	0.19	
1/16 MIC	2.06	1.0625	0.56	0.31	0.19	0.13	

Since *L. cubeba* fruit EO (containing approximately 70% citral) were demonstrated for antibacterial mechanism against *E. coli* [100], however, to the best of our knowledge, the mode of action of *L. cubeba* leaf EOs have never been reported previously. Therefore, *L. cubeba* leaf EOs was chosen to investigate the antibacterial mechanism against selected strain.

E. coli was selected as the model strain to further investigate the mechanism of action of two *L. cubeba* leaf EOs LC19 (1,8-cineole-rich EO) and BV27 (linalool-rich EO)

samples. Because it is a Gram-negative and rod-shaped bacterium and is considered the most important model organism. A model organism is a species that is extensively studied to understand a specific phenomenon, expecting that the knowledge gained can be applied to other species as well. Therefore, the data on cell size, cell cycle, DNA and membrane phenotypes … of *E. coli* was most finely understood.

2.2.6. Effect of *L. cubeba* leaf EOs (LC19 and BV27) on cell viability of *E. coli*

The plate colony-counting method was used to analyze the time-kill of the selected *L. cubeba* leaf EOs (LC19 and BV27). The EOs were prepared as described above (Section 2.2.4). Subsequently, the tubes with different EO concentrations (0.5 MIC, 1 MIC, 2 MIC) were inoculated with the freshly prepared culture of *E. coli* in order to maintain an initial bacterial concentration at 10^6 CFU/mL. The cultures were incubated 24 h and stirred under 120 rpm at 37°C. Total viable bacteria were enumerated by spreading 100 µL of culture on MHA plates after 0; 0.5; 1; 2; 4; 6; 8 and 24 h of incubation and the concentration was expressed as log CFU/mL. The control corresponded to the bacterial culture grown in sterile MHB without *L. cubeba* EO. Assays were carried out in triplicate [100]. The reduction of viability was calculated and expressed as follows:

Reduction of viability (%) = [$\log_{10}$ CFU ml^{-1} of treatment/$\log_{10}$ CFU ml^{-1} of control]*100

2.2.7. Effects of *L. cubeba* leaf EOs (LC19 and BV27) on membrane integrity and membrane permeabilization of *E. coli*

The effect of the selected *L. cubeba* leaf EOs (LC19 and BV27) on bacterial cell membrane integrity was evaluated using the LIVE/DEAD BacLight viability kit (Invitrogen™, Molecular Probes Inc., OR, USA). The kit consists of two stains, propidium iodide (PI) and SYTO 9, which both stain nucleic acids. Green fluorescing SYTO 9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged membranes. Once, the cell membrane integrity is ruptured, PI enters cells and competes with SYTO 9 for the same intracellular targets, causing the displacement of SYTO 9 and quenching SYTO 9 fluorescence by fluorescence energy transfer [24]. The persistence of SYTO 9 labeling suggests that EOs alone takes a longer time to damage the cell membrane, and as a consequence, PI enters cells more slowly (Fig 2.5). In addition, PI was also used to evaluate the effect on membrane permeability.

The cultures of *E. coli* were prepared in MHB media as described above with a

range of EO concentrations. Then, 5 mL of cell suspension were incubated with either LC19 or BV27 tested at different concentrations 0.5 MIC, 1 MIC and 2 MIC at 37°C. After 2 h of exposure, the cells were harvested by centrifugation at 10000g for 10 min (Sigma, Bioblock Scientific), then the supernatant was removed and pellets were re-suspended in 0.85% NaCl. After incubation at room temperature for 1 h (mixing every 15 min), samples were pelleted by centrifugation twice at 10000 g for 10 minutes. A 2X working stain solution was prepared by mixing equal volumes of SYTO 9 and PI and adding the mixture into 2 mL of deionized water. Subsequently, the mixture of SYTO 9 and PI was added into the suspension with the ratio 1:1. The dyes were let to react for 15min in the dark, at room temperature. Cell suspension with Tween 0.5% v/v and without EOs was used as control. Cells were examined under the microscope on 1% agarose (in water)-covered slides (CML, France). At least 20 photomicrographs were taken of different fields of view using a LEICA DM6000 photomicroscope equipped with an ORCA – ER C4742 – 80 camera (Hamamatsu, Japan) (Annex Fig. 8) as previously described [174]. The percentages of cells stained with PI (damaged cells) and SYTO 9 (viable cells) were determined and calculated using a program path (Image J). Assays were carried out in triplicate.

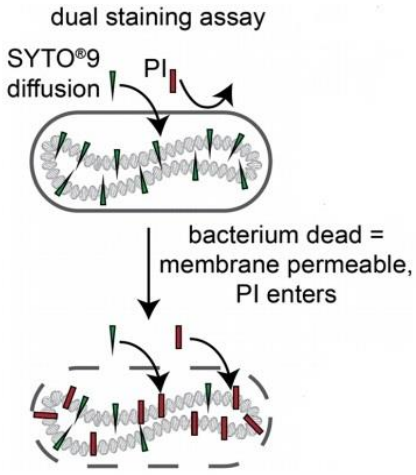


Figure 2.5: The principle of Live/Dead BacLight Kit

For complementary observations of the membrane integrity, the lipophilic fluorescent dye FM 4-64 (Invitrogen™, Molecular Probes Inc., OR, USA) at concentrations of 1 µg/mL was used and added directly in 10 µL of the suspension of untreated and treated with the leaf *L. cubeba* EO (LC19 and BV27) after 2 h of exposure at the same concentration as described above. The mixture was kept on ice for 1 min. The observation under fluorescence microscopy was taken as described above. The cell membrane was considered damaged when the dying fluorescence was different when

compared to non-treated cells. The following phenotypes have been considered as cell damaged membranes: discontinuous, non-homogenous, pore on bacterial membrane, disappearance of the fluorescent binding outside cell membrane. The number of cells having a damaged membrane was recorded. Assays were carried out in triplicate.

2.2.8. Effects of *L. cubeba* leaf EOs (LC19 and BV27) on cell size of *E. coli*

Digital photomicrographs were analyzed using Image J software. All cell lengths and widths were measured and recorded with and without EO treatment. Cell length and width were measured as the distance along the two axes (long and short) of the cell. The dividing cells that were not separated yet were counted and measured as one single cell. Cells were counted as two separated cells only when the constriction was completed. At least 1000 cell sizes were measured for each experiment. Filaments were considered when cell length was higher than 6 microns.

2.2.9. Effects of *L. cubeba* leaf EOs (LC19 and BV27) on DNA of *E. coli*

For DNA visualization, the fluorescent dye 4′,6-dia-mino-2-phenylindole (DAPI) was used [46]. 10 μL of cultures treated with the *L. cubeba* leaf EO samples LC19 and BV27 at the same concentration range as described above were treated using 3% DAPI solution (1 mg/mL DAPI in deionized water, Invitrogen^{TM}, Molecular Probes Inc., OR, USA) and kept in the dark for 10 min. Subsequently, cellular DNA of at least 1000 cells per treatment with EO and the control were observed. DNA metabolism was considered altered when the fluorescence pattern observed in treated cells was different from that of non-treated cells. The following phenotypes have been considered as altered DNA: discontinuous, non-homogenous signal, disappearance of the fluorescence intensity... The number of cells having an altered DNA metabolism was recorded. Assays were carried out in triplicate [46].

Fluorescence digital photomicrographs were analyzed using Image J software. Treated and untreated cells in suspensions were deposited onto a well slides, and images of many areas on the well were taken. The RGB images obtained were converted to grayscale (8 bits), and the threshold is found – the intensity value that allows everything below and above this value to be colored white and black, respectively. The ImageJ analysis algorithm can then be applied to count all of the black particles of a particular shape and size. The remaining non-standalone cells were counted by manual clicker [71].

2.2.10. Toxicity of bacterial pathogens in aquaculture

Different concentration of *V. parahaemolyticus* ND201 and *A. hydrophila* were tested in a pre-challenge experiment in order to validate the infective dose for the bacterial challenge.

The toxicity of *V. parahaemolyticus* ND201 was tested by bathing healthy shrimps of *L. vannamei* in salt water for 24 h. Batches of shrimp at a density of 10 juvenile shrimps were reared in aquarium containing salt water (4 liters salinity of 8‰) with varying concentrations of bacteria (10^6, 10^7, 10^8, 10^9, 10^{10} CFU/mL). Live shrimp were counted visually after 24 h of treatment.

The toxicity of *A. hydrophila* was tested by intra-peritoneal (i.p) healthy carp *C. carpio* for 24 h. The same broad concentrations of bacteria (10^6, 10^7, 10^8, 10^9, 10^{10} CFU/mL) was injected in carp and the survival carp were recorded after 24 h. The physicochemical characteristics of the rearing environment were keep at the best condition for carp and shrimp. LD50 was determined using the following formula:

$$DL_{50} = DL_{100} - \frac{\sum(a \times b)}{n} \qquad (2.6)$$

Where:

DL100: lower doses killing all animals

a: average of the sum of dead individuals to two consecutive doses

b: concentration difference between two successive doses

n: number of animals used per batch

The *in vivo* experiments were all carried out at the Department of Aquaculture – Vietnam National University of Agriculture (VNUA) in Hanoi, Vietnam.

2.2.11. Effects of *L. cubeba* fruit EO and OTC in shrimp assays

The juvenile shrimp of 3 – 3.5 cm (35 days Post-larvae) were purchased from a local shrimp farmer and acclimated in a composite tank for 1 month. *V. parahaemolyticus* ND201 strain isolated from AHPNS diseased shrimp *L. vannamei*. It was cultured at 30°C for 24 h on NB (Merck) plus 2% NaCl at a final concentration of 10^8 CFU/mL. Shrimps were infected with *V. parahaemolyticus* ND201 by immersing in water at LD50. After 2 days infection, shrimp expressed symptom of AHPNS were treated by immersing in 5 mins

with the antibiotics at $MIC_{oxytetracyclin}$ and with mixture of OTC and *L. cubeba* EO at FIC_{min}. The aquaria of four liters were inoculated with 5 juvenile shrimp per liter (20 shrimps per aquarium). The culture conditions were maintained at 27-28^{o}C; salinity of 8‰; pH 7-7.5; 5 - 6 ppm of dissolved oxygen levels. A partial exchange of approximately 30% of water was realized every two days. Feeding was carried out according to the standards applied by the farm protocols. Effects of the *L. cubeba* EO, OTC and the mixture of *L. cubeba* and OTC were investigated on the survival rate of juvenile shrimps in 10 days [132].

The following assays were performed in triplicate in a total of 15 aquaria:

1. C (-): the shrimp no infection, no treated (no EO, no antibiotic)
2. C (+): the shrimp infected with *V. parahaemolyticus*, no treated (no EO, no antibiotic)
3. OTC: the shrimp infected with *V. parahaemolyticus*, treated with OTC at 1 MIC (16.7 µg/mL) by adding OTC in water for 1 day
4. LC: the shrimp infected with *V. parahaemolyticus*, treated with *L. cubeba* fruit EO at 1 MIC 1.67 mg/mL = 1670 µg/mL or at ½ MIC 0.84 mg/mL = 840 µg/mL by adding EO in water for 1 day
5. FIC OTC-LC: the shrimp culture treated with OTC and *L. cubeba* fruit EO at FIC_{min}

OTC and *L. cubeba* fruit EO concentrations were 16.7 µg/mL (1 MIC) and 1667 µg/mL (1 MIC), respectively. The mixture of OTC and *L. cubeba* EO concentration were 4.2 µg/mL (1/4 MIC of OTC) and 316.7 µg/mL (1/5 MIC of EO) (corresponding to the lowest MIC of EO and antibiotics in the assay of antimicrobial activity).

Juvenile shrimp survival rate

The shrimp survival rate was recorded every day at 6 am for all the experiments. The survival rate (%) was calculated as described as follow [132]:

$$Shrimp\ survival\ (\%) = \frac{\text{Total number of live shrimps each day}}{\text{Initial number of shrimp}} x\ 100 \qquad (2.7)$$

At the end of experiment, death shrimp, survival shrimp and water from each aquarium was sampled. *L. vannamei* were crushed into 5 mL of sterile saline water. The homogenized sample was serially diluted in sterile saline solution. The water samples were diluted in sterile saline solution as well. A volume of 0.1 mL from each dilution was plated

onto TCBS agar for vibrios and incubated at 30°C for 24 h [132].

2.2.12. Experimental design in carp

2.2.12.1. Preparation of fish

Juveniles of common carp *Cyprinus carpio* (weight average 30.26 ± 1.56 g; total length average 13.25 ± 0.24 cm) were purchased from a local fish farmer and reared in a common tank for 2 weeks before their transfer in the experimental structures.

2.2.12.2. Preparation of L. cubeba leaf powder

Leaves of *L. cubeba* were harvested during the pre-flower phase, in Phu Tho – Vietnam. For fish meals, leaves were dried at 50°C for 48h and then crushed (by means of special meat grinder). The powder was then stored at 4°C in a closed and tided packaging until use.

2.2.12.3. Preparation of carp feed by enriching L. cubeba leaf powder

Experimental feeds were obtained adding respectively 0; 2; 4 and 8% (w/w) of *L. cubeba* leaf powder to a commercial fish feed (Brand: CJ VINA-Vietnam, 35% protein, 6% lipid, <16% fiber content). It was humidified with water and 3% of tapioca flour was added as a binder. After re-pelleting, the experimental feeds were air dried and then stored until use (Annex Fig. 11).

2.2.12.4. Feeding experiments and growth promoters

20 fish were randomly distributed in each aquaria containing a volume of 60 L of stagnant water and then each aquaria was randomly assigned to an experimental treatment (0; 2; 4 and 8% of plant enriched-diets). Assays were carried out in triplicate in a total of 12 aquaria.

Water parameters were regularly measured during the entire experiment to ensure the following characteristics: pH 7.56 ± 0.35; temperature 27 ± 0.5°C; O_2 4 mg/L; NH_4 0.62±0.21 mg/mL; NH_3 0.22 ± 0.23 mg/L (Annex Table 3, 4, 5). A partial exchange of approximately 30% of water was realized every two days.

Feed was distributed two times in the fish aquaria at a ratio of 3% of fish biomass per day during 21 days.

Growth performance and feed utilization were calculated as follows:

Weight Gain (WG in g) = final body weight (g) − initial body weight (g) (2.8)

Feed Conversion Ratio (FCR) = feed intake (g) / body weight gain (g) (2.9)

Specific Growth Ratio (SGR in %) = 100 × [(ln final weight - ln initial weight) / days of the experiment] (2.10)

2.2.13. Humoral immune responses induced of carp by plant materials

After 21 days of feeding regime, a subsample of fish (n=3) from each aquaria was anesthetized with clove oil 60 µL/L and then 1 mL of blood was collected from caudal vein with heparinized syringe with 0.1 mL of heparin (Sanofi Aventis, France). Blood was collected individually in a sterile eppendorf and centrifuged at 4°C 3000 g for 5 min.

Humoral immunological activity was evaluated using three different plasma parameters.

2.2.13.1. Lysozyme activity

Plasma lysozyme was determined by turbidometric assay according to Caruso and Lazard (1999) [34] with a minor modification consisting in the use of Phosphate Buffered Saline (PBS) instead of NaH_2PO_4. 20 µL of plasma were added to 180 µL of *Micrococcus lysodeikticus* (Sigma, France) suspended in PBS at pH 6.2. Two measures of absorption were carried out at 450 nm using Elisa reader (Infinite M200, Austria) immediately after mixing and after 4.5 min. One unit of lysozyme activity was defined as the amount of plasma lysozyme that causes a decrease in absorbance of 0.001/min at 450 nm. A series of dilutions was prepared for the standard lysozyme from hen egg-white (Sigma, France) in these conditions to establish the calibration curve.

2.2.13.2. Bactericidal activity

Bactericidal activity of plasma was determined using *A. hydrophila* ATCC 35654 strain according to Leano et al. (2003) [98]. Equal volumes (50 µL) of plasma and the bacterial suspension (10^4 CFU/mL) were mixed and incubated for 1 h at 30°C. A positive control was prepared by replacing blood plasma with sterile PBS. The mixture was then diluted using sterile PBS at a ratio of 1:10; and 100 µL were plated onto NA (Biokar, France) and then incubated at 30°C for 24 h. The result was expressed as count of CFU/mL.

The Bactericidal activity was determined as follows:

Bactericidal activity (%) = [1 – (Control count/Treated group count)] ×100 (2.11)

2.2.13.3. Alternative complement activity

Nonspecific haemolytic activity of plasma was carried out according to Caruso et al. (2005) [36] with a few modifications.

Rabbit red blood cells (RaRBC) were collected by auricular puncture of New Zealand rabbit using heparin as anticoagulant and then blood 1% was stocked in Alsever's solution (Sigma Aldrich, France) until use. An aliquot of this solution was centrifuged and pellets of blood cells were washed two times in sterile PBS and then suspended at 1% in PBS pH 7.4. Serial dilutions 1:2 of plasma were realized in PBS containing 10 mmol/L Mg^{2+} and 2 mmol/L Ca^{2+}. 50 µL of plasma dilutions were mixed in an Eppendorf tube with equal volume of RaRBC solution. A positive control giving 100% haemolysis was obtained using RaRBC and distilled water. The negative control corresponded to RaRBC and PBS containing 10 mmol/mL Mg^{2+} and 2 mmol/L Ca^{2+}. After incubation at 37°C for 1 h, the tubes were centrifuged at 2500 g for 5 min, and the OD of the supernatant was measured at 540 nm (Elisa reader, Infinite M200, Austria). Haemolytic activity was determined using the equation of linear regression obtained by plotting OD values (x) and dilutions (y). ACH50 unit corresponded to the volume of plasma giving 50% haemolysis and results were expressed as ACH50 units/mL.

2.2.14. Experimental infection of carp

A. hydrophila strain isolated from diseased catfish *P. hypophtalmus* was cultured at 30°C for 24 h on Tryptic Soy broth (TSB, Merck, France) at a final concentration of 10^8 CFU/mL. At the end of the 21 days feeding period, fish was lightly anesthetized with clove oil 60 µL/L, and then challenged with i.p. injection of 0.1 mL at 10^8 CFU/mL *A. hydrophila* per fish for a total of 208 fishes. Two fishes/aquaria were taken for the negative control and received 0.1 mL of sterile TSB. Fish were fed the same diets along the post-challenge period. Mortality was monitored daily (every 4 h) until no more mortality was observed and the trial was terminated on the 21 day post-challenge.

The Relative Percent of Survival (RPS) was determined using the following formula:

RPS = [1- (% mortality in treated group / % mortality in control group)] * 100 (2.12)

2.2.15. Statistical analysis

MIC and FIC values were compared by using ANOVA, Fisher LSD test ($P<0.05$). For comparison of cell length and width measures between EO treatments and controls,

analysis of variance was performed using ANOVA. Significant differences between mean values were determined by Fisher LSD test at the threshold of $P<0.05$. Same tests (ANOVA, Fisher LSD) were done for the comparison of percentage of cell viability, cell morphology, membrane, DNA defects between EO-treated and untreated cells.

Normality and equality of variance of aquaculture data were checked using Kolmogorof-Smirnov and Levene median tests respectively. The differences between treatments feeding were tested with analysis of variance (ANOVA) and significant differences were compared by Holm-Sidak method or Fisher's test with $\alpha=0.05$. Chi-square analysis was used to compare the mortality rates among 4 experimental treatments (0; 2; 4 and 8% plant) and Kaplan-Meier survival analysis was used for survival curves.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Screening of commercial EOs for antimicrobial activity

Nine essential oils (EOs) used in our studies including clove basil *O. gratissimum*, cajeput *M. leuladendron*, cinnamon *C. cassia*, cape yellowwood *Z. rhesta*, sweet wormwood *A. annua*, May Chang *L. cubeba*, basil *O. basilicum*, wormseed *C. ambrosioides*, corn mint *M. arvensis* were the common EOs in Vietnam. They are wide distributed, cultivated or even grew wild. The results of components analyses as well as antibacterial activities in solid and liqid phase were presented in Table 3.1, Table 3.2 and Table 3.3.

3.1.1. Chemical composition of commercial EOs

The results of the chemical analyses of the EOs of the different plants are presented in Table 3.1 (Annex Fig. 5). A large variety of components and amounts of the nine EOs tested was found.

Indeed, trans-cinnamaldehyde and estragole were the most abundant compounds in cinnamon *C. cassia* (87.69%) and basil *O. basilicum* (90.71%) EOs, respectively. Menthone (17.68%) and menthol (69.95%) were the 2 main compounds found in corn mint *M. arvensis* EO. Sweet wormwood *A. annua* EO was dominated by camphor 26.80%, 1,8-cineole (16.96%), β-caryophylene (8.61%) and γ-muurolene (10.41%). α-phellandrene (28.13%), α terpinene (21.31%), limonene (20.32%) was the dominant component of cape yellowwood *Z. rhetsa* EO. May chang fruit *L. cubeba* EO was dominated by 3 main compounds including limonene (17.13%), carveol (31.13%) and citral (39.25%) (Table 3.1), (Annex Fig. 5).

Table 3.1: Chemical composition of the nine commercial EOs used in this study

Essential oils	Main compounds	Percentage (%)
Clove basil *Ocimum gratissimum*	E-β-ocimene	15.24
	eugenol	48.50
	β-caryophyllen	6.47
	γ-muurolene	10.33
Cajeput *Melaleuca leucadendron*	1.8-cineole	34.50
	o-cymene	7.79

	terpinolene	8.33
	α-terpineol	8.01
Cinnamon *Cinnamomum cassia*	trans-cinnamaldehyde	87.69
Cape yellowwood *Zanthoxylum rhetsa*	α-phellandrene	28.13
	α terpinene	21.31
	limonene	20.32
Sweet wormwood *Artemisia annua*	1.8-cineole	16.96
	camphor	26.80
	β-caryophylene	8.61
	γ-muurolene	10.41
May Chang fruit *Litsea cubeba*	limonene	17.13
	carveol	31.13
	citral	39.25
Basil *Ocimum basilicum*	estragole	90.71
Wormseed *Chenopodium ambrosioides*	p-cymene	17.47
	α-terpinolen	57.34
	ascaridol	22.05
Corn mint *Mentha arvensis*	menthone	17.68
	menthol	69.95

The other EOs contained large amount of only compound (accounting ~ 50%) and the main minor compounds was normally less than 20%. Eugenol (48.50%), E-β-ocimene (15.24%) and γ-muurolene (10.33%) were the main compounds of clove basil *O. gratissimum* EO. Similarly, the mains component of wormseed *C. ambrosioides* EO was α-terpinolene (57.34%), p-cymene (17.47%) and ascaridol (22.05%). 1,8-cineole, o-cymene, terpinolene and α-terpineol were the dominant compound of cajeput *M. leucadendron* EO accounting for 34.50, 7.79, 8.33 and 8.01%, respectively.

3.1.2. Antibacterial activity (inhibition zones) of commercial EOs

The results of the antibacterial activity of the nine EOs are summarized in Tables 3.1 and Table 3.2 (Annex Fig. 7). Based on the inhibition zones and MIC values, all of the nine commercial EOs tested showed antibacterial effects against 10 bacterial strains. A broad variation in antimicrobial properties of the analyzed EOs was observed in the study.

In the most cases, the EOs tested by the disc diffusion method at a concentration of 10 μL/disc showed bacteriostatic activity except for basil *(O. basilicum)*, cajeput *(Z. rhesta)* and cape yellowwood *(A. annua)* against *S.* Typhimurium and *V. furnissii*. Indeed, the inhibition zones were ranged from 0-90.0 mm for nine EOs and were ranged from 10.3-32.0 mm and from 0-33.3 mm for AK30 and FOX30, respectively (Table 3.2) (Annex Fig. 7).

The EOs of cape yellowwood *Z. rhesta*, basil *O. basilicum* and sweet wormwood *A. annua* exhibited the lowest antibacterial activity in the disc diffusion method. These EOs did not affect *S.* Typhimurium and *V. furnissii*, while inhibition zones for other bacterial species were 10.7-23.3 mm; 9.3-32.0 mm and 8.0-16.7 mm, respectively. Good inhibition zones were also obtained for clove basil *O. gratissimum*, cajeput *M. leucadendron*, wormseed *C. ambrosioides* and corn mint *M. arvensis*, with 12.7-25.3 mm, 14.0-31.0 mm, 11.7-43.3 mm and 10.7-29.7 mm, repectively. It can be seen that EOs from cinnamon *C. cassia*, cajeput *M. leucadendron*, wormseed *C. ambrosioides* and May chang fruit *L. cubeba* EOs possess a higher antibacterial effect than AK30 and FOX30. May chang fruit *L. cubeba* and especially cinnamon *C. cassia* EO showed much larger inhibition zones than other EOs and two antibiotics tested.

The highest inhibition zone was observed in the case of May chang fruit *L. cubeba* EO against *B. cereus* with 90.0 mm. However, this EO showed mild inhibitory effect to *S.* Typhimurium with 9.0 mm. Among the 10 Gram-negative and Gram-positive bacteria, *S.* Typhimurium and *E. faecalis* were most resistant bacterial to nine commercial EOs.

AK30 and FOX30 were used as positive control in our study. The results obtained by the disc diffusion method of both antibiotics were agreed with Clinical & Laboratory Standards Institute (CLSI). As reported in this literature, inhibition zone of FOX30 against *S. aureus* ranged from 28.0-35.0 mm (D=31.7 mm in our study) and AK30 against *E. coli* corresponded to 21.5-26.0 mm (D=22.0 mm in our study) (CLSI, 2015).

Table 3.2: Antibacterial activity of nine commercial EOs (inhibition zones in mm) against 10 bacterial strain

Bacterial	***O. g.***	***M. l.***	***C. c.***	***Z. r.***	***A. a.***	***L. c.***	***O. b.***	***C. a.***	***M. a.***	**AK30**	**FOX30**
Gram-negative											
S. Typhimurium	15.3±1.2^{e}	13.0±1.0^{d}	28.7±0.6^{de}	R	10.3±1.2^{b}	9.0±1.0^{i}	R	14.7±0.6^{fg}	10.7±0.6^{c}	23.0±0.0^{de}	24.0±1.0^{b}
E. coli	18.0±0.0^{d}	15.3±4.5^{cd}	**30.0±5.3^{de}**	12.7±1.5^{cde}	12.0±0.0^{b}	19.0±1.0^{h}	11.0±1.0^{bc}	24.7±5.5^{cd}	14.7±3.5^{c}	22.0±0.0^{e}	25.3±2.5^{b}
A. hydrophila	25.3±1.5^{a}	**31.0±1.0^{a}**	**37.7±2.5^{bc}**	19.3±2.3^{ab}	16.0±2.6^{a}	29.7±0.6^{fg}	14.3±0.6^{b}	**43.3±1.2^{a}**	25.0±1.0^{ab}	27.7±0.6^{bc}	17.0±0.0^{cd}
V. parahaemolyticus	25.7±1.2^{a}	21.3±2.3^{b}	**54.7±4.2^{a}**	23.3±3.1^{a}	11.3±0.6^{b}	**37.0±2.6^{e}**	9.7±0.6b^{c}	29.3±1.2^{b}	21.7±0.6^{b}	22.7±2.1^{de}	23.7±1.5^{b}
V. furnissii	25.3±2.1^{a}	17.3±2.1^{bcd}	**39.7±3.5^{b}**	10.7±3.1^{e}	R	**32.0±2.0^{f}**	14.7±2.3^{b}	28.7±1.5^{bc}	29.3±1.2^{a}	19.7±1.5^{f}	19.0±0.0^{c}
Gram-positive											
B. subtilis	22.0±2.0^{bc}	18.0±3.6^{bc}	**33.7±0.6^{cd}**	16.0±4.4^{bc}	12.0±1.0^{b}	**50.3±1.2^{b}**	32.0±9.8^{a}	20.7±5.0^{de}	23.3±5.7^{b}	32.0±0.0^{a}	33.3±2.1^{a}
B. cereus	20.0±1.0^{cd}	21.7±1.5^{b}	**55.3±2.3^{a}**	15.3±1.2^{bcd}	16.7±0.6^{a}	**90.0±0.0^{a}**	15.0±1.0^{b}	17.0±1.0^{ef}	24.0±1.0^{b}	29.3±1.2^{b}	18.0±1.0^{cd}
L. innocua	20.3±0.6^{cd}	17.3±3.1^{bcd}	**57.3±3.1^{de}**	11.3±0.6^{cde}	16.3±0.6^{a}	**40.3±2.5^{d}**	13.3±1.2^{b}	11.7±1.5^{g}	12.3±1.5^{c}	24.0±1.0^{d}	16.0±1.0^{d}
E. faecalis	12.7±0.6^{f}	14.0±1.0^{cd}	28.0±0.0^{e}	11.3±2.1^{de}	10.3±2.1^{b}	28.7±1.2^{g}	9.3±1.2b^{c}	12.7±1.5^{fg}	22.0±5.3^{b}	10.3±1.2^{g}	R
S. aureus	23.3±2.1^{ab}	21.3±3.1^{b}	**41.0±4.6^{b}**	16.7±4.6^{bc}	8.0±1.0^{c}	**44.3±3.2^{c}**	14.7±3.5^{b}	25.7±2.1^{bc}	25.7±2.5^{ab}	26.0±0.0^{c}	31.7±1.5^{cd}

O.g.: Ocimum gratissimum, M. l.: Melaleuca leucadendron, C. c.: Cinnamomum cassia, Z. r.: Zanthoxylum rhetsa, A. a.: Artemisia annua, L. c.: Litsea cubeba, C. a.: Chenopodium ambrosioides, M. a.: Mentha arvensis.
Values are expressed by means ± SD of three replications. The inhibition zone expressed in mm includes the diameter of each disc (6 mm).
AK30: Amikacin 30 μg, FOX30: Cefoxcitin 30 μg was used as positive control. Values with different letters within a column are significantly different according to Fisher LSD test ($P<0.05$).

Table 3.3: Antibacterial activity (MIC and MBC in mg/mL, MBC/MIC) of nine commerical EOs against bacterial strains

Essential oils		***S*. Typhimurium**	***E. coli***	***A. hydrophila***	***V. parahemolyticus***	***B. cereus***	***S. aureus***
Ocimum grastissimum	MIC	5.3 ± 1.8^{b}	5.3 ± 1.8^{b}	8.4 ± 3.7^{ab}	6.3 ± 0^{b}	6.3 ± 0^{b}	10.5 ± 3.7^{a}
	MBC	10.5 ± 3.7^{c}	10.5 ± 3.7^{c}	25.3 ± 0^{ab}	25.3 ± 0^{ab}	21.1 ± 7.3^{bc}	33.7 ± 14.6^{a}
	MBC/MIC	2.0	2.0	3.0	4.0	3.3	3.2
Melaleuca leucadendron	MIC	11.3 ± 0^{ab}	11.3 ± 0^{ab}	11.3 ± 0^{ab}	11.3 ± 0^{ab}	7.5 ± 3.3^{b}	15.0 ± 6.5^{a}
	MBC	45.1 ± 0^{a}	$> 45.1\pm0^{a}$	$> 45.1\pm0^{a}$	45.1 ± 0^{a}	22.6 ± 0^{b}	45.1 ± 0^{a}
	MBC/MIC	4.0	4.0	4.0	4.0	3.0	3.0
Cinnamomum cassia	MIC	0.4 ± 0^{d}	1.1 ± 0.5^{b}	0.7 ± 0.2^{cd}	0.8 ± 0^{bc}	0.8 ± 0^{bc}	6.6 ± 0^{a}
	MBC	3.3 ± 0^{ac}	5.5 ± 1.9^{c}	2.8 ± 1.0^{a}	2.2 ± 1.0^{a}	22.2 ± 7.7^{d}	13.3 ± 0^{b}
	MBC/MIC	8.0	5.0	4.0	2.7	26.7	2.0
Zanthoxylum rhetsa	MIC	34.9 ± 12.1^{a}	21.0 ± 0^{b}	21.0 ± 0^{b}	10.5 ± 0^{c}	14.0 ± 6.0^{bc}	10.5 ± 0^{c}
	MBC	$> 41.9\pm0^{a}$	$> 41.9\pm0^{a}$	$> 41.9\pm0^{a}$	41.9 ± 0^{a}	41.9 ± 0^{a}	41.9 ± 0^{a}
	MBC/MIC	1.2	2.0	2.0	4.0	3.0	4.0
Artemisia annua	MIC	23.4 ± 0^{b}	23.4 ± 0^{b}	23.4 ± 0^{b}	23.4 ± 0^{b}	39.0 ± 13.5^{a}	23.4 ± 0^{b}
	MBC	$> 46.8\pm0^{a}$	$> 46.8\pm0^{a}$	$> 46.8\pm0^{a}$	$> 46.8\pm0^{a}$	$> 46.8\pm0^{a}$	$> 46.8\pm0^{a}$
	MBC/MIC	2.0	2.0	2.0	2.0	1.2	2.0
Litsea cubeba	MIC	5.5 ± 0^{b}	5.5 ± 0^{b}	9.2 ± 3.2^{a}	5.5 ± 0^{b}	3.7 ± 1.6^{b}	5.5 ± 0^{b}
	MBC	11.1 ± 0^{c}	11.1 ± 0^{c}	22.1 ± 0^{b}	18.4 ± 6.4^{b}	$> 44.2\pm0^{a}$	7.4 ± 3.2^{c}
	MBC/MIC	2.0	2.0	2.4	3.3	12.0	1.3

Ocimum basilicum	MIC	24.1 ± 0^{a}	20.1 ± 7.0^{a}	24.1 ± 0^{a}	24.1 ± 0^{a}	32.1 ± 13.9^{a}	20.1 ± 7.0^{a}
	MBC	$> 48.2\pm0^{a}$	$> 48.2\pm0^{a}$	$> 48.2\pm0^{a}$	$> 48.2\pm0^{a}$	$> 48.2\pm0^{a}$	$> 48.2\pm0^{a}$
	MBC/MIC	2.00	2.40	2.00	2.00	1.50	2.40
Chenepodium ambrosioides	MIC	3.1 ± 0^{d}	6.2 ± 0^{bc}	8.2 ± 3.6^{b}	12.3 ± 0^{a}	5.1 ± 1.8^{cd}	6.2 ± 0^{bc}
	MBC	10.3 ± 3.6^{c}	12.3 ± 0^{c}	49.4 ± 0^{a}	24.7 ± 0^{b}	10.3 ± 3.6^{c}	49.4 ± 0^{a}
	MBC/MIC	3.33	2.00	6.00	2.00	2.00	6.00
Mentha arvensis	MIC	18.8 ± 6.5^{a}	5.6 ± 0^{b}	22.6 ± 0^{a}	5.6 ± 0^{b}	18.8 ± 6.5^{a}	11.3 ± 0^{b}
	MBC	45.1 ± 0^{a}	15.0 ± 6.5^{b}	45.1 ± 0^{a}	11.3 ± 0^{b}	37.6 ± 13.0^{c}	$> 45.1\pm0^{a}$
	MBC/MIC	2.4	2.7	2.0	2.0	2.0	4.0

MIC: Minimum Inhibitory Concentration expressed in mg/mL. MBC: Minimum Bactericidal Concentration expressed in mg/mL. Results are means of three different experiments ± SD. Values with different letters within a row are significantly different according to Fisher LSD test ($P<0.05$).

3.1.3. Antibacterial activity (MIC and MBC values) of nine commercial EOs

After screening the antibacterial activity of nine EOs from Vietnam against 10 bacterial species by disc diffusion method, the inhibitory effect of these EOs was tested against some relevant pathogenic bacteria for food and aquaculture including: *S.* Typhimurium, *E. coli, V. parahaemolyticus, A. hydrophila, B. cereus* and *S. aureus* by microdilution method. The MIC and MBC values were presented in Table 3.3. The lower MIC and MBC values, the higher antibacterial activity of EOs and vice versa.

The MIC and MBC ranged from 0.4-38.7 mg/mL and 2.2-49.4 mg/mL for nine EOs, respectively. The most sensitive strain was Gram-negative *S.* Typhimurium (lowest MIC values) and the most resistant strain was *A. hydrophila* (highest MIC values).

As same as inhibitory effect found in disc diffusion method, cinnamon *C. cassia* possessed a highest activity (MIC = 0.4-6.6 mg/mL) while cape yellowwood *Z. rhesta*, basil *O. basilicum* and sweet wormwood *A. annua* exhibited the lowest antibacterial activity (MIC = 10.5-39.0 mg/mL). A moderate inhibitory effect was found for clove basil *(O. gratissimum),* cajeput *(M. leucadendron),* wormseed *(C. ambrosioides)* and corn mint *(M. arvensis)* (MIC = 3.1-22.6 mg/mL).

In addition, *O. basilicum, A. annua, Z. rhetsa* and *M. leucadendron* did not have a bactericidal effect with MBC values > 50 μL/mL (out of the tested concentration range).

To determine the nature of the antibacterial effect of EOs, the MBC/MIC ratio was used. When the ratio MBC/MIC<4, the EO was considered as a bactericidal and when the ratio MBC/MIC>4, it was considered as a bacteriostatic. In our study, *O. gratissimum, A. annua, Z. rhesta, C. ambrosioides, O. basilicum, M. arvensis* EOs showed a bactericidal effect against tested strains in the most cases. Whereas, the other EOs including *M. leucadendron, C. cassia* were bacteriostatic except for *M. leucadendron* against *B. cereus* and *S. aureus;* and *C. cassia* against *V. parahaemolyticus* and *S. aureus.*

Several factors such as temperature, strain and EOs origin, composition or concentration could affect the inhibition zone values. For instance, *Mentha* spp. *(*including *M. piperita* and *M. spicata)* and *O. basilicum* EO from Serbia at the concentration of 1.0 μg/mL against the human pathogenic bacteria *B. subtilis*, *Enterobacter cloacae*, *E. coli* O157:H7, *Micrococcus flavus*, *P. mirabilis*, *P. aeruginosa*, *Salmonella* Enteritidis, *S. epidermidis, S.* Typhimurium and *S. aureus* showed the same inhibitory effect with D =

10.0-25.0 mm and D = 8.0-23.0 mm, respectively [153]. Antimicrobial activity of higher amount (40 μL) of four EOs from Romanian against *E. coli*, *S. aureus*, *S.* Typhimurium, *B. cereus*, *P. aeruginosa* were reported [146]. Thyme *T. vulgaris* EO exhibits the best inhibitory activity against all bacteria evaluated followed by by basil *O. basilicum*, lovage *Levisticum officinale*, and parsley *Petroselinum crispum* EOs [146]. *A. annua* L. EO from Bosnia, consisting artemisia ketone (30.7%) and camphor (15.8%) as major components, inhibited tested microorganisms with the inhibition zones ranged from 15.0-50.0 mm. *A. annua* EO showed higher antimicrobial activity compared to thymol and ampicillin at the same concentration (10 mg/mL) [37]. Our study reported a larger zone of inhibition in the case of *M. leucadendron* from Pakistan. The inhibition zones against *Bacillus spizizenii, S. aureus, Enterobacter aerogenes, E. coli, K. pneumoniae*, *P. aeruginosa* and *Salmonella enterica* were ranged from 11.7-14.7 mm, 11.0-16.7 mm and 11.2-13.3 mm at concentration 8 μg/mL for *M. leucadendron*, *M. fulgens* and *M. bracteata*, respectively [151]. 5μL/disc of *M. leucadendron* from Egypt possessed a zone of inhibtion ranged from 11.7-13.3 mm against *B. subtilis* and *E. coli* [65].

EOs investigated in our study showed higher antibacterial activity than other common EOs used in Vietnam such as citrus, perrila EOs [3, 4]. The inhibition zones of *Citrus grandis* (Bac Giang), *C. sinensis* (Hoa Binh), *C. limonia* (Bac Giang) were ranged from 7.8-8.8 mm against same broad of strain [4]. Similarly, 20 μL of perrila leaves *Perilla frutescens* showed a moderate activity (D= 9.0-22.0 mm) against *S. aureus, E. coli, S. typhi, B. subtilis, B. cereus, P. fluorescens* and *Streptococcus feacium.* However, this EOs showed no inhibitory effect against *P. aeruginosa* [3].

Disc diffusion assays are simples, low cost and rapid methods which require rather small amounts of plant extract. Moreover, it is ability to test enormous numbers of microorganisms and antimicrobial agents. However, diffusion methods present some disadventages. First, using volatile compounds could lead to reduced zones of inhibition, since they could evaporate very quickly. Also, poorly soluble compounds do not diffuse uniformly through the agar medium plates. Secondly, for non-polar extracts and EOs, diffusion technique is not suitable for testing.

Moreover, the outputs reported from the different studies are difficult to compare directly by diverse bacterial strains, culture media, and antimicrobial sample sources. Generally, disc diffusion method is regularly used as preliminary tests and qualitative data

for the antimicrobial activity of a large number compounds, in order to select the ones with larger inhibitory zones (higher inhibitory activity).

In this study, the antibacterial activity of nine EOs was evaluated in solid phase (disc diffusion) and liquid (liquid phase). The antibacterial activity of EOs determined in disc diffusion and microdilution method were correlated. According to our results, *C. cassia* EO was also found the most active whereas *Z. rhetsa, O. basilicum* and *A. annua* EO were the weakest active in both methods.

A difference in the constitution or in the concentration of the constituents of the EO can directly influence the antimicrobial activity [142]. Some researchers reported that there is a relationship between the chemical structures of the most abundant in the tested EO and the antimicrobial activity.

Among 28 commercial EOs from Canadian, *Corydothymus capitatus* which contained 76% carvacrol (monoterpenoid phenol) was the most active, followed by *C. cassia* (65% cinnamaldehyde), *Satureja montana* (43% thymol), *Eugenica caryophyllus* (78% eugenol), *C. citratus* (78% citral) and *Thymus mastichira* (47% 1,8-cineole) against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium [119]. In addition, *Cinnamomum camphora* EO and *Eucalyptus globulus* EO showed weakest activity, wheares cinnamon *C. zeylanicum* EO showed strongest activity in inhibiting some tested bacterial strains of *B. subtilis, S. aureus, E. coli, K. pneumoniae, P. aeruginosa* and *P. vulgaris* [128]. Camphor, 1,8-cineole and cinnamaldehyde were the major compounds found in *C. camphora, E. globulus* and *C. zeylanicum*, respectively.

Trinh et al. (2015) analyzed the composition of cinnamon *C. cassia* from Vietnam and showed that this EO contained 90.08% of trans-cinnamaldehyde. The same inhibitory effect was observed between *C. cassia* and its main component, trans-cinnamaldehyde, with the MIC and MBC values against *L. innocua* were 0.32 mg/mL and 2.70 mg/mL, respectively [164]. However, the lower activity of cinnamon *C. cassia* EO from China, which accounting 68.52% trans-cinnamaldehyde, was observed. The MIC values ranged from 2.5-10.0 mg/mL against *S. aureus, B. subtilis, S.* Typhimurium and *E. coli* [79]. The difference amount of trans-cinnamaldehyde might be led to reduce of antibacterial activity.

Anna et al. (2014) investigated the chemical diversity of *A. annua* from several countries. Artemisia ketone was the main component of *A. annua* EOs from China (64%), India (11.5-58.0%), France (2.8-55.0%) and North - America (35.6-68.0%), followed by

1,8-cineole and germacrene D [27]. On the other hand, camphor, germacrene D and 1,8-cineole were the major components found in the *A. annua* EO from Vietnam and India. Our finding confirmed the results obtained from the samples from Vietnam with the addition of 10.48% γ-muurolene in the main composition [27]. The antibacterial activity of *A. annua* found in our study was correlated with the previous studies [27, 131, 172]. *A. annua* EO from Iran, which contained 48% camphor and 9.39% 1,8-cineole, had the MIC values 20, 32 and 64 mg/mL against *B cereus* [27], *S. aureus* and *E. coli* [172]. In addition, the higher antibacterial activity of *A. annua* from Serbia was observed with the MIC values was ranged from 5.0-20.0 mg/mL, in which artemisia ketone 35.7% and α-pinene 16.5% were the main component of this EO [131].

In our study, the main component of *O. gratissimum* and *O. basilicum* were eugenol 48.50% and estragole 90.71% and the MIC values ranged from 5.3-10.5 mg/mL and from 20.1-32.1 mg/mL, respectively. Previous studies showed the same inhibitory effect of *Ocimum* EO [14, 123]. Pandley et al. (2014) reported the chemical diversity and antibacterial activity of *Ocimum* EO from Bangladesh, Brazil, Italia, India and Thailand. For instance, estragole (methyl chavicol), linalool, methyl eugenol and methyl cinnamate were the four main chemotype found in *O. basilicum* [123]. Whereas, *O. gratissimum* EO found 2 main chemotype: eugenol - rich type and thymol - rich type. Anand et al. (2011) compared the antimicrobial activity of two EOs *O. gratissimum* (53.89% eugenol) and *O. basilicum* (70.04% estragole). *O. gratissimum* showed lower MIC values (3.13-6.25 mg/mL) compared to MIC values of *O. basilicum* (6.25-12.5 mg/mL) against *E. coli* and *S. aureus,* respectively [14].

M. leucadendron EO from Pakistan, which contained 95.4% eugenol methyl ether, showed lower MIC and MBC values 4-8 μg/mL compared to our study [151]. In addition, *M. leucadendron* EO rich in 1,8-cineole (55.8%) had higher MIC and MBC values, correspondence in 1067 and 1191 μg/mL against *Paenibacillus larvae*, respectively [15].

Variability of MIC values and composition from *L. cubeba* EO were claimed in previous study. The same MIC values (1.25-10 mg/mL) were reported for *L. cubeba* fruit EO rich in citral (83.85%) from India. However, two *L. cubeba* fruit EOs containing mainly citronellal and citronellol possessed a lower antimicrobial activity (MIC = 5-20 mg/mL) against the same broad of bacterials [140]. Wang and Liu (2010) [176] found citral (63.75%) and limonene (7.38%) as the main constituents with zone inhibition and

MIC values ranged from 14.1-34.5 mm and 100-700 μg/mL, respectively.

Weak antibacterial activity of *C. ambrosoides* EO from Brazil which contained α-terpinene (40.73%), p-cymene (21.81%) and trans-ascaridole (12.49%), were reported with the MIC values ranged from 62.5-250 μL/mL against *S. aureus, L. monocytogens, E. coli* and *S.* Cholerauis [142]. Owolabi et al. (2009) showed difference amount of composition of *C. ambrosioides* from Nigeria, including α-terpinene (63.1%), p-cymene (26.4%) and ascaridole (3.9%) [120]. However, this species presented no antibacterial activity against bacteria *B. cereus, S. aureus* and *E. coli*. The author emphasized that, in addition to the classes to which the constituents belong, other factors must be taken into account in assigning the antibacterial activity, such as isomerism and the synergism between the components [142].

In our study, the antibacterial potential of EOs tested in disc diffusion and microdilution methods can be presented as: *A. annua* < *O. basilicum* < *Z. rhesta* < *M. arvensis* < *M. leucadendron* < *O. gratissimum* < *L. cubeba* < *C. ambrosioides* < *C. cassia*. The antimicrobial activity of EOs depends on their chemical composition special on the major compounds. However, EOs minor compounds may exhibit interactions that could lead to either synergistic or antagonistic effects [83]. As reported in the literature, the antibacterial of terpene can be presented as: hydrocarbons < ethers < alcohols < ketones < aldehydes < cinnamic aldehyde < phenols [88, 153]. The strong antibacterial effect of trans-cinnamaldehye [92], eugenol [53]; linalool [133] and 1,8-cineole [49] were reported in previous studies.

A broad constituents showed variation in antimicrobial activities against 25 bacterial strains including *E. coli*, *Salmonella* Pullorum, *P. aeruginosa*, and *Brochothrix thermosphacta* [59]. From this, the component with the widest spectrum of activity was found to be thymol followed by carvacrol, α-terpineol, terpinen-4-ol, eugenol, linalool, (-)-thujone, δ-3-carene, citral, nerol, geraniol, menthone, β-pinene, limonene, α-pinene, α-terpinene, sabinene, γ-terpinene, citronellal, terpinolene, 1,8-cineole, carvacrol methyl ether, myrcene, β-caryophyllene, α-phellandrene, α-humulene, β-ocimene, aromadendrene, p-cymene, in that order [59].

Comparing the MIC values, the EOs investigated showed better activity against Gram-positive than Gram-negative bacteria. Some authors have reported that Gram-negative micro-organisms are slightly more sensitive to EOs compared to Gram-positive.

The Gram-positive and Gram-negative microorganisms differ in several aspects other than with respect to the structure of their cellular walls, mainly with regard to the presence of lipoproteins and lipopolysaccharides in Gram-negative bacteria that form a barrier to hydrophobic compounds. Indeed, the cell wall of Gram-positive bacteria is composed basically of peptidoglycan (approximately 90-95%), which forms a thick, fibrous layer. The hydrophobic molecules easily penetrate the outer and cytoplasmic membranes to reach their site of action.

Whereas, the cell wall of Gram-negative bacteria is more complex. It has a thin peptidoglycan layer (2–3 nm thick), and composes approximately 20% of the dry weight of the cell. An outer membrane (OM) lies outside of the thin peptidoglycan layer. The presence of an OM which composed of a double layer of phospholipids that is linked to inner membrane by lipopolysaccharides (LPS), is mainly the difference between Gram-negative and Gram-positive bacteria. Small hydrophilic solutes are able to pass through the OM via abundant porin proteins that serve as hydrophilic transmembrane channels, and this is one reason that Gram-negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs (Fig 2.1) [113].

Although cinnamon *C. cassia* EO was the most potent antimicrobial agent among nine tested EOs, however, this EO has been deeply investigated in the world [79, 92] and in Vietnam [164]. In order to find out a new plant from Vietnam having potential of exploited at industrial scale, addition with the promising of the antibacterial activity and the limitation of the research number, *L. cubeba* was selected for further research. For instance, China is the greatest producer of *L. cubeba* EO, it has been widely used in this country for centuries and the price of this EO increased about three times from 2010 to 2011 (from 7.71 USD/kg to 22.41 USD/kg) [39]. To the best of our knowledge, *L. cubeba* was poorly studied in Vietnam. It has potential growth in the waste land, newly cleared fields and in mountain from 100 – 500 m of elevation [39]. In Vietnam, *L. cubeba* plants are distributed many provinces such as Lao Cai, Son La, Cao Bang, Lang Son, Tuyen Quang, Bac Can, Thai Nguyen, Phu Tho, Vinh Phuc, Hanoi, Ninh Binh, Nghe An, Thua Thien Hue, Da Nang, Khanh Hoa, Kon Tum, Gia Lai, Lam Dong. Consequently, Vietnam has a rich potential for exploitation of this plant. In the last two decades, Vietnamese people have been exploiting the fruit of *L. cubeba* to distillate EOs. Hence, some places started to cultivate this plant to secure long-term material. In some districts, there is some area planting *L. cubeba* up to several hectares such as Bao Loc, Di Linh (Lam Dong), An

Khe (Gia Lai), Vinh Thach (Binh Dinh) and Tra My (Quang Nam). For this reason, *L. cubeba* has a great potential to apply as an industrial crop model.

In conclusion, after screening the antibacterial activities of the nine EOs from Vietnam, Litsea cubeba was selected to further investigate the chemical composition diversity, antibacterial activity, mechanism of action and application in aquaculture.

3.2. Chemical compositions and antibacterial activities of *L. cubeba* EO

Since the biological activities and therapeutic effects may vary based on the chemical components of the plant material, determination the chemical compositions diversity and antibacterial activities of *L. cubeba* EO was very important before used.

As reported in the literature, the chemical compositions and antimicrobial activities of different part from *L. cubeba* in China were reported including roots, stems, alabastra (flower buds), flowers, leaves and fruits. Among those, EO from leaves and fruits showed higher antimicrobial activity compared to the others [176]. In addition, the main compounds (citral) and the antibacterial activity of *L. cubeba* fruit EOs from China, India, Taiwan… were reported in some previous researchs [101, 140, 176]. However, the major components of *L. cubeba* leaf EOs were change depends on the origin of the plant and leading to the change in the biological activity. Therefore, our research will focus to investigate the combination effect of *L. cubeba* fruit EO as well as the chemical composition and antibacterial activites diversity of *L. cubeba* leaf EOs.

3.2.1. Synergy study of *L. cubeba* fruit EO and other antibacterial agents

To enhance its antibacterial activity, the *L. cubeba* fruit EO was used in combination with other antibacterial agents. The FIC indices ranging from 0.23 to 1.50 for the combined antimicrobial substances tested were listed in Table 3.4 and Table 3.5. Overall, no antagonistic effect was observed for any of the tested combination. The FIC values showed that the antimicrobial agents provided a synergistic effect against the majority of tested microorganisms. In fact, out of 22 EO-EO, and antibiotic-EO pairs tested, 15 of them showed synergistic effect ($FIC \leq 0.5$), 3 of them showed addition effect ($0.5 < FIC \leq 1.0$) and 4 of them showed indifference effect ($1.0 < FIC \leq 4.0$) (Table 3.4, Table 3.5).

Table 3.4: FIC values and the combinations effects of L. cubeba fruit EO and other EOs against pathogenic bacterial

Strains	Antimicrobial agents	MIC (mg/mL)		FIC	Sum FIC	Interaction
		Alone	Combination			
V. parahaemolyticus ATCC 17802	*L. cubeba*	5.53	0.69	0.13	0.38	Synergy
	C. cassia	2.21	0.55	0.25		
	L. cubeba	5.53	0.69	0.13	0.25	Synergy
	C. ambrosioides	12.34	1.54	0.13		
	L. cubeba	5.53	5.53	1.00	1.25	Indifference
	M. leucadendron	11.28	2.82	0.25		
E. coli ATCC 25922	*L. cubeba*	5.53	0.69	0.13	0.25	Synergy
	C. cassia	5.54	0.69	0.13		
	L. cubeba	5.53	0.69	0.13	0.25	Synergy
	C. ambrosioides	6.17	0.77	0.13		
	L. cubeba	5.53	2.77	0.50	1.50	Indifference
	M. leucadendron	11.28	11.28	1.00		

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).

Among nine EOs tested in our study, *C. cassia*, *C. ambrosioides*, *M. leucadendron* and *L. cubeba* showed higher antibacterial activity than the others. The combination of *L. cubeba* with *C. cassia* or *C. ambrosioides* showed synergistic effects whereas the combination of *L. cubeba* with *M. leucadendron* lead to the indifference effect against both of tested bacteria. The results obtained against *E. coli* and *V. parahaemolyticus* demonstrated the occurrence of synergism since *C. cassia* and *C. ambrosioides* EO enhanced the action of *L. cubeba* at a lower dose (0.69 mg/mL) compared its use alone (5.53 mg/mL). However, the MIC values of *L. cubeba* were maintained or decreased only two-fold in combination with *M. leucadendron* compared it used alone against *V. parahaemolyticus* and *E. coli*, respectively (Table 3.4).

Table 3.5: FIC values and combinations effects of L. cubeba fruit EO and antibiotics against pathogenic bacteria

Strains	Antimicrobial agents	MIC (mg/mL)		FIC	Sum FIC	Interaction
		Alone	**Combination**			
V. parahaemolyticus ATCC 17802	*L. cubeba*	5.53	0.69	0.13	0.23	Synergy
	Nalidixic acid	$2.67.\ 10^{-3}$	$0.27.\ 10^{-3}$	0.10		

	L. cubeba	5.53	0.61	0.11	0.28	Synergy
	Oxytetracycline	2.67. 10^{-3}	0.45. 10^{-3}	0.17		
Vibrio sp. 2S4	*L. cubeba*	1.15	0.77	0.67	1.17	Indifference
	Nalidixic acid	64.0. 10^{-3}	32.0. 10^{-3}	0.50		
	L. cubeba	1.15	0.96	0.83	1.33	Indifference
	Oxytetracycline	16.0. 10^{-3}	8.0. 10^{-3}	0.50		
Vibrio sp. 2N38	*L. cubeba*	2.30	0.97	0.42	0.92	Addition
	Nalidixic acid	53.3. 10^{-3}	26.7. 10^{-3}	0.50		
	L. cubeba	2.30	0.76	0.33	0.83	Addition
	Oxytetracycline	8.0. 10^{-3}	4.0. 10^{-3}	0.50		
V. parahaemolyticus ND201	*L. cubeba*	1.67	0.22	0.13	0.30	Synergy
	Nalidixic acid	8.0. 10^{-3}	1.4. 10^{-3}	0.17		
	L. cubeba	1.67	0.32	0.19	0.44	Synergy
	Oxytetracycline	16.7. 10^{-3}	4.2. 10^{-3}	0.25		
V. parahaemolyticus TB81	*L. cubeba*	1.75	0.37	0.21	0.38	Synergy
	Nalidixic acid	9.3. 10^{-3}	1.6. 10^{-3}	0.17		
	L. cubeba	1.75	0.19	0.11	0.32	Synergy
	Oxytetracycline	16.0. 10^{-3}	3.4. 10^{-3}	0.21		
E. coli ATCC 25922	*L. cubeba*	5.53	0.44	0.08	0.33	Synergy
	Nalidixic acid	4.0. 10^{-3}	1.0. 10^{-3}	0.25		
	L. cubeba	5.53	1.38	0.25	0.40	Synergy
	Oxytetracycline	1.67. 10^{-3}	0.25. 10^{-3}	0.15		
E. coli 9C48	*L. cubeba*	1.38	0.08	0.06	0.14	Synergy
	Nalidixic acid	16.0. 10^{-3}	1.3. 10^{-3}	0.08		
	L. cubeba	1.38	0.08	0.06	0.14	Synergy
	Oxytetracycline	32.0. 10^{-3}	2.6. 10^{-3}	0.08		
E. coli 11C123	*L. cubeba*	2.30	0.58	0.25	0.50	Synergy
	Nalidixic acid	21.3. 10^{-3}	5.3. 10^{-3}	0.25		
	L. cubeba	2.30	0.97	0.42	0.92	Addition
	Oxytetracycline	21.3. 10^{-3}	10.7. 10^{-3}	0.50		

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).

Various bacterial species were tested to evaluate the combination effects of *L. cubeba* EO and an antibiotic (OTC or nalidixic acid). The results obtained will be used for

treating antibiotic resistant bacteria in aquaculture. MIC values ranging from 1.15-5.53 mg/mL; 1.7-32.0 μg/mL and 2.67-64.0 μg/mL for *L. cubeba*, OTC and nalidixic acid against *Vibrio* spp. and *E. coli* (2 references strains and 6 isolated strains), respectively. For both reference strains, a concentration of 1.7 and 4.0 μg/mL antibiotics could inhibit the growth of *E. coli* and *V. parahaemolyticus*, respectively. Whereas, a 16 times and 6 times higher concentrations were needed to inhibit the same population of isolated strains. Interestingly, *L. cubeba* fruit EO had higher inhibitory effect against isolated strains than reference strains. Indeed, the MIC values were lower 2.4-4.8 times (corresponding to 1.15-2.30 mg/mL) compared to MIC values (5.53 mg/mL) against reference strains (Table 3.5).

A majority of the combinations of *L. cubeba* fruit EO and nalidixic acid or OTC provided a synergistic effect against tested bacteria. The best combination effect was found between *L. cubeba* fruit and nalidixic acid or OTC against *E. coli* 11C123, for which FIC values were 0.14 and showed a clear synergistic effect (MIC reduced by 12 to 17 fold). A considerable 4 to 10 times and 4 to 13 times reduction in the MIC of the antibiotics and *L. cubeba*, were observed in the other combinations with FIC indexes ranging from 0.23 to 0.50, respectively (Table 3.5).

Generally, a higher amount of antibiotic was required when treating bacterial resistance to antibiotics. However, the effect of EOs against isolated bacteria or resistant bacteria was variable. In our study, *L. cubeba* showed higher antibacterial effect against isolated than reference strains. The inhibitory effect of clove *S. aromaticum* and citronella *C. nardus* against references and isolated bacteria were reported [177]. Indeed, higher MIC values ranging from 0.015-0.062 μg/mL were found against 32 bacterial strains including *Vibrio* spp., *Edwardsiella* spp., *Aeromonas* spp., *E. coli*, isolated from aquatic animals compared to reference strains (MIC = 0.015 μg/mL). Otherwise, the higher inhibitory effects were observed when comparing the MIC values of *C. cassia, C. verum, Origanum compactum, Thymus capitatus* and *T. vulgaris thymoliferum* against *L. monocytogenes*, *S.* Typhymurium and *E. coli* isolated from meat than references strains [107].

The interaction between two antimicrobial agents can produce four possible types of effects: indifferent, additive, antagonistic, or synergistic effects. Combinations of *L. cubeba* and antimicrobial agents (EO, antibiotic) were synergistic for the majority, then followed by indifferent and additive effects. Several studies reported the combined antimicrobial effect of EOs. For instance, oregano *O. vulgare* in combination with thyme *T. vulgaris* EOs possessed indifferent effect against *E. coli*, *L. monocytogenes*, *P.*

aeruginosa, and additive against *P. fluorescens* and *L. innocua* [72, 73]. Lv et al. (2011) found additive effect of the combination of oregano *O. vulgare* and basil *O. basilicum* against *E. coli* [102]. *Lippia multiflora, M. piperita* and *O. basilicum* in pair combination showed synergistic effect regarding the inhibition of *E. coli* with FIC values ranging from 0.19-0.29 [23]. The combinations of *C. citratus* and *C. giganteus* exerted synergistic, additive and indifferent antibacterial effects against *S. aureus, S. typhimurium*; *E. coli* and *E. faecalis,* respectively [22].

In many cases, natural antimicrobial compounds from plants possess relatively weak activity, compared to synthetic antibiotics. In our study, the combination effect of EO with antibiotic were investigated. Several studies have described various interaction effects for antibiotics and EOs. Lemongrass *C. citratus* EO and its main component, citral, combined with streptomycin and kanamycin exhibited synergistic or additive effect (FIC = 0.28-1.00) against *S. typhimurium* [149]. Oregano *O. vulgare* EO in combination with doxycycline, florfenicol or sarafloxacin possesed synergistic effects against *E. coli* isolated from chickens (FIC=0.38–0.5) [150]. Shiraz oregano (*Zataria multiflora*) EO showed synergy with vancomycin against Methicillin-sensitive *S. aureus* (MSSA) and 12 clinical isolates of Methicillin-resistant *S. aureus* (MRSA) [103]. Tea tree *M. alternifolia* EO combinations with gentamicin or tobramycin had a synergistic effect against *E. coli* and *S. aureus* [47]. The author suggested that aminoglycosides antibiotics inhibit protein synthesis and tea tree EO damages the cytoplasmic membrane of bacteria; possibly this combination possessed multi-target synergy [96].

The decrease of MIC values was observed when they were used in combination and this could be explained by the presence of various antimicrobial components. Three hypothesis could be proposed to explain the synergistic effect between 2 antimirobial agents against a microorganism: (i) the antibacterial mechanism 2 antimicrobial agents might be different; they act on the different targets of bacteria; (ii) the synergistic effect could be due to the similarity of their mechanism; and (iii) the synergistic effect occurs only when they inhibit together bacteria [22].

OTC, an important member of tetracyclines, is a broad-spectrum antibiotic that acts inhibit bacterial protein synthesis by binding to the 30S subunit [42]. Whereas, nalidixic acid inhibit a subunit of DNA gyrase [52]. In addition, *L. cubeba* fruit EO destroyed the *E. coli* cell membrane with the presence of manys holes and gaps on the treated cells [100]. It seems likely that the synergistic effects of *L. cubeba* EO and antibiotics (OTC or nalidixic

acid) might be due the fact that EO disintegrated the inner and outer membrane of *E. coli*, making it easier for antibiotics to enter the cytoplasm and inhibit proteins or DNA synthesis, leading to the death of the cells. Since many EO or EO components affected the cell membranes of bacteria and antibiotics have specific targets for protein or DNA synthesis, 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 enhanced the diffusion of other antimicrobials [170].

The efficacy of combinations appears to be related to chemical composition of combined EOs and to possible interactions between their major components. As reported in the literature, combination of EOs possessing compounds with similar structures may exhibit additive rather than synergistic effect. Antagonistic effect has been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons [22]. Carvacrol and thymol in combination with antibiotic expressed more synergistic effect than eugenol against drug-resistant strains of *S.* Typhimurium, *E. coli* and *S. aureus* [122]. The combination could be increased the ingression of antibiotics via permeabilized membranes and/or inhibition of protective enzymes [122]. This difference in performance may be due to the ring of delocalized electrons present in carvacrol and thymol but lacking in eugenol, which is purported to assist in the positioning in the hydrophobic membranes.

Zhou et al. (2007) proposed two hypotheses to explain synergistic effects of cinnamaldehyde with carvacrol or thymol (phenolic coumpounds) against bacteria (i) Thymol or carvacrol could increase the permeability of the cytoplasmic membrane, and probably enable cinnamaldehyde to be more easily transported into the cell. (ii) Thymol or carvacrol could increase the number, size or duration of existence of the pores created by the binding of cinnamaldehyde to proteins in the cell membrane, so that a synergistic effect is achieved when these two components are used in combination [190]. Therefore, cinnamaldehyde, which contains a prop-2-enal side group to the benzene ring, was synergistic with fewer of the antibiotics than the phenols components [122], which could provide some initial indications on the mode of action of these EO components. The combination of the oxide 1,8-cineole with aromadendrene (hydrocarbons) or limonene (hydrocarbons) were found to have additive and synergistic effects, respectively [22].

The present study demonstrated the potential of the combination of L. cubeba fruit EO and other antimicrobial agents (EO, antibiotic) to increase antibacterial activity effect. Most of

pair combination showed synergistic effect and the best synergistic effect was 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 antibiotics as well as EOs in the future therapy. The results obtained could be a potential and promising application for sustainable therapy in aquaculture.

3.2.2. Chemical composition diversity of *L. cubeba* leaf EOs

The samples and their location of *L. cubeba* leaves from seven provinces of North Vietnam are listed in Table 3.6 and Fig 3.1.

Table 3.6: List of L. cubeba leaf EOs samples collected

Location	Code	Collection date	GPS	EO yield % (v/w)	Altitude sea level (m)
Ha Giang	HG 01	February 2015	22°48 N 105°1' E	0.500	1064
	HG 11			0.600	
Yen Bai	YB 03	November 2014	21°43' N 104°54' E	0.100	274
	YB 04			0.010	
	YB 05			0.013	
	YB 07			0.013	
	YB 09			0.200	
	YB 10			0.300	
	YB 12	August 2014		0.300	
	YB 16			0.300	
Tam Dao	TD 18	August 2014	21°25' N 105°34' E	0.700	914
	TD 21	October 2014		0.300	
	TD 26			0.050	
Thai Nguyen	TN 08	February 2015	21°41' N 105°30' E	0.008	140
Phu Tho	PT 13	January 2015	21°24' N 105°4' E	0.250	28
	PT 15			0.100	
	PT 20			0.150	
	PT 22			0.200	
	PT 23			0.150	
Lao Cai	LC 17	October 2014	22°18' N 104°10' E	0.800	1140
	LC 19			1.500	

	LC 24			0.040	
Ba Vi	BV 06	November 2014	21°5' N 105°23' E	0.025	336
	BV 25			0.700	
	BV 27			0.700	

All samples were harvested during the pre-flower phase, from August 2014 to February 2015. During this phase, the plant grows faster and the yield of leaves EOs is the highest. A total of 25 samples from seven provinces (Fig 3.1) was collected including Lao Cai (n=3), Thai Nguyen (n=1), Tam Dao (n=3), Ba Vi (n=3), Phu Tho (n=5), Ha Giang (n=2), Yen Bai (n=8) (Table 3.6). The EOs yield of 25 samples ranged from 0.008 – 1.500% w/w (Table 3.6).

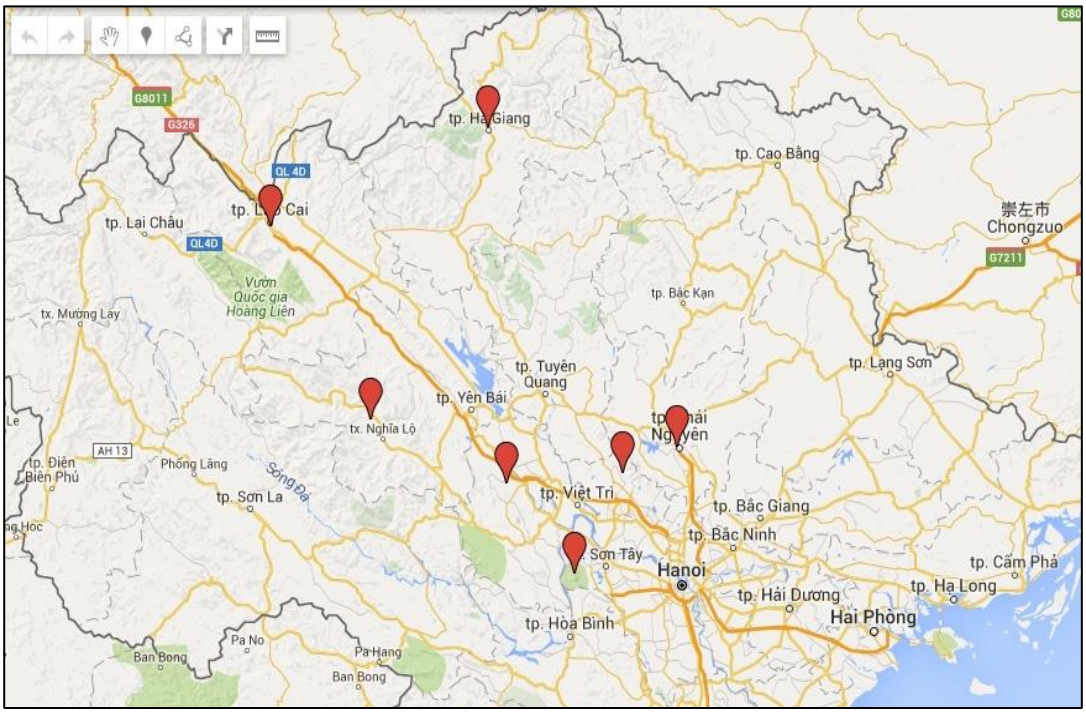


Figure 3.1: Map for sample collections

The chemical composition of 25 samples of EOs isolated from *L. cubeba* leaves is presented in Table 3.7 (Annex Fig. 6). A total of 49 individual compounds (14 monoterpenes, 13 oxygenated monoterpenes, 9 sesquiterpenes, 5 oxygenated sesquiterpenes and 8 non-terpene compounds) were identified while two monoterpenes and one sesquiterpene were unknown. The identity of the subclasses of the three unknown compounds was based on their mass-spectral fragmentation patterns in addition to their retention indices. The high content of 1,8-cineole or linalool (oxygenated monoterpenes) of EOs were detected. Indeed, the samples collected in Tam Dao (TD), Phu Tho (PT), Ba Vi

(BV) yielded EOs with a large amount of linalool 91.97; 94.93 and 93.98%, respectively (corresponding to 863.3; 877.8 and 861.9 g/L) (Fig 3.2). The main minor compounds such as D-limonene (1.41-2.52%), isocaryophyllene (0.85-2.46%) and α-terpineol (0.40-0.62%) accounted for less than 10% of the mixture. The others minor compounds were present at a range of 0.02 to 0.58%.

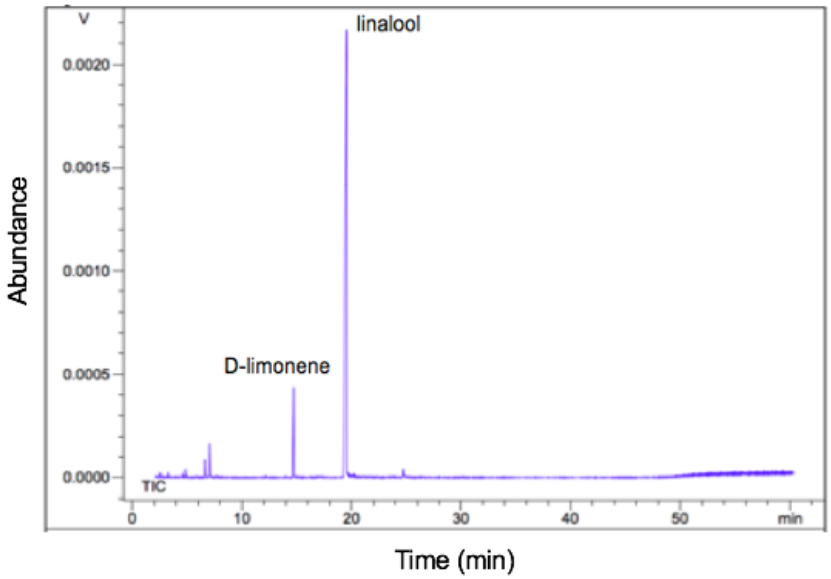


Figure 3.2: Chromatography of L. cubeba leaf EO BV27

On the other hand, the composition of samples from Ha Giang (HG), Yen Bai (YB) and Lao Cai (LC) was dominated by 1.8-cineole accounting for 48.09; 48.00 and 49.58%, respectively (Fig. 3.3). The others main compounds in these 3 locations were sabinene (14.29-15.38%), α-terpineol (9.65-13.71%), α-pinene (4.81-5.60%) and β-pinene (4.54-4.02%).

The data on the 25 *L. cubeba* leaf EO compositions were also examined using principal component analysis (PCA) (Fig. 3.4). Together the first two principal components represented more than 92% of the data set variance and showed four discrete clusters, as labelled in Fig. 3.4. The clusters 1 - linalool type EOs - included PT13, PT15, PT20, PT22, PT23, BV06, BV25, BV27, TD18, TD21, TD26. The clusters 2 - 1,8-cineole type EOs including HG01, HG11, YB03, YB04, YB05, YB07, YB09, YB10, YB12, YB16, LC17, LC19, LC24. In addition, two others cluster from Yen Bai region (YB05) and Thai Nguyen region (TN08) were found in our study with high amount of linalool and m-cymene or 1,8-cineole, respectively. The leaf EOs of *L. cubeba* analysed in this work can thus be classified as “1,8-cineole-type” or “linalool-type”.

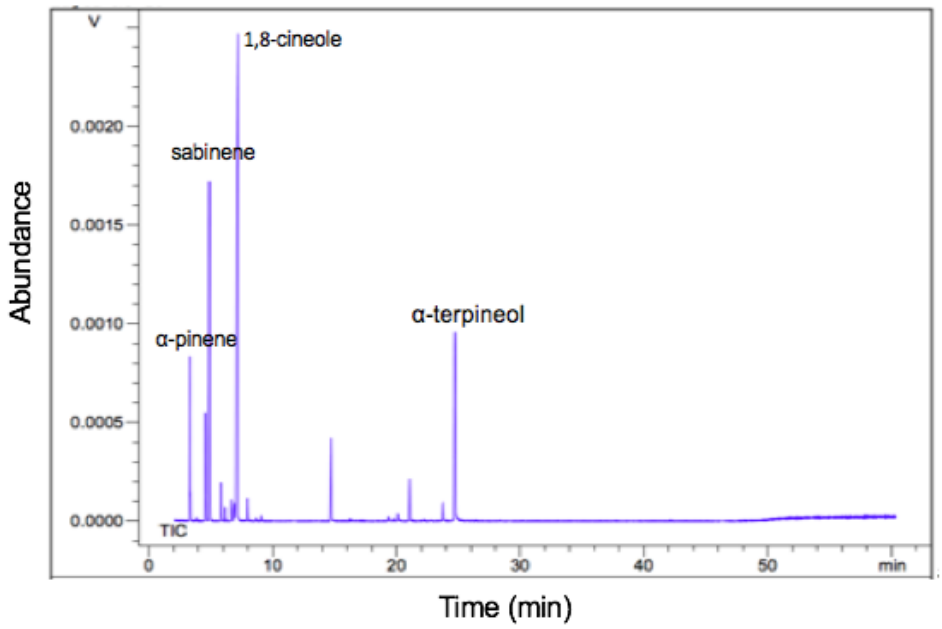


Figure 3.3: Chromatography of L. cubeba leaf EO LC19

Previous studies showed that *L. cubeba* EO can be extracted from different part of the plant, as flower, fruit and leaves have a higher yield of EOs [176]. The results obtained in our study (0.008-1.5%) was lower than thoese obtained in other research [159, 176]. For example, the yield of EOs leaves of *L. cubeba* was 1.3% in China [176]; 2.76-9.33% in Indonesia [159] and 0.3-6.6% in Vietnam [26]. The reported variations in the yields of EOs of *L. cubeba* and other botanically identical plant species such as *R. officinalis* L., *S. officinalis* L., and *Eucalyptus globulus* have been reported [94, 187]. This variations could be attributed to various interactions factor such as genetic, physiological state of the plant, geographical factors as well as the external environment of the growing plants [94].

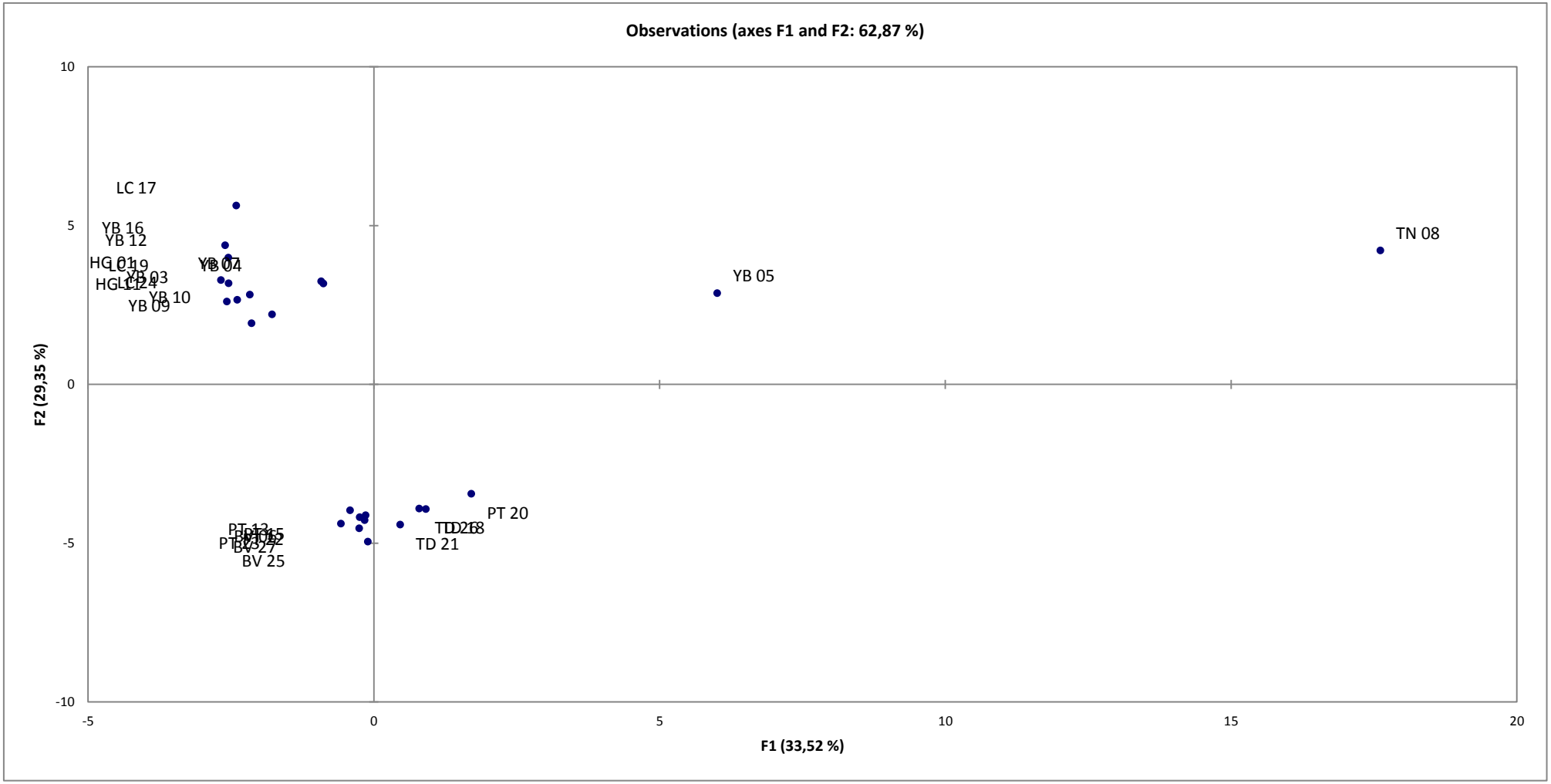


Figure 3.4: PCA of chemical compositions of L. cubeba leaf EOs (n=25)

Table 3.7: Chemical composition of L. cubeba leaf EOs samples from different provinces of North Vietnam

Class/compound	RI (Exp)	RI (Lit)	Ha Giang (%, n=2)	Yen Bai (%, n=8)	Lao Cai (%, n=3)	Thai Nguyen (%, n=1)	Phu Tho (%, n=5)	Ba Vi (%, n=3)	Tam Dao (%, n= 3)
Monoterpene hydrocarbons									
α-pinene	1022	1027	5.38±0.19	4.81±0.73	5.60±0.04	0.68	0.12±0.08	0.19±0.04	0.25±0.02
camphene	1067	1075	0.14±0.00	0.12±0.05	0.14±0.02	0.08	0.03±0.05		
β-pinene	1112	1113	4.45±0.07	4.02±0.40	4.54±0.06	0.76	0.12±0.07	0.16±0.05	0.14±0.01
sabinene	1118	1123	**15.38±0.08**	**14.29±0.97**	**15.13±0.38**	2.34	0.02±0.04	0.37±0.09	0.09±0.01
3-carene	1158	1148		0.04±0.11			0.02±0.05		
myrcene	1184	1176	1.50±0.02	1.55±0.18	1.72±0.04	0.34	0.58±1.29	0.07±0.06	0.13±0.01
α-terpinene	1186	1178	0.37±0.01	0.45±0.19	0.71±0.12	0.18			
D-limonene	1199	1201	3.70±0.03	1.85±0.51	1.57±0.34	1.41	1.52±1.00	1.41±0.36	2.52±0.48
β-phellandrene	1207	1209	1.16±0.01	0.89±0.44	0.44±0.76	0.25			
cis-β-ocimene	1231	1234		0.22±0.19	0.06±0.10	0.48	0.17±0.12	0.16±0.06	0.32±0.03
γ-terpinene	1237	1238	0.70±0.03	0.79±0.33	1.18±0.16	0.43			
trans-β-ocimene	1244	1242		0.10±0.06	0.05±0.09	0.17	0.02±0.05		0.08±0.07
m-cymene	1257	1267	0.09±0.01	0.59±0.96	0.15±0.02	0.18			
α-terpinolen	1269	1275	0.16±0.00	0.26±0.13	0.31±0.04	0.15			
Oxygenated monoterpenes									
1,8-cineole	1215	1213	**48.09±1.61**	**48.00±4.96**	**49.58±2.38**	**13.69**		1.72±0.89	0.36±0.00
cis-sabinene hydrate	1470	1465	0.39±0.09	0.27±0.13	0.21±0.10				0.04±0.08
linalool	1557	1551	2.02±1.52	2.10±2.40	0.30±0.04	**50.08**	**94.93±2.41**	**93.98±1.43**	**91.97±1.06**
terpinen-4-ol	1601	1593	1.48±0.00	2.28±0.29	2.50±0.23	1.50			
β-cyclocitral	1607	1598				0.13			
β-terpineol	1638	1646		0.05±0.05	0.11±0.01		0.24±0.54		
terpenes unknown	1681		1.11±0.03	0.84±0.37	1.09±0.02	0.70			
neral	1683	1690					0.03±0.06		
α-terpineol	1708	1711	**13.35±0.40**	**9.65±4.12**	**13.71±0.47**	5.74	0.54±0.31	0.62±0.32	0.40±0.03
geranial	1736	1744		0.03±0.05		0.42	0.03±0.06		
citronellol	1780	1786				0.21			

isogeraniol	1810	1812			0.05±0.08	0.37	0.04±0.08		
geraniol	1859	1862		0.01±0.04		0.47	0.08±0.18		0.10±0.09
Sesquiterpenes									
β-elemen	1569	1570	0.10±0.02	0.17±0.04	0.14±0.02	0.42			
isocaryophyllene	1578	1570	0.42±0.09	1.30±1.62	0.68±0.11	**10.37**	1.27±0.94	0.85±0.34	2.46±0.09
aromandendrene	1593	1600				0.17			
α-caryophyllene	1652	1663		0.09±0.23		1.05	0.04±0.08		0.17±0.02
γ -selinene	1661	1682		0.06±0.18		0.60			
γ-muurolene	1687	1681				0.33	0.03±0.06		0.32±0.16
β-selinene	1694	1711		0.04±0.13		0.41			
germacrene D	1719	1722		0.06±0.11		0.17			
α-gurjunene	1745	1760		0.04±0.12		1.05			
Oxygenated sesquiterpenes									
sesquiterpenes unknown	1950	1962		0.03±0.08		0.08			
caryophyllene oxide	1957	1962		0.15±0.42		1.10			0.06±0.10
humulene oxide	2008	2015		0.03±0.07		0.12			
nerolidol	2030	2054		0.02±0.05		0.18			
juniper camphor	2198	2205		0.30±0.68	0.03±0.05	2.51	0.08±0.11	0.05±0.09	0.29±0.07
Others									
ethanol	936	929		0.01±0.02			0.04±0.05	0.14±0.12	0.03±0.05
6-methyl-5-heptene-2-one	1328	1319						0.06±0.10	0.15±0.26
2-hexenal. (E)-	1220	1209				0.07			
1-hexanol	1355	1354		0.07±0.08		0.09	0.02±0.04	0.08±0.07	0.07±0.12
3-hexen-1-ol	1382	1386				0.41			
(E)-2-hexen-1-ol	1407	1400		0.07±0.07		0.09	0.05±0.07	0.16±0.19	0.05±0.09
terpenes unknown	1644			0.28±0.52					
terpinyl acetate	1686	1700		4.09±7.57					

RI (Exp): Retention Index Experiment. RI (Lit): Retention Index Literature. Means values ± SD of samples

Great variations have been reported in literature on the chemical profiles of *L. cubeba* leaf EOs sampled from a wide range of geographical locations. Some studies have reported as few as 16 compounds [159] while others have reported over a 65 compounds in *L. cubeba* EOs [140]. Despite these differences, main compounds that have been classified are linalool, 1,8-cineole, sabinene, linalool/citronellal. The major compounds of *L. cubeba* leaf EO found in this study were linalool, 1,8-cineole and sabinene, which is consistent with others studies [26, 140, 159]. *L. cubeba* EOs linalool-rich were found in India (78% linalool) [111] and in Ba Vi – Vietnam (91.1% linalool) [26]. *L. cubeba* EOs 1,8-cineole-rich were found in Yen Bai, Lang Son, Thanh Hoa – Vietnam [26] and Indonesia [159]. In addition, *L. cubeba* EOs sabinene-rich (>50%) were found in Dien Bien – Vietnam [26] and in Dibrugarh, Sibsager, Assam – India [140]. Our findings confirmed the result obtained by Bighelli et al. (2005) for only samples collected from Ba Vi and Yen Bai in North Vietnam [26]. However, others studies have reported different compositions containing either (*Z*)-citral (neral) 32.9%, sabinene 14.2%, linalool 9.5%, limonene 9.2%, terpinen-4-ol 4.0% from Hue province (central region of Vietnam) [155], or a mixture of 1,8-cineole (13.97%), γ-elemene (8.27%), caryophyllene (8.04%), linalool (6.94%), limonene (6.78%) from samples collected in China [176].

Many factors such as the geographical origin, the genetic factors, the plant material and the season of harvesting may be responsible for the variability of the EOs chemical composition [187]. Altitude has been described as one of the most important factors affecting the composition and yield of EOs [106]. It could influence the enzymatic activities in certain plant species and then the biosynthesis of certain secondary metabolites. In our study, 1,8-cineole-rich EOs were mostly found in Ha Giang, Lao Cai and Yen Bai at a higher altitude (1000 m) than linalool-rich samples which were found at a relative low altitude of 300 m in Tam Dao, Ba Vi and Phu Tho (Table 3.6). Similar observations have been reported in the literature for saro *C. fragans* EO from Madagascar [133] and for thyme *Thymus vulgaris* EO from Spain [162].

3.2.3. Antibacterial activity of *L. cubeba* leaf EOs

Table 3.8 shows the antibacterial activities of 8 *L. cubeba* leaf EOs from six regions, and pure 1,8-cineole and linalool against tested strains. The MIC values ranged from 2.8 to 12.3 mg/mL for the four 1,8-cineole type samples (HG01, YB12, LC17, LC19) and from 0.3 to 5.8 mg/mL for the four linalool type samples (TD18, TD21, PT13, BV27).

The most sensitive strains were Gram-negative, *A. hydrophila*, *E. tarda*, *V. furnissii* and *E. coli*. In most cases, considering the ratio MBC:MIC was <4 [147], the majority of EOs exerted a bactericidal effect against tested strains. The 1,8-cineole type samples (YB12, LC17, LC19, HG01) showed a bacteriostatic effect against *S. garvieae*.

MIC and MBC values indicated that leaf *L. cubeba* EO samples had different inhibitory effects against the tested bacteria. In most cases, MIC values were lower against all tested strains than their respective pure major component, linalool or 1,8-cineole, suggesting synergistic effects among the components of EOs. EOs components may exhibit interactions that could lead to either synergistic or antagonistic effects. Lemongrass (*C. flexuosus*) and basil EOs (*O. basilicum*) have been shown to be less effective than their main compound, citral and linalool, respectively [7, 152]. In contrast, other studies have shown that the tea tree oil (*M. alternifolia*) and basil oil were similar effects to those of their major compound, terpinene-4-ol and α-terpineol, respectively or were more efficient than 1,8-cineole [33].

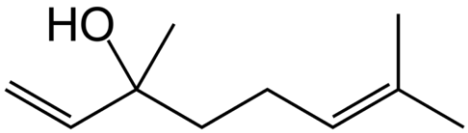
Table 3.8: Antibacterial activity of L. cubeba leaf EOs, 1,8-cineole and linalool against pathogenic bacterial strains

EO	*A. hydrophila*		*B. subtilis*		*E. coli*		*S.* Typhimurium		*V. parahaemolyticus*		*V. furnissii*		*E. tarda*		*S. garvieae*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
1,8-cineole – type																
HG01	3.1^{c}	6.1^{c}	3.1^{abc}	12.2^{b}	6.1^{d}	6.1^{c}	3.1^{a}	24.4^{d}	6.1^{b}	24.4^{c}	3.1^{c}	6.1^{b}	3.1^{c}	3.1^{c}	6.1^{b}	$>48.8^{d}$
YB12	6.2^{d}	6.2^{c}	6.2^{e}	12.3^{b}	3.1^{c}	3.1^{b}	6.2^{b}	24.6^{d}	12.3^{c}	24.6^{c}	6.2^{d}	12.3^{c}	3.1^{c}	3.1^{c}	6.2^{b}	$>49.3^{d}$
LC17	3.0^{c}	3.0^{b}	6.1^{e}	12.1^{b}	3.0^{c}	3.0^{b}	6.1^{b}	12.1^{c}	12.1^{c}	24.2^{c}	6.1^{d}	12.1^{c}	1.5^{b}	3.0^{c}	3.0^{a}	$>48.5^{d}$
LC19	2.9^{c}	2.9^{b}	2.9^{ab}	11.4^{b}	2.8^{abc}	5.7^{c}	2.9^{a}	22.9^{d}	5.7^{ab}	45.7^{d}	2.9^{c}	11.4^{c}	5.7^{d}	5.7^{d}	5.7^{b}	$>45.7^{d}$
Linalool – type																
TD18	0.8^{ab}	0.8^{a}	3.1^{abc}	6.1^{a}	1.5^{ab}	1.5^{a}	3.1^{a}	12.3^{c}	3.1^{ab}	6.1^{a}	0.8^{a}	3.1^{a}	1.5^{b}	1.5^{b}	3.1^{a}	6.1^{c}
TD21	0.7^{ab}	0.7^{a}	2.9^{ab}	5.7^{a}	2.9^{bc}	2.9^{b}	5.8^{b}	11.5^{bc}	2.8^{ab}	5.7^{a}	0.7^{a}	2.9^{a}	0.7^{a}	0.7^{a}	5.7^{b}	5.7^{bc}
PT13	0.7^{ab}	0.7^{a}	2.9^{ab}	5.8^{a}	0.7^{a}	1.4^{a}	2.9^{a}	5.8^{ab}	1.4^{a}	5.8^{a}	0.7^{a}	2.9^{a}	0.7^{a}	1.4^{b}	2.9^{a}	5.8^{bc}
BV27	0.3^{a}	0.3^{a}	2.7^{a}	5.4^{a}	2.7^{abc}	2.7^{b}	5.4^{b}	5.4^{a}	2.7^{ab}	21.8^{c}	1.4^{b}	2.7^{a}	0.7^{a}	0.7^{a}	2.7^{a}	5.4^{b}
1,8-cineole	2.9^{c}	5.8^{c}	23.0^{f}	46.1^{c}	11.5^{e}	11.5^{d}	23.0^{c}	23.0^{d}	46.1^{d}	$>46.1^{d}$	11.5^{e}	11.5^{c}	1.4^{b}	1.4^{b}	5.8^{b}	$>46.1^{d}$
Linalool	1.4^{b}	1.4^{ab}	5.4^{d}	5.4^{a}	5.4^{d}	5.4^{c}	5.4^{b}	5.4^{a}	5.4^{ab}	10.9^{b}	5.4^{d}	5.4^{ab}	1.4^{b}	1.4^{b}	2.7^{a}	2.7^{a}

Values with different letters within a column are significantly different according to Fisher LSD test ($P<0.05$).

In general, EOs contain two distinct groups of chemical constituents: hydrocarbons which are made up almost exclusively of terpenes (monoterpenes, sesquiterpenes, and diterpenes) and the oxygenated compounds (oxygenated terpenoids) which are mainly esters, aldehydes, alcohols, phenols, ethers, ketones and oxides. The main component of *L. cubeba* leaf EO, 1,8-cineole belonging to ethers group and linalool belonging to alcohols group, are well-known for their antibacterial activities (Fig 3.5) [99, 101]. Others minor constituents found in our study (Table 3.7) may contribute to the overall antimicrobial activity, such as α-terpineol, α-pinene [188]. Oxygenated terpenoids such as alcoholic and phenolic terpenes such as eugenol and linalool were reported to have higher antimicrobial activity than the other compounds, ranking the activity as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons [88]. In this context, in agreement with others studies [133, 188], our results demonstrated higher activity for linalool-type (alcohol) compared to 1,8-cineole-type (ether) samples.

1,8-cineole



linalool

Figure 3.5: Structure of 1,8-cineole and linalool

Similar ranges of MIC and MBC values have been reported for EOs containing similar main compounds. For example, *Cinnamomum longepaniculatum* EO, which is rich in 1,8-cineole, showed similar MICs values ranging from 3.13-6.25 μL/mL against *E. coli, S. aureus* and *S.* Enteritidis [99]. In addition, the MIC of linalool-rich EOs from *O. gratissimum* and *O. basilicum* ranged respectively from 0.2-1.6 mg/mL against *A. hydrophila* [158] and 0.8-2.6 mg/mL against *E. coli, B. subtilis* [80]. Reports in the suggest that the activity of *L. cubeba* leaves EO may vary with its composition. *L. cubeba* samples originating from Assam (India), which are rich in sabinene showed lower activity (MIC range 2.5-20 mg/mL) [140] than samples from China (MIC=0.15-1 mg/mL) [176].

In conclusion, this study showed the importance of chemical composition analysis of leaf EOs of L. cubeba from North Vietnam since two main chemotypes, linalool and 1,8-cineole were confirmed. Both EOs were more effective against tested bacteria compared to their

major compound suggesting a synergistic effect between their constituents. Moreover, the linalool-rich type showed higher antibacterial activity than the 1,8-cineole-rich one.

3.3. Antibacterial mechanism of *L. cubeba* leaf EOs

E. coli was selected as the model strain to further investigate the mechanism of action of LC19 (1,8-cineole-rich EO) and BV27 (linalool-rich EO) samples. Because it is a Gram-negative and rod-shaped bacterium and is considered the most important model organism. A model organism is a species that is extensively studied to understand a specific phenomenon, expecting that the knowledge gained can be applied to other species as well. Therefore, the data on cell size, cell cycle, DNA and membrane phenotypes … of *E. coli* was most finely understood.

Additionally, since the antibacterial mechanism of *L. cubeba* fruit EO (containing approximately 70% citral) against *E. coli* were reported for [100], however, the mode of action of *L. cubeba* leaf EOs have never been reported previously, to the best of our knowledge. Due of the above reasons, our research focused to investigate the antibacterial mechanism of *L. cubeba* leaf EOs against *E. coli*.

3.3.1. Effect of *L. cubeba* leaf EOs LC19 and BV27 on viability of *E. coli*

Time-kill experiments were performed using LC19 and BV27 samples on *E. coli*. In both cases, a concentration-dependent reduction of viability was observed (Fig. 3.6 and Fig. 3.7). Notably, after 2 h of incubation with LC19, a reduction of 1.46; 1.94 and 4.49 $\log_{10}$ CFU/mL of *E. coli* was observed at 0.5, 1 and 2 MIC, respectively. Maximal loss of viability was reached after 8 h of incubation, with about 2.12 and 3.29 $\log_{10}$ CFU/mL reduction at 0.5 and 1 MIC, and no colony was detected after 4 h at 2 MIC (Fig. 3.4). In the case of BV27 treated cells, the viability kinetics at 0.5 MIC was similar to that of 1 MIC LC19, with a reduction in viability of 2.16 $\log_{10}$ CFU/mL after 2h of incubation and a plateau reached after 8h. At concentrations of 1 and 2 MIC, no living cells were detected after 2 and 1 h of incubation, respectively (Fig. 3.6).

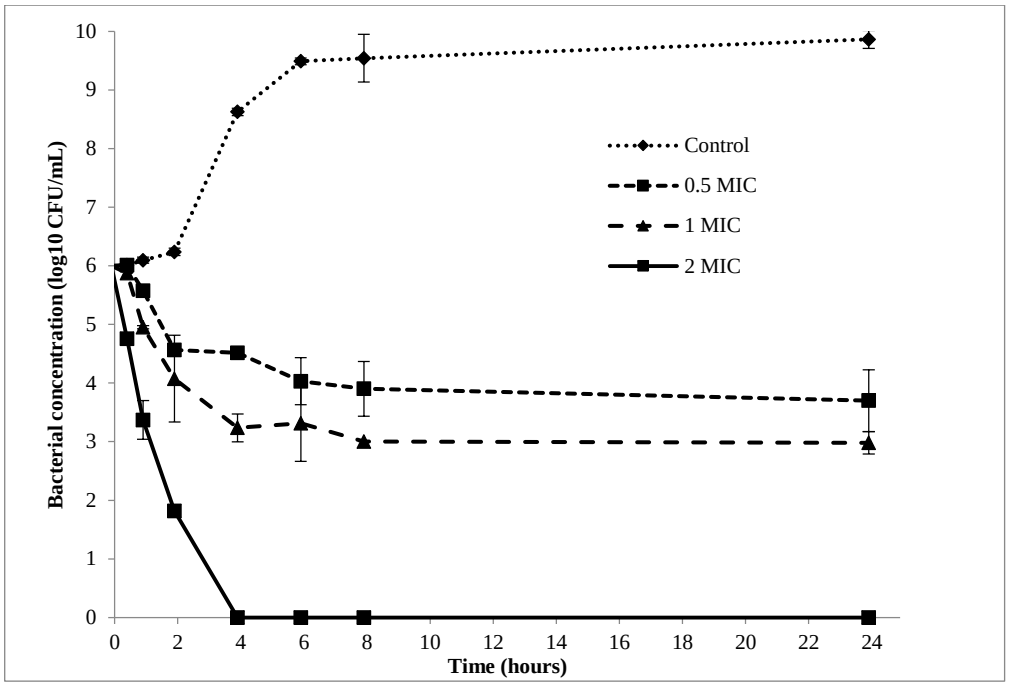


Figure 3.6: Effects of L. cubeba leaf EO LC19 (1,8-cineole-type) on viability of E. coli ATCC 25922

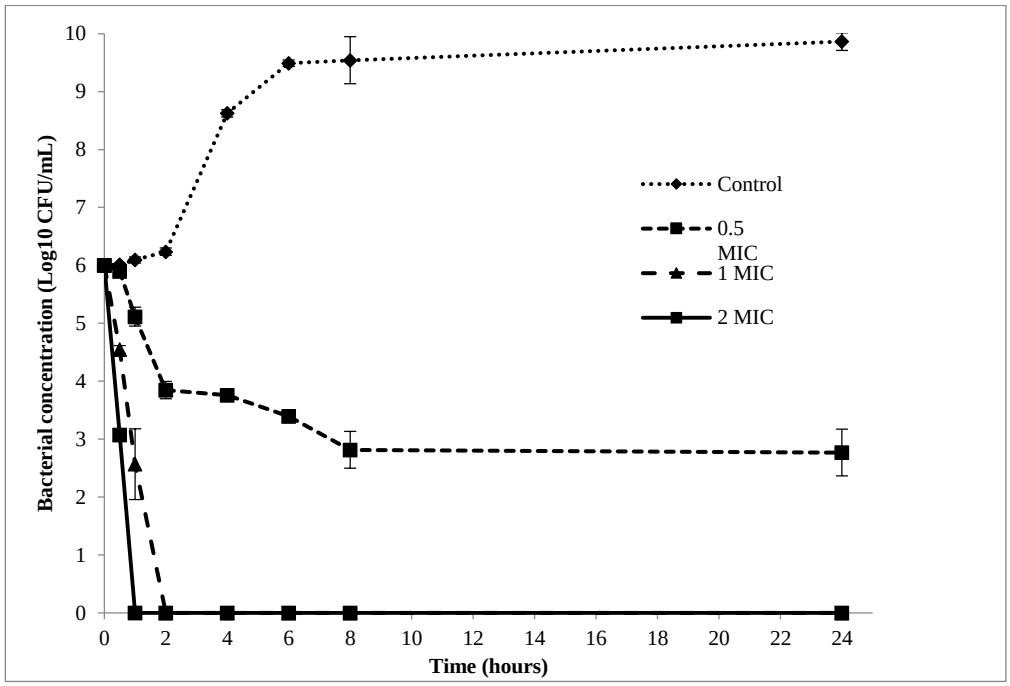


Figure 3.7: Effects of L. cubeba leaf EO BV27 (linalool-type) on viability of E. coli ATCC 25922

Both *L. cubeba* extracts showed a concentration-dependent effect but with apparent different kinetics. Cell viability was also estimated using a LIVE/DEAD BacLight viability kit. A total of 98% of the control cells exhibited green fluorescence (SYTO 9) indicating viable and intact cells throughout the incubation period. Red-stained (PI-positive) cells (about 2%) were occasionally observed in the control cultures, most likely corresponding to senescent bacteria (Fig 3.8, Fig 3.10).

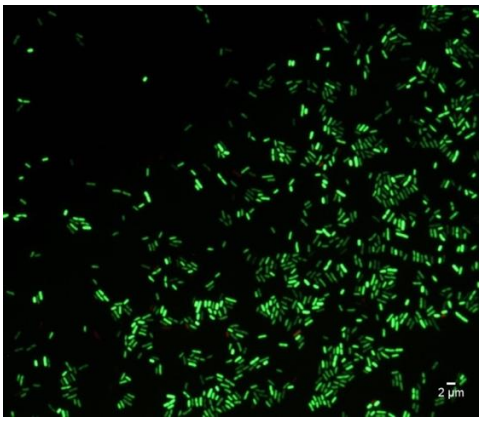
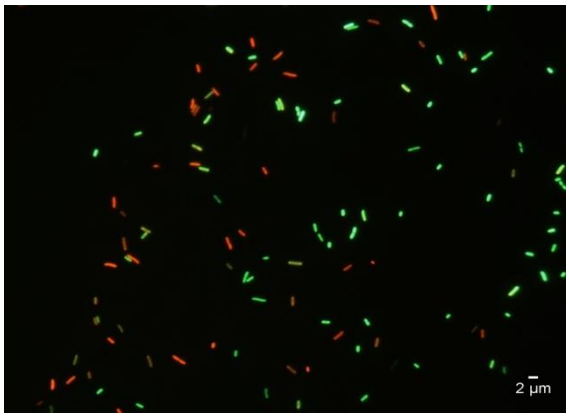
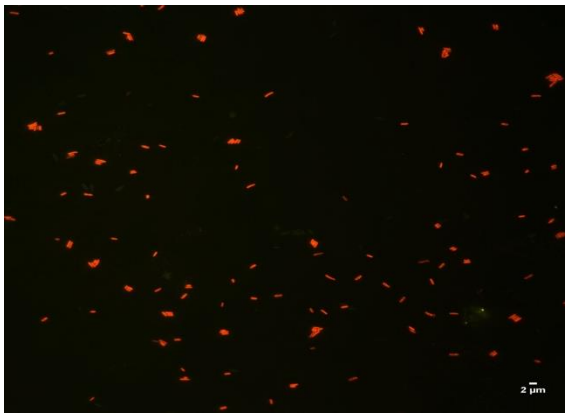


Figure 3.8: Fluorescence microscopic images with LIVE/DEAD Baclight kit of E. coli ATCC 25922 control cells (without EO) after 2 h of incubated. Scale bars represent 2 µm

A decrease in the number of intact cells with a concomitant increase in the number of PI-positive cells (red cells) was observed upon addition of the EO. The percentage of LC19-treated cells positive for PI was 34.57; 56.12 and 97.52% at 0.5, 1 and 2 MIC, respectively (Fig 3.9, Fig 3.10, Table 3.9). In the case of BV27, the percentage of cell membrane damaged after 2 h of exposure was higher than for LC19 ($p<0.0001$). In the presence of BV27 at 0.5 MIC, the percentage of damaged cells reached almost 100% of the total population and 100% at 1 MIC and 2 MIC were labeled with only PI (Fig 3.9, Fig 3.10, Table 3.9). These data are consistent with time-kill experiments described above even if the expected fraction of red cells after 2 h should have been much higher for LC19 at 0.5 and 1 MIC. We cannot exclude the possibility during the course of the experiment (2 h) dead cells lysed and disappear from the counts. Another possible explanation is that the red cells did not correspond to dead cells but rather to dying cells, or cells with compromised membranes. In all, the effect of the two EOs on viability and bacterial cell physiology appear to be different.





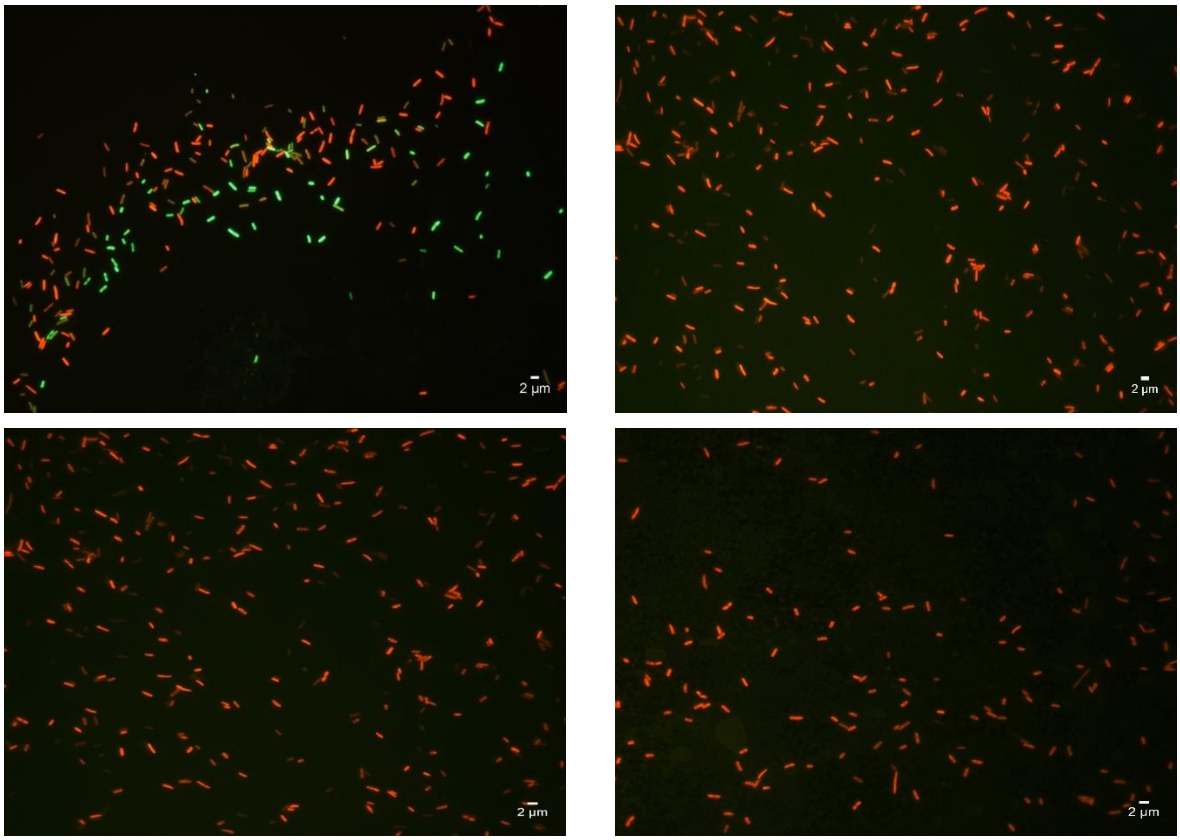


Figure 3.9: Fluorescence microscopic images with LIVE/DEAD Baclight kit of E. coli ATCC 25922 after 2 h of exposure to L. cubeba leaf EOs (LC19 and BV27) at different concentrations

L. cubeba leaf EOs (LC19 left and BV27 right) 0.5MIC top, 1 MIC center and 2 MIC bottom. Viable cells are indicated by green fluorescence, whereas cells with damaged membranes are indicated by red fluorescence. Scale bars represents 2µm

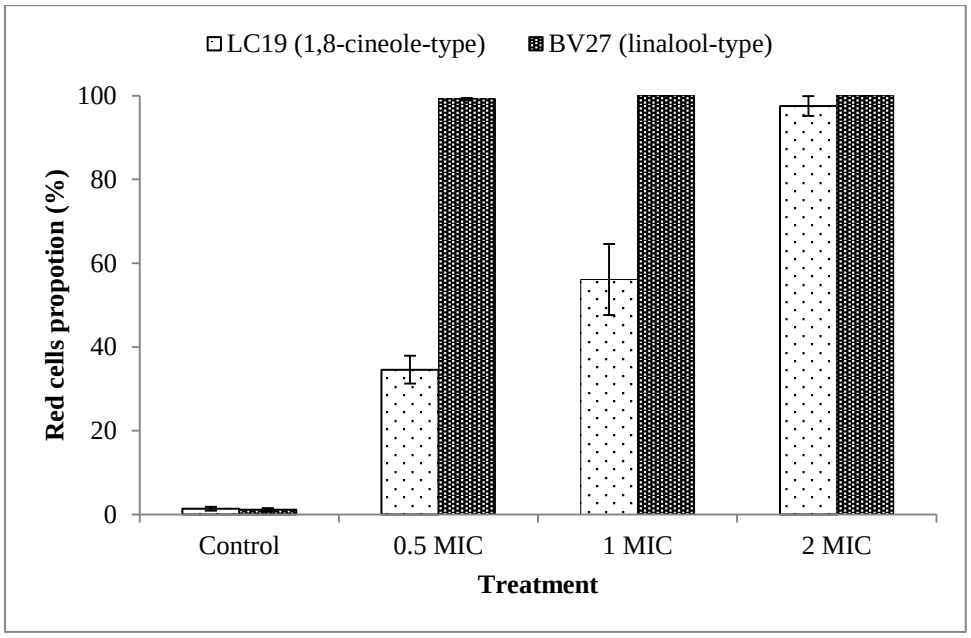


Figure 3.10: Percentage of red-stained (PI-stained) E. coli ATCC 25922 cells after 2 h of exposure with two L. cubeba leaf EOs LC19 (1,8-cineole type) and BV27 (linalool type) at 0.5 MIC, 1 MIC and 2 MIC

3.3.2. Integrity of *E. coli* cell membranes after exposure to *L. cubeba* leaf EOs LC19 and BV27

To test whether EOs could affect membrane integrity, we observed stained membranes of EO-treated and untreated cells using fluorescence microscopy. Upon contact with LC19, a significant fraction of *E. coli* cells showed abnormal membrane staining. This fraction increased together with EO concentration up to about 20% at 2 MIC in a dose-dependent manner (Fig. 3.11, Fig 3.12, Table 3.9). The membrane defects observed corresponded to either an abnormal diffusion of fluorescent dye in the cell membranes or to the presence of non-fluorescent region (holes) in the cell membranes compared to the control cell membranes (Fig. 3.10).

Under BV27 treatment, the cells with visibly altered membrane stain represented 29.99% at 0.5 MIC and increased to 100% at 1 MIC (Fig. 3.11, Fig 3.12, Table 3.9). Notably, specific defects affecting the morphology (up to 2% of cells at 2 MIC) and apparent condensation or cell permeabilization to the dye were observed (Fig. 3.12). Cell permeabilization to the dye enables diffusion of the dye into the cell, thereby reducing the staining contrast between the inner and outer side of the cells. This phenotype was observed for 5; 94 and 95% of the cells treated at 0.5; 1 and 2 MIC respectively (Fig. 3.11, Fig 3.12).

Taken together, our data showed that membrane integrity was affected in cells exposed to both LC19 and BV27 EOs. While only a fraction of LC19-treated cells showed membrane defects, a majority of BV27-treated cells showed defective membrane stain. This suggests that LC19 and BV27 have different mode of action toward *E. coli*, BV27 having a significant effect on membrane integrity and permeabilization.

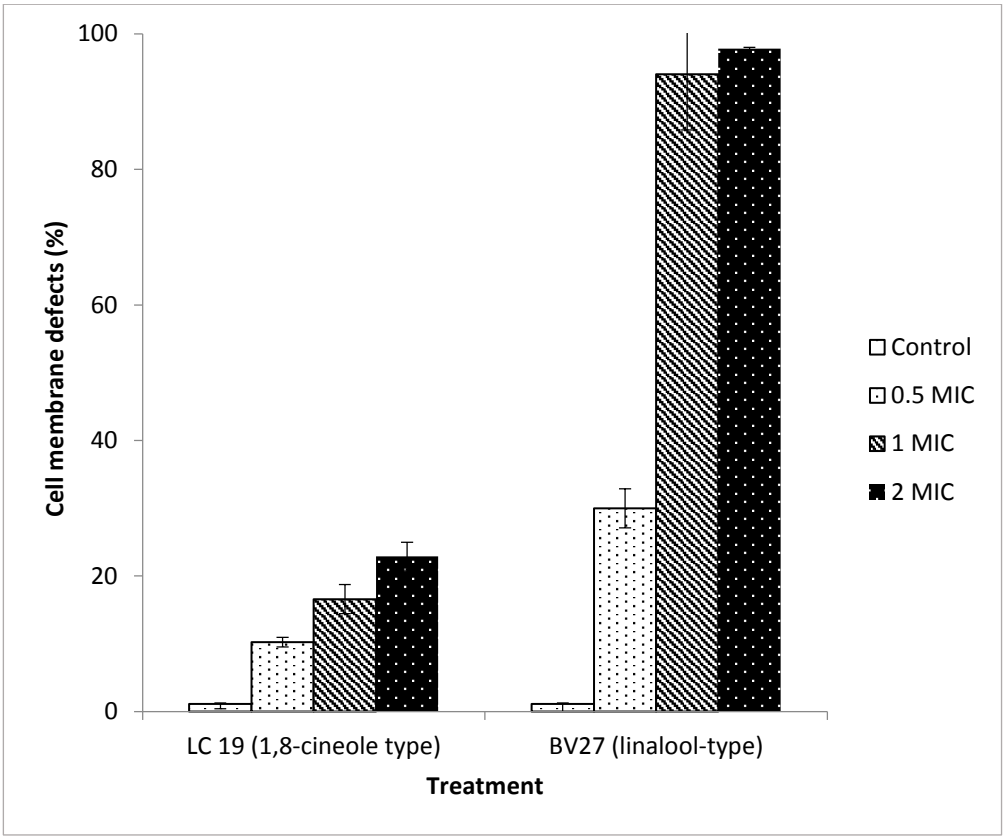


Figure 3.11: Effects of two L. cubeba leaf EOs LC 19 (1,8-cineole type) and BV 27 (linalool type) on E. coli ATCC 25922 cell membranes using FM 4-64 stain

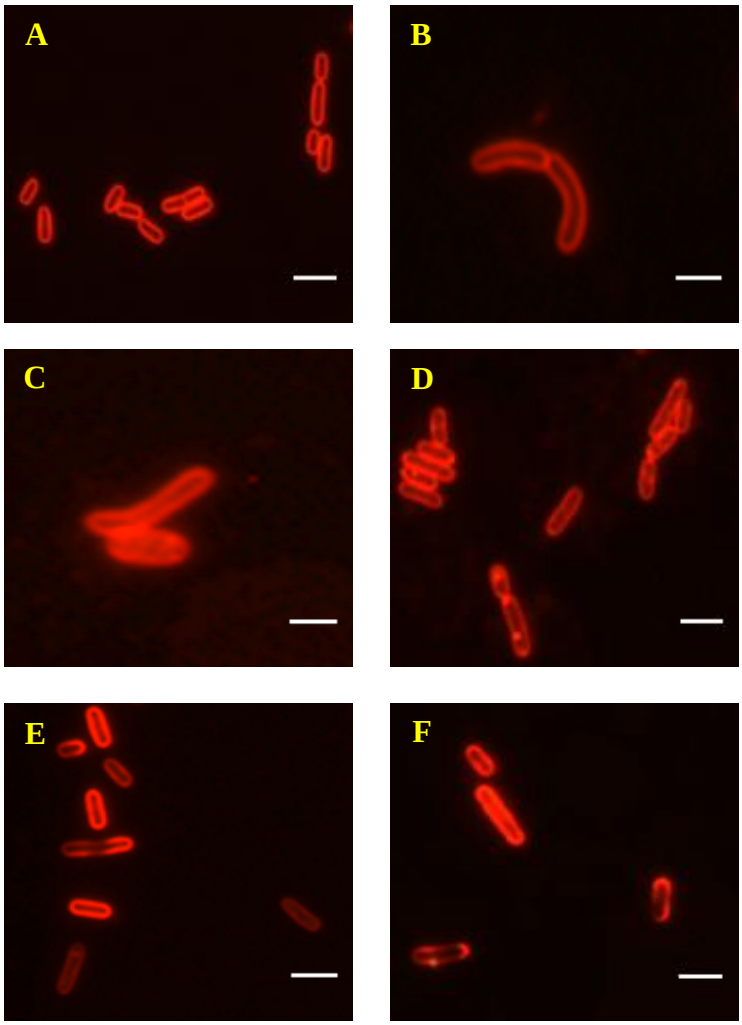


Figure 3.12: Fluorescence microscopic images with FM 4-64 of membrane phenotypes of E. coli ATCC 25922 cells after 2 h of exposure to L. cubeba leaf EOs at different concentrations

(A) Control cells; (B) Change of morphology (0.39; 0.78 and 2.11% at 0.5 ; 1 and 2 MIC of BV27, respectively); (C) Cell permeabilization (5.72; 94.0 and 95.5% at 0.5 ; 1 and 2 MIC of BV27, respectively) wheareas 0.13% of control cell was permeabilized; (D) Condensation of fluorescence dye (22.33; 0 and 0% at 0.5; 1 and 2 MIC of BV27, respectively); (E) Diffusion of fluorescence dye (8.0; 10.9 and 10.33% at 0.5; 1 and 2 MIC of LC19, respectively); (F) Damage cell membrane (1.04; 4.07 and 11.82% at 0.5; 1 and 2 MIC of LC19, respectively). Scale bars represent 2 µm

Table 3.9: Effects of two L. cubeba leaf EOs (LC19 and BV27) on viability, size, membrane and DNA integrity of E. coli ATCC 25922 cells

Treatments		Defect stained cells (%)			Viability reduction (%)	Cell filamentation (%)	Length (μm)	Width (μm)	Length/width
		PI	DAPI	FM4-64					
Control		1.37 ± 0.42^{a}	1.48 ± 0.14^{a}	1.12 ± 0.13^{a}		<0.001^{a}	2.00 ± 0.09^{c}	0.66 ± 0.03^{d}	3.02 ± 0.25^{a}
LC19	0.5 MIC	34.57 ± 3.33^{b}	32.95 ± 1.03^{b}	10.24 ± 0.73^{b}	26.79	1.070^{c}	2.92 ± 0.07^{a}	1.00 ± 0.01^{b}	2.93 ± 0.03^{a}
(1,8-cineole – type)	1 MIC	56.12 ± 8.50^{c}	45.53 ± 3.75^{c}	16.59 ± 2.14^{c}	34.65	1.431^{d}	3.02 ± 0.18^{a}	1.03 ± 0.05^{b}	2.95 ± 0.06^{a}
	2 MIC	97.52 ± 2.38^{d}	81.80 ± 2.63^{d}	22.91 ± 2.06^{d}	80.57	0.138^{ab}	3.04± 0.04^{a}	1.01 ± 0.05^{b}	3.01 ± 0.11^{a}
BV27	0.5 MIC	99.23 ± 0.26^{d}	43.55 ± 2.02^{c}	29.99 ± 2.88^{e}	38.28	0.305^{b}	2.39 ± 0.05^{b}	0.87 ± 0.02^{c}	2.72 ± 0.03^{b}
(linalool – type)	1 MIC	100 ± 0.0^{d}	98.10 ± 0.58^{e}	94.05 ± 8.22^{f}	100	0.035^{a}	2.46 ± 0.05^{b}	1.15 ± 0.01^{a}	2.16 ± 0.03^{c}
	2 MIC	100 ± 0.0^{d}	97.94 ± 1.44^{e}	97.82 ± 0.19^{f}	100	0.000^{a}	2.50 ± 0.04^{b}	1.17 ± 0.01^{a}	2.14 ± 0.01^{c}

Values are expressed in means values ± SD of three different experiments. Values followed by different letters within a column are significantly different by Fisher LSD test ($P<0.05$).

3.3.3. Variation of *E. coli* cell length treated with *L. cubeba* leaf EOs LC19 and BV27

As cell morphology and sizes appeared to be affected by EO treatment, the length and width of the cells were measured on images of treated and untreated cells. The mean length of *E. coli* cells exposed to either LC19 or BV27 increased after 2 h as a function of the concentration of the EO compared with those of the green control cells whose mean length were 2.00 µm ($p<0.0001$) (Fig. 3.13, Table 3.9). Significant cell filamentation (cell lengths >6 µm) was observed under all treatment ($p<0.0001$) except for 1 and 2 MIC BV27. No cell filament was observed in the control populations. A higher percentage of cell filaments was observed at the lethal dose of LC19 treated cells compared to BV27 ones ($p<0.0001$). Strikingly, the percentage of cell filaments decreased according to the concentration of BV27, while it was 40 and 15 times higher for LC19 at 1 and 2 MIC, respectively (Fig. 3.13, Table 3.9). These observations suggest that LC19 and BV27 have different effects on *E. coli* cell cycle.

Under LC19 treatment, the mean cell length increased by about 50% in red cells compared to control red cells (2.00 µm vs 2.92; 3.02; 3.04 µm at 0.5; 1 and 2 MIC, respectively) showing that a fraction of cells (maybe at a specific stage in the cell cycle) were affected (Fig. 3.13, Table 3.9). The length of the green treated-cell increased significantly ($p<0.0001$), slightly more moderately than the red stained cells ($p<0.0001$) (Fig. 3.13). An increase from 36 to 63% of LC19 green treated cells compared to control green cells (1.89 µm vs 2.57; 2.68; 3.09 µm at 0.5; 1 and 2 MIC, respectively (Fig 3.13). Since 2 MIC of LC19 corresponded to the MBC (lethal dose) we can argue that all 2 MIC - green cells underwent the same fate as the red cells. In this regard, cell death could be caused by cell filamentation and lysis.

Under treatment with BV27, mean lengths of red and green cells increased moderately (by about 25%) compared to control cells ($p<0.0001$). There was no significant difference in the lengths of red cells at 0.5; 1 and 2 MIC between the three treatments as mean length were 2.39; 2.46 and 2.50 µm, respectively (Fig. 3.13 and Table 3.9). Almost no green cells were detected among treated cells, even at 0.5 MIC, and their respective mean lengths were significantly higher (3.71 µm vs 2.00 µm than the control cells, p value < 0.0001) (Fig. 3.13).

Considering that MIC and MBC were equal in BV27, all the red cells might be dead. BV27 can induce cell filamentation but to a much lesser extent than LC19.

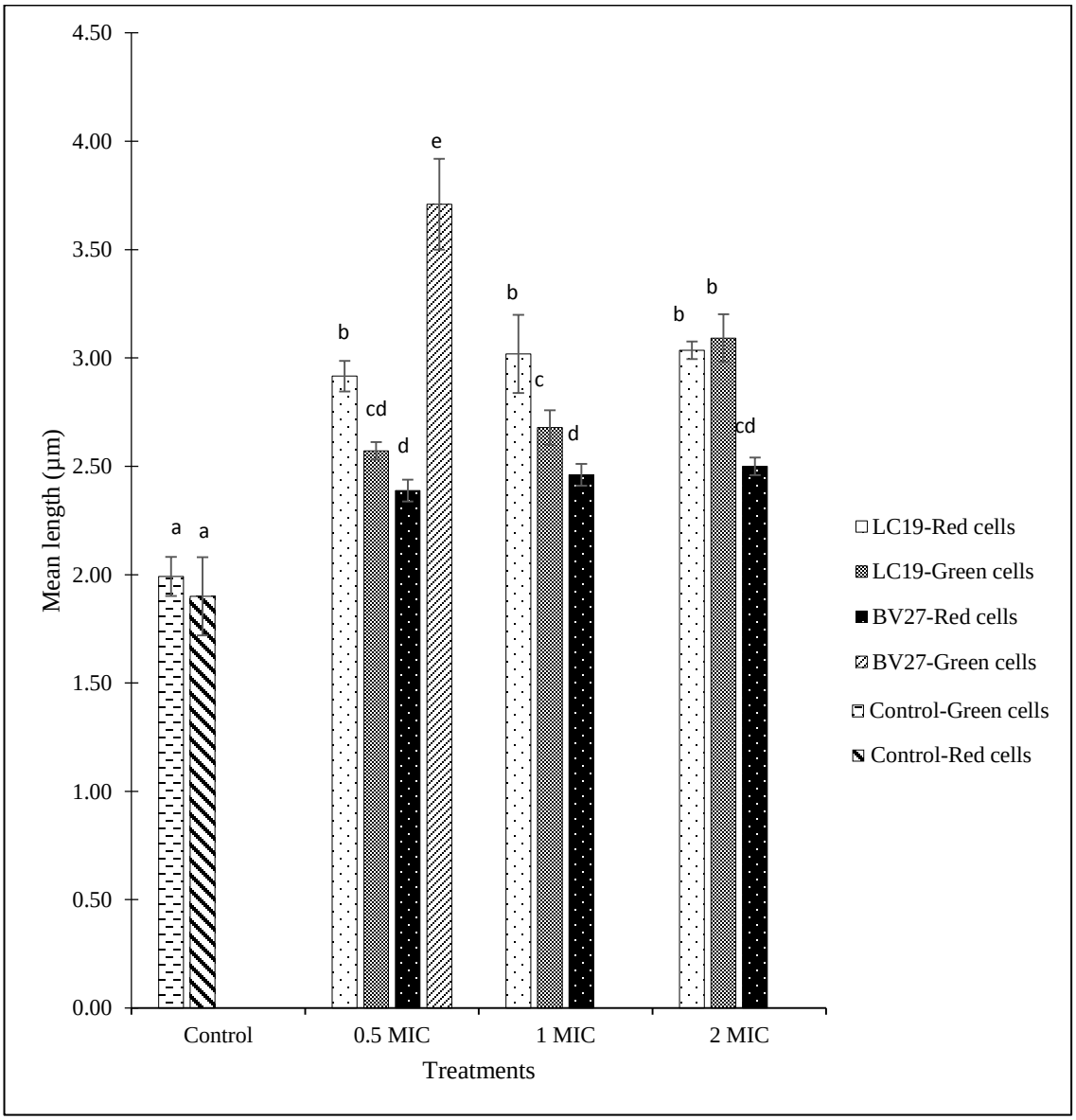


Figure 3.13: Effects of two L. cubeba leaf EOs LC19 (1,8-cineole type) and BV27 (linalool type) on the length of green-stained and red-stained E. coli ATCC 25922 cells after 2 h of exposure

Values are expressed as Means±SD of three different experiments. Values followed by different letters are significantly different arccording to Fisher LSD test (P<0.05)

A significant increase in cell width was observed in cells treated with either EO compared to untreated cells. The cells were slightly swollen (the diameter of treated cells was slightly larger, p value<0.0001). Interestingly, while the ratio cell length:cell width was

maintained in cells treated with LC19, the ratio was significantly higher in cells treated with BV27 (Table 3.9). This could be due to an effect on the cell cycle and/or cell wall synthesis.

3.3.4. Effect of *L. cubeba* leaf EOs LC19 and BV27 on DNA of *E. coli*

DAPI is a fluorescent dye that can penetrate the bacterial cell and integrate with DNA. In control cells, staining with DAPI revealed the DNA of the cells to be a central uniform mass, or nucleoid. During cell division, the nucleoid shape appears transiently bilobed as a result of DNA replication that duplicates the genetic material (chromosome) of the cell. At the end of the cell division process, the divided cells possess two DNA masses or nucleoid located close to the cell poles. In our experiments, 1.48% of untreated cells showed uneven DNA staining corresponding to 1.37% of red-stained cells and 1.12% of cells with membrane damaged or senescent cells (Fig. 3.14, Fig. 3.15, Table 3.9). The intensity of fluorescence of *E. coli* treated with both EOs was lower than in the control, but the effects of LC19 (1,8-cineole type) and BV27 (linalool type) on *E. coli* differed. In the case of treatment with LC19, 32.95; 45.53 and 81.80% of the cells lost DNA at 0.5; 1 and 2 MIC, respectively (Fig. 3.14, Fig. 3.15, Table 3.9) indicating either cell leaking (i.e. death) and/or defects in DNA organization, replication and/or segregation in daughter cells. On the other hand, 43.55; 98.10 and 97.94% of the cells at 0.5; 1 and 2 MIC exhibited a change of the nucleoid structure in BV27 treated cells, respectively (Fig. 3.14, Fig. 3.15, Table 3.9). The DNA of BV27-treated cells was unevenly stained with spots and had undergone compaction (Fig. 3.15), as if treated with some antibiotics (cf chloramphenicol, that stops gene expression by inhibiting a RNA polymerase subunit). Cell filaments contained unusually long DNA-fibers that crossed the whole cell, indicating that DNA structure itself was affected.

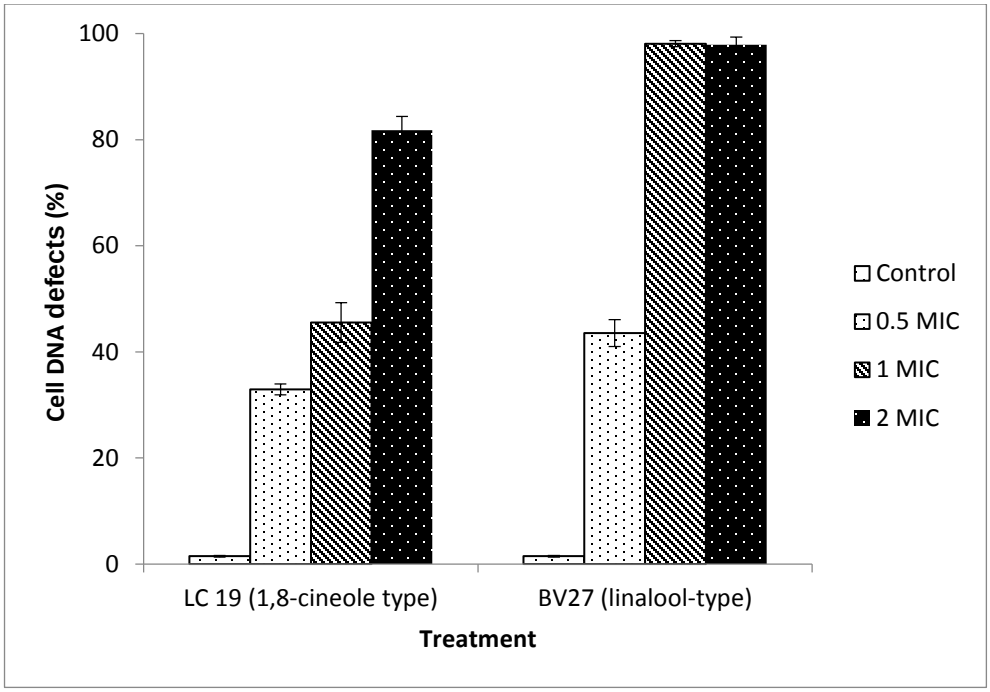
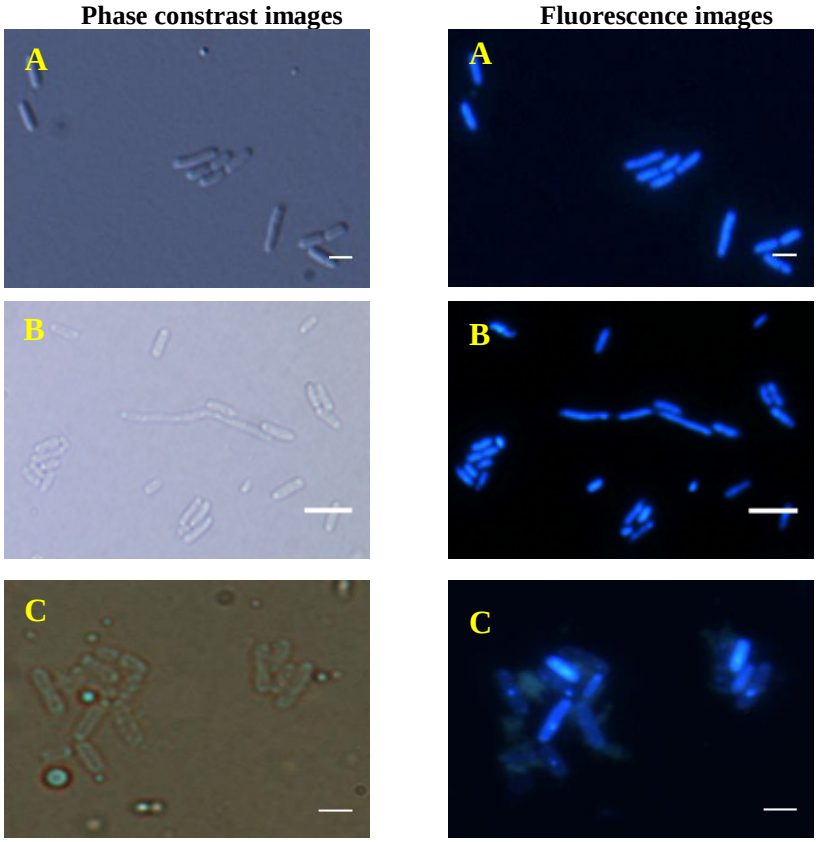


Figure 3.14: Effect of two L. cubeba leaf EOs LC19 (1,8-cineole type) and BV27 (linalool type) on DNA of E. coli ATCC 25922 using DAPI staining



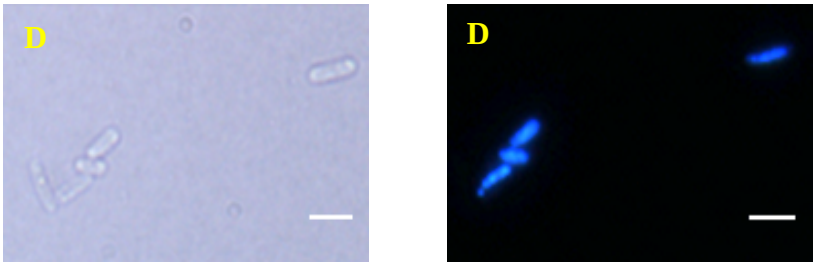


Figure 3.15: Fluorescence microscopic images with DAPI of DNA phenotypes of E. coli ATCC 25922 cells after 2h of exposure to L. cubeba leaf EOs at different concentrations

(A) Control cells; (B) Cell filaments (1.07; 1.43 and 0.14% at 0.5 ; 1 and 2 MIC of LC19; 0.3; 0.035 and 0% at 0.5 ; 1 and 2 MIC of BV27, respectively); (C) Cell porous and slightly swollen (0; 98.10 and 97.94% at 0.5 ; 1 and 2 MIC of BV27, respectively); (D) Loss of DNA from cell (32.95; 45.53 and 81.80% at 0.5 ; 1 and 2 MIC of LC19, respectively). Scale bars represent 2 µm (Phase constrast and fluorescence images)

In general, EOs has been reported that active components of antibacterial EOs might bind to the bacterial cell surface and then penetrate the phospholipid bilayer of the cytoplasmic membrane and/or membrane bound enzymes. In this way they can accumulate in cell membranes and increase their permeability, leading to the leakage of vital intracellular constituents (including DNA) and ultimately to cell death [79]. In our study, the reduction in the viability of *E. coli* after a short exposure to LC19 and BV27 confirms the strong effect of both EOs. Similarly, at 4 mg/mL, *O. basilicum* was shown to kill all *E. coli* cells within 1 h of exposure [182]. In addition, *Cinnamomum longepaniculatum* and 1,8-cineole have been shown to have a bactericidal effect after 4 h and 12 h, respectively [99]. Using the membrane-binding fluorescent dye FM4-64, we observe damages to *E. coli* cell membrane integrity. Although the LIVE/DEAD Baclight Bacteria viability Kit only enables differentiation between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between active and dead cells. Microscopic assessment of LIVE/DEAD-stained cells is usually simplified to either “green”-labled (live) or “red”-labeled (dead) cells [24]. In our study, membrane permeabilization was demonstrated by the uptake of the fluorescent probe, propidium iodide. de Sousa et al. (2012) reported that treated with 1,8-cineole at 1 MIC (10 µL/mL), *P. fluorescens* presented 45% green cells and 55% red cells after 15 min of treatment [49]. Their study indicated that the treatment with 1,8-cineole at sub-lethal concentration led to the loss of cell wall (leading to cell shape/morphology/width alterations) and cytoplasmic membrane integrity, causing cell death even after only a short exposure.

Many stress conditions such as low pH (4.3), low water activity (0.95), low temperature, high concentration of NaCl (5%) or high hydrostatic pressure can cause cell elongation of foodborne pathogens, often without substantial changes in the numbers of viable cells. Cell filamentation was observed in all treatments with both LC19 and BV27. At present, we do not know whether this phenomenon was reversible in our case. However, it is remarkable that a much higher filamentation rate was observed in LC19-treated cells. These filaments contained abnormal DNA staining, suggesting that DNA itself could be affected by LC19 and lead to cell filamentation as a response to the induced stress (SOS response). Cinnamaldehyde caused cell elongation of *E. coli* O157:H7 and *B. cereus* occurring during a period of ≤5 h and 1 h of exposure, respectively [92, 174]. Treated cells were elongated, showing filamentous structures and inhibition of cell separation. The septa were present between the elongated cells, but its formation was incomplete. These results led to the hypothesis that the exo-polysaccharide on the outer membrane of cells became detached and released or that the peptidoglycan and cytoplasmic membrane were perturbed [113]**.** The change in cell morphology may be due to the effect of EOs on the cell membrane, which could result in the destruction of the cell wall, leading to the loss of intracellular dense materials in treated cells [79].

Generally, gross membrane damage occurs after prolonged exposure to EOs which is quantified by the loss of intracellular material that absorbs at 260nm, corresponding to DNA. Cui et al. reported that *S. sclarea* EO, which contain 74.56% linalyl acetate and 13.26% linalool, decreased *E. coli* DNA: the DNA content in treated cells was reduced by 50.77% compared to that in the control [46]. The EOs of *O. vulgare* L. and *R. officinalis* L., which are rich in carvacrol and 1,8-cineole respectively, led to alteration of the cell wall structure of *P. fluorescens* after 2 h, with rupture of the plasmatic membrane, shrinking of the cells, condensation of the cytoplasmic content and leakage of the intracellular material [46]. Moreover, there was an increase in the permeability of the cell membrane, resulting in cell death after only 15 min exposure. Concerning EO components, Zengin et al. (2014) [188] reported that the combination of 1,8-cineole and linalool or 1,8-cineole and α-terpineol led to release of constituents including DNA from *E. coli* O157:H7, *S.* Typhimurium and *S. aureus* after 2 h of exposure. Among the components tested, 1,8-cineole had greater effects than the others terpenes on the leakage of cellular material [188]. Regarding the mechanism of action

of 1,8-cineole against *E. coli*, once the phenolic compound crossed the microbial cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, changed in the membrane permeability due to the rupture of the cell membrane, affecting cellular activity. Whereas, linalool, alcohol components, caused permeability alteration of the outer membrane, alteration of cell membrane function and leakage of intracellular materials [188]. Similarly, Tyagi and Malik (2010) [167] reported that exposure to a combination of four different EO vapors containing a high amount of 1,8-cineole, and negative air ions, led to complete leakage of the cytoplasmic material in *P. fluorescens* within 4 h of exposure.

Taken together, our data suggest that LC19 may act directly or indirectly on the DNA metabolism causing DNA loss, correlated with a reduction in viability (Table 3.9). DNA loss could be caused by compaction and/or segregation defects that failed to deliver duplicated DNA to daughter cells. DNA loss may be also due to membrane defects causing loss of intracellular contents including DNA. It could also be a combination of the two hypotheses. In the same way for BV27, the changes observed in the shape of the nucleoid and condensation could be a consequence of action on DNA metabolism or gene expression. While LC19 showed a progressive, concentration dependent effect on membrane and DNA structures, BV27 had a moderate effect at 0.5 MIC but compromised nearly 100% of cells at 1 MIC and above, indicating a "threshold" concentration between 0.5 and 1 MIC.

In conclusion, the investigation of their mode of action against E. coli revealed that the change in morphology, the loss of integrity and permeability of cell membrane and the loss of intracellular content (DNA) could be the mechanisms behind the action of L. cubeba leaf EOs. However, the effects of both leaf EOs on E. coli differed. LC19 moderately affected cell membrane but led to significant cell filamentation rate and changes in cell width that suggest an effect on cell wall synthesis [165]. It also exhibited many cells without DNA, suggesting a general effect on cell cycle and, to a lesser extent, on membrane permeabilization. BV27 rapidly damaged cell membrane integrity and led to cell permabilization in addition to change in DNA morphology even at sublethal concentration, indicating a strong antibacterial activity based on disruption of membrane integrity. It also upset the ratio of cell length to width, suggesting an effect or an interference with peptidoglycan synthesis machinery [189] as previously suggested [100].

3.4. Application of *L. cubeba* plant extract in aquaculture

Literature review indicated that the herbal therapy in aquaculture could be used as raw plant material, plant extracts, EO or plant powder [136]. Although the raw material is easy to use at small-scale farmers, they are difficult to maintain the biological effects in aquatic animal. The plant extract may be more effective than raw material and favorable for industrial scale. However, the using of organic solvent product can be more expensive to prepare extraction and remove the solvent. Therefore, EO (water plant extract) or crude dry powder can be provided more convenient than the others. Our research decided to conduct the *L. cubeba* fruit EO in shrimp experiment and *L. cubeba* leaf powder in fish experiment.

3.4.1. Effect of *L. cubeba* fruit EO on whiteleg shrimp *L. vannamei*

In the current study, the preliminary tests aimed to evaluate the effect of *L. cubeba* fruit EO on the vibrio concentration and the survival rate of post-larval shrimp PL12-15 (12 – 15 days). The shrimp *L. vannamei* PL 12 – 15 were purchased from Hai Phong and transferred to VNUA – Hanoi. Shrimps were acclimated at 24 – 27^{o}C for 1 week for preliminary test. After infection with *V. parahaemolyticus* at concentration of 10^7 CFU/mL, shrimp were divided into aquarium at 20 larval/aquarium (4 liters). Each test was performed in triplicate (section 2.2.11), including control (-), control (+), larvae treated with OTC at 1 MIC (16.7 μg/mL), larvae treated with *L. cubeba* at ½ MIC (840 μg/mL) and 1 MIC (1670 μg/mL).

The EO and antibiotic was added to the bath aquarium. The vibrio concentration in water and in shrimp muscle were measured.

However, there was some important notice during shrimp experiment:

- During transformation and acclimation, a number of larvae died due to stress.
- Larvae were dead after 1 h and 2 h of exposure to *L. cubeba* EO at 1 and 1/2 MIC, respectively. These concentrations were probably toxic to larvae

Therefore, based on these preliminary tests, we needed to modify the protocol and the condition of the lab work. The modifications included:

- Change of the larvae purchase near Hanoi to reduce the time of transportation. Indeed, a private laboratory from Bac Ninh was choosen.
- Change the larval by juvenile shrimp (PL35, length about 3-3.5 cm)

- Change from adding directly EO in water by immersing shimp in EO

The modified experiment was carried out as described above in section 2.2.11.

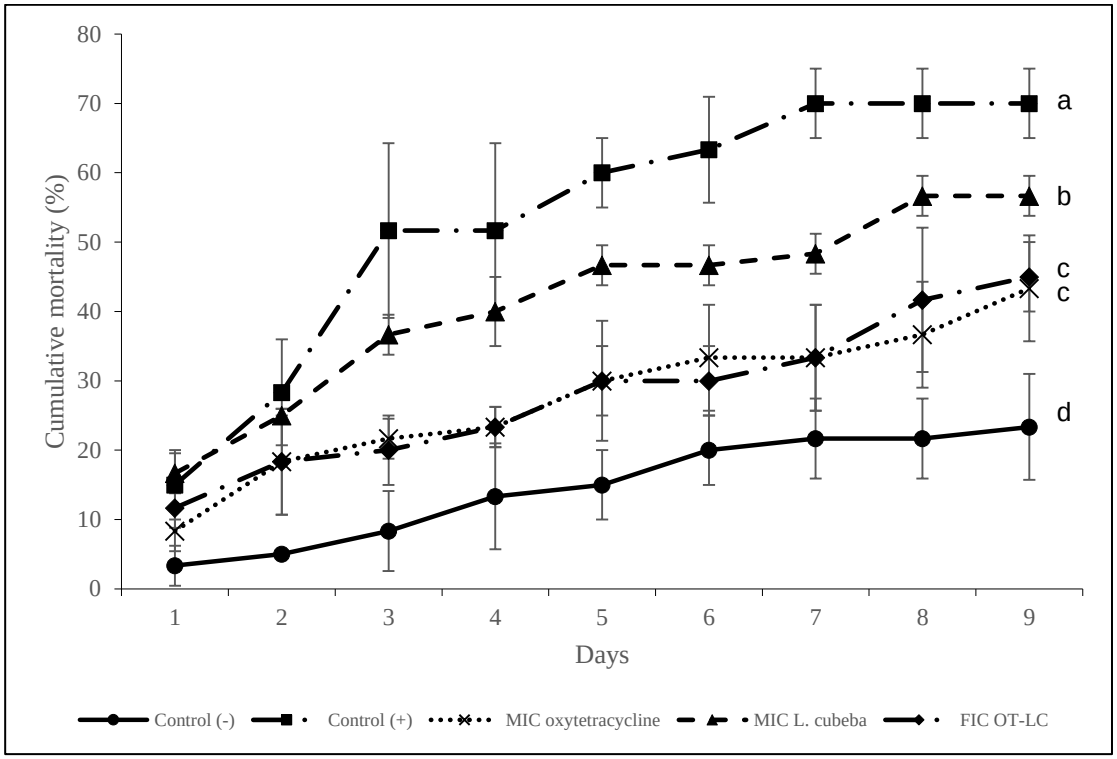


Figure 3.16: Effect of L. cubeba fruit EO, oxytetracycline and their combination on the survival rate of whiteleg shrimp

The cumulative mortality of shrimps and the vibrio concentration in both water and shrimp were determined for the five assays (C (-), C (+), FIC OTC-LC, OTC and LC) as shown in Fig 3.16 and Table 3.10. The *L. cubeba* fruit EO was less active than OTC and the combination of *L. cubeba* fruit EO and OTC regarding protection of shrimp against infection by the pathogenic bacteria. Indeed, the survival of *L. vannamei* reached 76.7% in C (-), followed by antibiotic (OTC) (56.7%), FIC OTC-LC (55.0%) and LC (43.3%), while the C (+) shown the lowest survival rate (30.0%). The current study explored only the vibrio concentration at the end of experiments. The highest vibrio concentration was found in death shrimp, followed by survival shrimp and bath water in all of treatment. The bacterial concentration of both water and shrimp in OTC, LC, and FIC OTC-LC aquarium were lower than in the C (+). The vibrio concentrations in both survival and death shrimp in C (-) were lowest among the 5 treatments. Whereas, the bacterial concentration in water culture of C (-) assay was higher than the treatments with EO, OTC or mixture oF OTC-LC. Inversely, the

survival in three treatments (FIC OTC-LC, LC and OTC) assays was higher than in the C (+) and lower than C (-) (Fig. 3.16).

Table 3.10: In-vivo antimicrobial activity of L. cubeba fruit EO and oxytetracycline on whiteleg shrimp

Treatment	Cumulative mortality (%)	Bacterial concentration (Log CFU/g)		
		Water	Survival shrimp	Death shrimp
Control (-)	23.3	$1.8.\ 10^4$	$1.0.\ 10^4$	$2.3.\ 10^4$
Control (+)	70.0	$1.5.\ 10^6$	$5.0.\ 10^6$	$6.3.\ 10^7$
LC	56.7	$1.1.\ 10^4$	$3.8.\ 10^5$	$1.7.\ 10^6$
OTC	43.3	$1.9.\ 10^3$	$2.0.\ 10^6$	$2.0.\ 10^7$
FIC LC-OTC	45.0	$7.3.\ 10^3$	$0.9.\ 10^6$	$1.1.\ 10^7$

Similar inhibitory effect of plant extract on the bacterial concentration and survival rate of shrimp were reported in previous study [43, 132]. As reported in the literature, a vibrio concentration lower $6.5.\ 10^3$ CFU/mL did not affect shrimp survival, whereas a population of 10^7 CFU/mL vibrios caused negative effect for shrimp and water bath [132]. Guava (*P. guajava*) was able to eliminate luminous bacteria from black tiger shrimp (*P. monodon*) more effectively than OTC [43]. An increasing of the shrimp larvae (*P. monodon*) survival concomitant with a decrease in bacterial concentration were observed when *C. fragrans* EO was added directly to the water tank [132]. Interestingly, even at higher concentration (734 μg/mL) and smaller larvae (Nauplii) than our study, *C. fragans* EO did not cause negative effect to shrimp. Similarly, two EOs of *C. fragrans* (B8: linalool-type and B143: 1,8-cineole-type) reduced the total heterotrophic aerobic bacteria and the *Vibrio* concentrations in the rearing water of *P. monodon* shrimp larvae [144].

Several medicinal plants have been used in shrimp feed which include *C. fragans, Aegle marmelos, A. sativum, Azadirachta indica, Catharanthus roseus, Curcuma longa, Cynodon dactylon, Eclipta alba, Lantana camara, Melia azedarach, Mimosa pudica, Momordica charantia, Morus alba, Ocimum americanum, Phyllanthus amarus, Phyllanthus emblica, Picrorhiza kurooa, P. guajava,* and *Solanum nigrum* [57]. These can be administered as whole plants or parts (leaf, root, seed, twig) or extract (methanol, ethanol, n-hexan, water, EO) products; they can be applied in aqueous solution as feed additives, single or in

combination, or as a mixture with prebiotics or other immunostimulants [8]. They are commonly administrated through oral (diet); injection (intraperitoneal or intramuscular) or bath immersion. Recently, AftabUddin et al. (2017) investigated the effect of methanol, petroleum ether and n-hexan extracts composed of nine herbs including *A. vera, Andrographis pariculata, Annona squamosa, Azadirachta indica, Citrus aurantifolia, Coriandrum sativum, Ocimum sanctum, Ollium cepa* and *P. guajava* on growth, survival rate and immune-protection against pathogenic *V. harveyi* in the tiger shrimp *P. monodon* [8]. The shrimps fed on diets with methanolic extraction of 2.5 mL/kg had higher survival rate, specific growth rate (SGR) and better food conversion ratio (FCR) than other groups. The alcoholic and organic solvents were more efficient for the extraction of bioactive metabolites (polar and non-polar) having antimicrobial and immunostimulant activities, compared to water-based methods. However, during processing with organic solvent extraction, the material must be further concentrated in a rotary evaporator under reduced pressure to remove the solvent. Preparing plant extracts with water can be easily conducted, and the byproducts obtained in this series of processes can readily be directly administered as a dietary supplement [110]. The hot-water extract and EO isolated from leaf and twig of stout camphor tree *Cinnamomum kanehirae* decreased the survival rate of whiteleg shrimp *L. vannamei* after injection with *V. alginolyticus* (10^6 CFU/shrimp) at the levels of 2 μg/g shrimp [184]. More recently, Gracia-Valenzuela et al. (2014) reported the positive effect of feed mixed with oregano EOs (containing mainly carvacrol or thymol) on infected shrimps *L. vannamei.* In fact, the vibrio concentration was significantly lower in tissues from shrimps that were administered supplemented-feed with oregano oil than control [70].

3.4.2. Effect of *L. cubeba* plant powder on common carp *C. carpio*

3.4.2.1. Enhancement of common carp growth promotion

Experimental feeds were obtained adding respectively 0; 2; 4 and 8% (w/w) of *L. cubeba* leaf powder to a commercial fish feed (Brand: CJ VINA-Vietnam, 35% protein, 6% lipid, <16% fiber content). The re-pellet of carp feed enriched with *L. cubeba* leaf powder was air dried, cut in small piece and stored in 0^oC until use (Fig 3.17).

Figure 3.17: Carp feed enrich with L. cubeba leaf powder

No adverse effect was observed for all fish batches during the 21 days feeding period before the experimental infection. As shown on Table 3.11, the weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were improved by supplementation of *L. cubeba* in a dose-related manner; a significant difference appeared for the highest dose of leaf powder (8%) ($P < 0.029$).

Table 3.11: Growth parameters of C. carpio after 21 days of feeding with different doses of L. cubeba leaf powder

L. cubeba	**Initial weight (g)**	**Final weight (g)**	**SGR (%)**	**WG (g)**	**FCR (g/g)**
0%	30.40±2.31	34.12±1.68	$0.56±0.08^{a}$	$3.72±0.37^{a}$	$4.70±0.62^{a}$
2% w/w	30.00±0.52	33.99±0.16	$0.60±0.04^{a}$	$3.99±0.21^{a}$	$4.28±0.27^{a}$
4% w/w	30.43±2.98	36.42±1.91	$0.87±0.14^{a}$	$5.99±0.73^{ab}$	$3.03±0.51^{a}$
8% w/w	30.20±0.26	37.20±1.24	$0.99±0.07^{b}$	$7.00±0.57^{b}$	$2.48±0.20^{b}$

SGR, specific growth rate; WG, weight gain; FCR, feed conversion ratio. Data are expressed as mean ± SEM (One-way Analysis of Variance and Holm–Sidak method $P < 0.05$). Values followed by different letters within a column are significantly different by Holm–Sidak method ($P < 0.05$)

3.4.2.2. Improvement of common carp immunostimulation

Effects on non-specific immunity of plasma were shown in Fig. 3.18, Fig 3.19, Fig

3.20. The increase of these parameters appeared clearly dose-dependent for plasma lysozyme ($P < 0.001$). The control revealed the lowest value of 442.4 U/mL. The highest significant lysozyme activity was recorded after feeding for 21 days with 8% doses of *L. cubeba* (1202.7 U/mL) followed by 4 and 2% doses of *L. cubeba* (913.0 and 633.3 U/mL, respectively) (Fig 3.18).

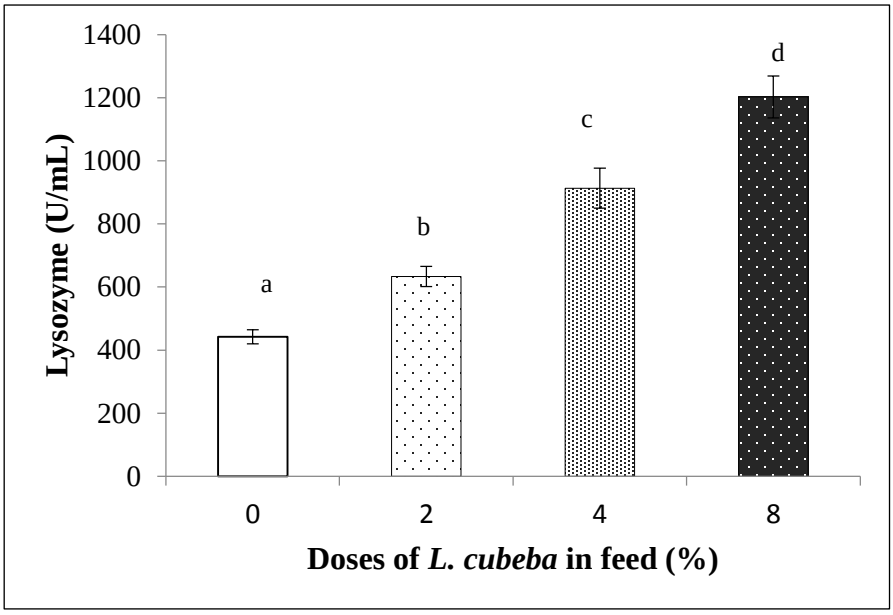


Figure 3.18: Plasma lysozyme (U/mL) of common carp C. carpio fed with different amounts of L. cubeba leaf powder

Data are expressed as mean ± SEM. Values followed by different letters are significantly different by Holm - Sidak method ($p<0.05$)

Bactericidal activity was significantly higher for the 4 and 8% dose *L. cubeba* ($P < 0.029$). The lowest bactericidal activity was after feeding for 21 days with 0% dose of *L. cubeba* (control) with the rate 21.8% inhibition CFU of *A. hydrophila* compared to the positive control (PBS only). A higher bactericidal activity was recorded when fish was fed with 2% dose of *L. cubeba* (26.7%) but no significant differences compared to control. Conversely, the highest bactericidal activity was found for 4 and 8% doses of *L. cubeba* (37.8 and 42.1%, respectively) (Fig 3.19).

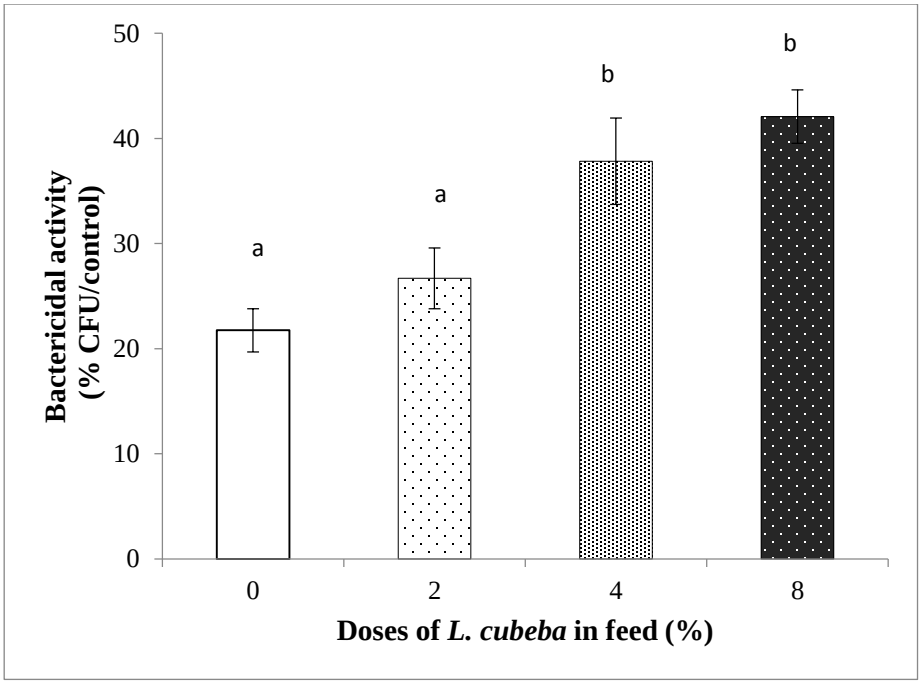


Figure 3.19: Bactericidal activity of plasma (% CFU/control) of common carp C. carpio fed with different amounts of L. cubeba leaf powder
Data are expressed as mean ± SEM. Values followed by different letters are significantly different by Holm - Sidak method ($p<0.05$)

For haemolysis activity, the increase was significant for the fish group fed with 8% plant powder only ($P < 0.001$). Indeed, the use of 8% dose of *L. cubeba* led to highest level of complement activity (ACH50 was 183.3 units/mL), followed by 4% and 2% doses (137.3 and 106.3 units/mL, respectively) ($P<0.001$) (Fig 3.20). The control revealed the lowest value (98.8 units/mL) as compared with the treatment groups.

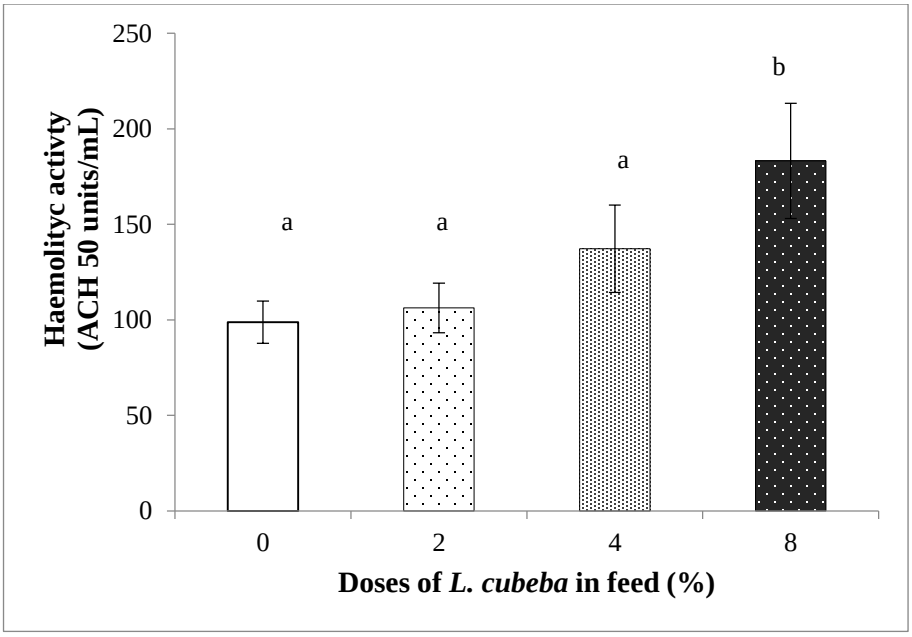


Figure 3.20: Haemolytic activity of plasma (CH50 units/mL) of common carp C. carpio fed with different amounts of L. cubeba leaf powder
Data are expressed as mean ± SEM. Values followed by different letters are significantly different by Holm - Sidak method ($p<0.05$)

3.4.2.3. Effect on common carp survival

Following use of herbal fed for 21 days, fish infected with *A. hydrophila* started to die after 4 days of the experiment (Fig. 3.21). A second strong peak of mortality at day 14 has particularly affected the control and fish fed with the lowest amount of *L. cubeba* leaf powder (2%). Cumulated mortalities observed for all treatments after 21 days post-infection were significantly different (Chi square 21.334 with 3 d.f. $P < 0.001$). Significant differences of mortality among treatments were confirmed by Kaplan –Meier survival analysis (25.751, with 3 d.f. $P < 0.001$). Pairwise multiple comparisons (Holm-Sidak method) showed that survivals of fish fed with 4 and 8% doses (63 and 66%, respectively) were significantly higher than those fed with 2% and the control (36 and 27%, respectively) (Fig. 3.21). Mortality rates and the Relative Percent Survival (RPS) increased according to the addition of *L. cubeba* leaf powder in feed (Table 3.12).

Table 3.12: Mortality and Relative Percent Survival of C. carpio fed with different doses of L. cubeba leaf powder

Parameter	***Litsea cubeba* doses in feed (%)**

	0% (n=52)	2% (n=55)	4% (n=43)	8% (n=58)
Mortality (%)	73.1±12.1	63.6±12.7	37.2±14.4	34.5±12.2
RPS (%)	0	28	49	53

Mortality percentages are presented with their respective Interval of confidence (IC) with α=0.05.

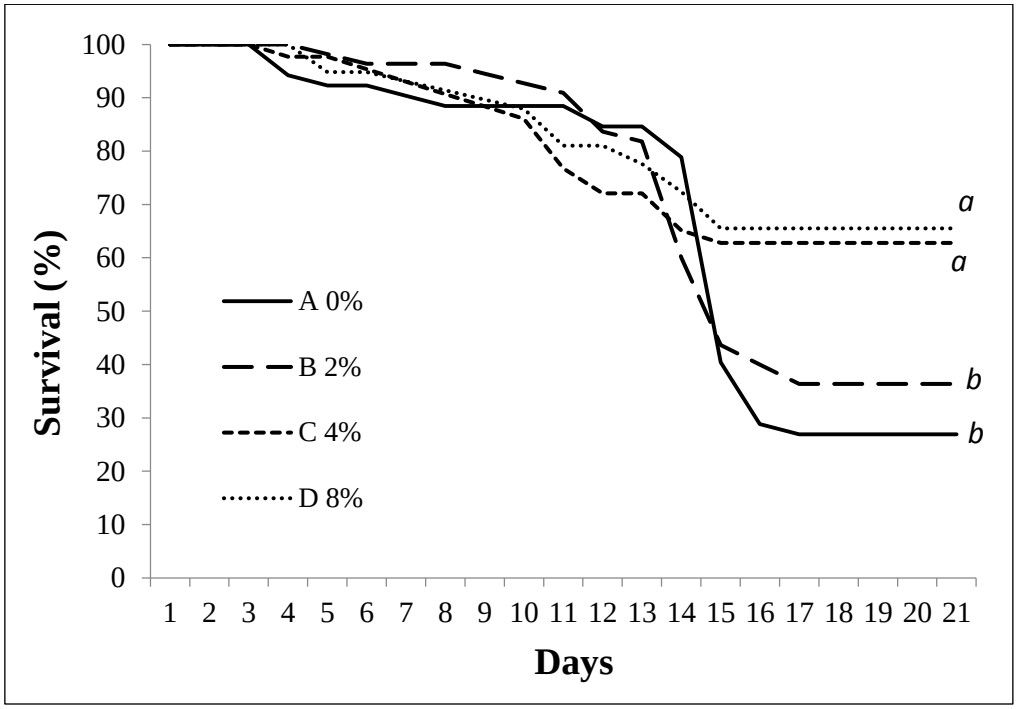


Figure 3.21: Survival rate of common carp fed with different doses of L. cubeba leaf powder Kaplan-Meier survival analysis. Different letters (a, b) indicate significant differences between treatments (p<0.001, n=208)

Positive effects of herbal supplementation on fish growth performance have often been reported. Nya and Austin (2009) [115] recorded increases in SGR and FCR in rainbow trout fed with different doses of ginger. *C. zeylanicum* which belongs to the Lauraceae family such as *L. cubeba* had a positive effect on growth performance and feed utilization (including SGR, FCR, feed efficiency ratio, protein efficiency ratio, apparent protein utilization, energy utilization) of Nile tilapia (*O. niloticus*) [9]. Shalaby et al. (2006) showed that food intake, SGR and final weight of Nile tilapia increased when garlic (*A. sativum*) was incorporated in the diet [148]. Moreover, the WG of common carp (*C. carpio*) was improved after 4 weeks of feeding with fed containing 1.5% garlic powder [105]. Of interest, Mahdavi et al. (2013) declared that incorporation of *A. vera* ethanolic extract 0.5 and 2.5% into fish diets of common

carp enhanced WG, FCR, SGR, Food Conversion Efficiency (FCE) [104]. Jian and Wu (2004) [86] reported an increase in body weight of Jain carp after feeding with a Traditional Chinese Medicine (TCM) formulation of Astragalus root (*Radix astragalin*) and Chinese angelica root (R. *angelicae sinensis*) at a ratio of 5:1. Several hypotheses, including stress reduction, increase of feed intake or appetence, have been suggested to explain the growth promotion induced by herbal therapy. Common carp fed with 150 mg/kg of a mixture of soapbark tree *Quillaja saponins* has shown a better WG, a higher energy retention and a significant protein utilization values without any change in metabolic rate and oxygen consumption [66]. Furthermore, the growth-promoting effect in carps has been observed for several substances possessing antibacterial properties [40, 58]. It has been suggested that the reduction in the intestine microbiota might decrease maintenance's costs and immune responses in gut; thus, the animal can spare energy for anabolic processes [68].

Feeding with *L. cubeba* led to higher fish growth promotion compared to the control, and this may be explained that *L. cubeba* leaf powder enhanced the nutrient digestibility leading to improved nutrient utilization and to a higher protein synthesis [136] or attributed to the active ingredients of the plants. The effect of basil *O. basilicum* which is rich in linalool was also investigated in fish. El-Dakar et al. (2015) indicated that the growth parameters and feed utilization indices (WG, FCR, SGR, Protein efficiency ratio (PER), Protein Productive Value (PDV), Energy utilization) of sea bream (*Sparus aurata*) were improved when fish fed diet containing dried basil seed, but not significant difference was recorded with basil leaves when compared to the control [61]. The author explained that seeds contain much higher content of proteins, lipids and active compounds such as planteose, mucilage, polysaccharides and fixed oil relative to leaves [61]. Ji et al. (2007) suggested that the improvement of growh promotion could be caused by a lower plasma triglyceride and high plasma HDL-CHO (high-density lipoprotein cholesterol) levels in the herbal diets [85]. On the other hand, the increase in the protein content upon addition of plant extract could also explained the enhancement of fish weight [93].

Adverse rearing conditions and chronic stress in fish are immune suppressive. Thus, the enhancement of both specific and nonspecific immune responses may be a relevant health management strategy to reinforce fish against stress and diseases.

By definition, an immunostimulant is a “substance that enhances the immune system by interacting directly with cells of the system activating them”. The use of immunostimulants in fish diets has been accelerated in order to obtain natural substances which can improve non-specific immunity. Generally, there are two main approaches to evaluate the efficacy of an immunostimulant:

- Measurement of the efficiency of innate cellular and humoral immune mechanisms, namely lysozyme, complement, bactericidal activity, phagocytosis, respiratory burst, leucocyte proliferation and count, monocyte/lymphocyte/granulocyte count, total protein, myeloperoxidase and antiprotease.

- Direct protection tests against fish pathogen infection

Generally, stressful conditions in carp, such as starvation, low dissolved oxygen or high salinity induce a decrease in lysozyme activity [74]. Lysozyme is a mucolytic enzyme excreted by leucocytes and it is also found in a large variety of fish secretion such as mucus, and in many tissues including blood. In mammals this enzyme splits the linkages between N-acetylmuramic acid and N-acetyl glucosamine of the cell walls (peptidoglycan layers) of Gram-positive bacteria. However, in fish, lysozyme has a stronger antibacterial activity, extended to Gram-negative [145]. Moreover, lysozyme is recognised to be an opsonin, and activates the complement system and phagocytes. Therefore, lysozyme plays an important role in the host defense mechanisms against infectious diseases. The results of this study revealed a significant increase of the lysozyme activity in all treatment groups after feeding for 21 days, especially in fish receiving 8% dose of *L. cubeba*. Similarly, several herbal compounds have been reported to increase the lysozyme level in carps [6, 17, 186]. In common carp, the lysozyme activity significantly increased in fish that received feed supplemented with oat *Avena sativa* extract at concentration of 5, 10 and 20 g/kg [17]; mixture of 4 plant ethanol extract of *O. basilicum, C. zeylanicum, Juglans regia* and *M. piperita* at concentration of 0.25, 0.50, 0.75, 1.0 and 1.25 g/kg [6]. Moreover, *Oliviera decumbens* and *Satureja khuzestanica* used alone or in combination [11]; *Astragalus radix, Ganoderma lucidum* and combination of them [186], mixture of *Angelica membranaceus* and *A. sinensis* [86] enhanced innate immunity indices in both vaccinated (using vaccine developed against *A. hydrophila*) and non-vaccinated carp. In addition, the methanolic soluble fractions of *Lawsonia inermis* (henna) at

60 and 600 mg/kg enhanced some non-specific immune parameters such as serum lysozyme, bactericidal activity and respiratory burst activity of common carp that were i.p. injected with plant extract [108]. Feeding rohu for 14 days with 0.5% prickly chaff-flower led to enhancement in lysozyme activity [171]. Ardó et al. (2008) recorded elevation in lysozyme levels of Nile tilapia fed for 7 days with Chinese herbs, *A. membranaceus* and *Lonicera japonica*, alone or mixed [16].

Various humoral factors involved in innate and/or adaptive immunities are elevated in the serum to protect the host from infection. Serum bactericidal activity is a mechanism noted for killing and clearing of pathogenic organisms in fish [171]. The increased bactericidal activity of plasma of *L. cubeba* treated groups suggested that various humoral factors involved in innate immunity have been enhanced in the serum to protect the host against *A. hydrophila* infection. Increased bactericidal activity has been reported in Indian carp (*Labeo rohita*) fed for 5 weeks with *Achyrantes aspera* [171]. Similarly, serum bactericidal activity was significantly increased in common carp following the use of greater amounts of herbal extracts (mixture of elecampane inula *Inula helenium*, coltsfoot *Tussilago farfara*, mustard *Brassica nigra*, purple coneflower *Echinacea purpurea* and greater celandine *Chelidoniume majus*) in diets [108]. Punitha et al. (2008) reported significant increases of the serum bactericidal activity in grouper juveniles administered with diets containing a herbal mixture (Bermuda grass *Cynodon dactylon;* long pepper *Piper longum;* Stonebreaker *Phyllanthus niruri;* coat buttons, *Tridax procumbens* and ginger *Zingiber officinalis*) [130].

Indeed, the haemolytic reaction is a simple and convenient *in vitro* assay to assess the complement activity of the serum. Complement is a humoral factor of innate immunity. It plays an important role in the immune surveillance and clearance of invading pathogens such as parasites, fungi, bacteria and virus and it promotes several biological activities that are essential for fish immunity [74]. Among the three different pathways to activate the complement, the Alternative Complement Pathway (ACP) is the most important one in teleost fish. However, to the best of our knowledge, few studies have reported effects of herbal therapy on ACH50 in carp. For instance, the hydro-alcoholic extracts of *Stachys lavandulifolia* Vahl (Laminaceae) significantly increased the complement activity [12]. Knowing that stress has been reported to reduce the complement activity in carps [74], strengthening this capacity using *L. cubeba* leaf powder as shown here is a promising result.

The results from this study have certainly reinforced the view that plant materials have a role in fish disease control strategies. All treatment except 2% dose led to protection against *A. hydrophila* infections of fish fed for 21 days, as compared to controls. Although mortality in experimental challenge diseases on *C. carpio* may vary greatly, the RPS values observed with *L. cubeba* at 4 and 8% were higher than those reported in other studies using Chinese medicinal plants [186]. Increased survival of carps fed with high doses of *L. cubeba* might be due to the enhancement of nonspecific parameters such as lysozyme, bactericidal and haemolytic activities of plasma as it has been supported by several works [186]. Our results showed that the increase in humoral non-specific immunity parameters was dose-dependent with a significant difference for all tested parameters at the highest dose of *L. cubeba* (8%). Such additive effect of these immunological parameters may explain the dose-related survival. Thus, for example, feeding common carp with oat *A. sativa* extract at 0.5, 1.0 and 2.0% showed that the highest dose enabled better protection against challenge with *A. hydrophila* [17]. Also, Soltanian et al. (2016) noticed an inverse relationship between the mortality rate after challenge with *A. hydrophila* and the percentage of henna *L. inermis* in common carp diet, i.e. the higher the percentage of *L. inermis* in the diet, the less the level of mortality after challenge [108].

Literature review indicated that oral administration of plant extracts are the most suitable for fish culture assays because it is non-stressful and less labour intensive when compared to i.p. injection [136]. Generally, studies on herbal therapy in fish used extracts from plants. Although these extracts may be more concentrated and effective than raw plant material, they are generally unaffordable for small-scale farmers and may be complex to be incorporated in fish feed. The use of the crude dry leaf powder here provides a simple and inexpensive preparation of the herbal-enriched diets. Regarding the sensory characteristics, the dry powder of leaves is more suitable for the diet supplementation than EOs which are volatile substances or organic extracts that may be expensive and require more technicity. Other studies involving dry leaves have shown positive effects against bacterial diseases of fish. *P. guajava* leaf powder administered in the diet at the ratio of 1 : 4 (w/w) reduced the mortality of *A. hydrophila*-infected tilapia and did not cause any adverse effect on fish [121]. Yilmaz et al. (2013) reported that the mortality induced by *Streptococcus iniae* in *O. mossambicus* significantly decreased by addition of 1% dry leaves of thyme, rosemary or fenugreek in feed

[185]. Similar to our study, a dose-dependent reduction of mortality of Nile tilapia (*O. niloticus*) infected by *Streptococcus agalactiae* was obtained with feed containing dry leaf powder of *Andrographis paniculata* [135].

In brief, natural plant products (essential oil and powder) were improved act as growth promotion, immunostimulation and antimicrobial properties in common carp and shrimp larval in our study. We suggest that the addition of 4–8% of L. cubeba dry leaf powder in the feed may be an ecological way to improve carp farming through better efficiency of feed; immune stimulation and increased resistance to bacterial disease. Moreover, the high potential application of L. cubeba EO in aquaculture have demonstrated. However, their application is limited due to their own unique flavor, its toxicity, solubility and stability during the process. Microencapsulation L. cubeba could be a very good alternative to protect EO and to reduce the side effect of EO on the aquatic organisms. Furthermore, due to its wide distribution in East Asia, and considering the potential to produce a wide range of chemicals from different parts of the plant, this species has been proposed to be tested as an industrial crop model [39]. Its application in aquaculture may be a further opportunity giving an added value to the local biodiversity as this plant is readily found and easily grown in North Vietnam.

CONCLUSION AND PROSPECTS

CONCLUSION

1. All of nine tested EOs showed antibacterial effects against several bacteria including pathogens with various levels due to the difference in the composition of EOs. The inhibition zones and MIC of EOs were ranged from 0-90 mm and 0.4-39.0 mg/mL, respectively. *L. cubeba* was chosen for further study due of its antibacterial activity and its distribution.
2. *L. cubeba* fruit EO in combination with other EOs (*C. cassia, C. ambrosioides, M. leucadendron*) or antibiotics (nalidixic acid, OTC) showed synergistic effects against bacteria including pathogen strains (FIC = 0.14-1.50). The MIC of *L. cubeba* fruit EO, other EOs and antibiotic in combination was decreased from 1-17 times compared to used alone. The EOs of *L. cubeba* leaf collected in seven provinces in North Vietnam (n=25) were characterized by their high content in either 1,8-cineole or linalool. Linalool-type EO samples (MIC=0.3-5.8 mg/mL) were more effective against the tested bacterial strains than 1,8-cineole-type (MIC=2.8-12.3 mg/mL) samples.
3. *L. cubeba* LC19 (50% 1,8-cineole) and BV27 (94% linalool) leaf EOs showed a strong bactericidal effect against *E. coli* after 4 h and 2 h of exposure. Both EOs triggered changes in cell morphology, loss of integrity and permeability of the cell membrane, and loss of intracellular content (DNA) in treated cells. However, the effects of both leaf EOs against *E. coli* were distinct. LC19 affected cell membrane and DNA, led to cell filamentation and perturbation of cell width. BV27 damaged cell membrane integrity leading to cell permeabilization, and changed the DNA morphology.
4. The cumulative mortality of infected shrimp with *V. parahaemolyticus* was decreased after administration of *L. cubeba* fruit EO (56.7%) or mixture of *L. cubeba* and OTC at FIC values (45.0%) in the bath culture compared to control (70.0%). WG, SGR and FCR were improved by supplementation of *L. cubeba* leaf powder to the feed in a dose-related manner, and a significant difference appeared at the highest dose (8%) when compared to the control. Feeding common carp for 21 days with dietary supplement, i.e 2, 4 and 8% food diet of *L. cubeba* leaf powder, enhanced bactericidal, complement, lysozyme activities and survival rate of fish. The higher doses (4% and

8%) led to significant effects in terms of enhancement of the non-specific immunes system and resistance to *A. hydrophila* infection.

PROSPECTS

1. The current study has enabled finding two chemotypes of *L. cubeba* leaf EOs in seven provinces of Vietnam, 1,8-cineole and linalool. It is essential to identify the chemical compositions of *L. cubeba* leaf EOs from other provinces as well as the part of *L. cubeba* to enrich the knowledge of chemical constituent of *L. cubeba* from Vietnam. Furthermore, in order to commercialize herbal extract or plant products, it is important to standardize the active ingredients to maintain the clinical efficacy of the product.
2. The mechanism of action study revealed that both of *L. cubeba* leaf EOs (1,8-cineole and linalool type) affected on the integrity of cell membrane and cell permeabilization, as well as affected the cell length and DNA metabolism of *E. coli*. This requires further studies involving experiments such as potential membrane, proton motive force, genes expression, protein synthesis as conducted in a previous research [164, 189]. As well as potential targets in cell cycle might be investigated by working on synchronized bacterial populations.
3. The current study evaluated the effect of *L. cubeba* on aquatic animal at lab scale. Further research will be required at larger scale such as farmer or aquaculture industry to evaluate the effect on the environment of aquatic animal. In parallel, the microencapsulation of EOs would be developed to reduce their negative effect on aquatic animals as well as the loss of bioactivity during the process.

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PUBLICATIONS

1. **Nguyen Hai Van**, Caruso Doménico, Lebrun Marc, Nguyen Ngoc Tuan, Trinh Thi Trang, Meile Jean-Christophe, Chu-Ky Son, Sarter Samira (2016) *Antibacterial activity of Litsea cubeba (Lauraceae) and its effects on the biological response of common carp Cyprinus carpio challenged with Aeromonas hydrophila*. Journal of Applied Microbiology, **121**(2), pp. 341-351

2. **Hai Van Nguyen**, Thu Trang Vu, Son Chu-Ky, Samira Sarter (2017) *Interaction effect of essential oil and antibiotic on antibacterial against pathogenic bacteria in aquaculture*. Journal of Science and Technology, **55**(5A), pp. 66-73

3. **Nguyen Hai Van**, Meile Jean-Christophe, Lebrun Marc, Caruso Doménico, Chu-Ky Son, Sarter Samira (2017) *Litsea cubeba leaf essential oil from Vietnam: chemical diversity and its impacts on antibacterial activity*. Letters in Applied Microbiology, **66**, pp. 207-214

APPENDIX

TABLE

Table 1: Retention time and Kovats index of series alkane

Retention time	Kovats index
2.25	900
3.041	1000
4.502	1100
6.828	1200
9.943	1300
13.535	1400
17.313	1500
21.05	1600
24.712	1700
28.217	1800
31.585	1900
34.824	2000

Table 2: Final concentration of two component in checkerboard method

	2 MIC A	**1 MIC A**	**1/2 MIC A**	**1/4 MIC A**	**1/8 MIC A**	**1/16 MIC A**
2 MIC B	2:2	2:1	2:1/2	2:1/4	2:1/8	2:1/16
1 MIC B	1:2	1:1	1:1/2	1:1/4	1:1/8	1:1/16
1/2 MIC B	1/2:2	1/2:1	1/2:1/2	1/2:1/4	1/2:1/8	1/2:1/16
1/4 MIC B	1/4:2	1/4:1	1/4:1/2	1/4:1/4	1/4:1/8	1/4:1/16
1/8 MIC B	1/8:2	1/8:1	1/8:1/2	1/8:1/4	1/8:1/8	1/8:1/16
1/16 MIC B	1/16:2	1/16:1	1/16:1/2	1/16:1/4	1/16:1/8	1/16:1/16

Table 3: pH water of aquarium

Treatment	Day																				
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**	**D13**	**D14**	**D15**	**D16**	**D17**	**D18**	**D19**	**D20**	**D21**
A1	7.75	7.50	7.50	7.50	7.25	7.75	7.75	7.50	7.50	7.25	7.25	7.50	7.75	7.25	7.25	7.25	7.75	7.75	7.75	7.50	7.50
A2	7.50	7.50	7.50	7.50	7.75	8.50	8.00	7.75	7.50	7.25	8.00	8.00	7.50	7.50	7.50	7.50	8.00	7.75	7.25	8.00	7.50
A3	7.75	7.50	7.50	7.00	7.00	7.50	7.25	7.00	7.25	7.75	7.50	7.50	7.25	7.50	7.25	7.50	7.75	7.25	7.50	7.00	7.75
Mean	7.67	7.50	7.50	7.33	7.33	7.92	7.67	7.42	7.42	7.42	7.58	7.67	7.50	7.42	7.33	7.42	7.83	7.58	7.50	7.50	7.58
SD	0.14	0.00	0.00	0.29	0.38	0.52	0.38	0.38	0.14	0.29	0.38	0.29	0.25	0.14	0.14	0.14	0.14	0.29	0.25	0.50	0.14
B1	7.75	7.75	7.50	7.50	7.50	7.75	8.25	7.25	7.50	7.25	7.50	7.50	7.75	7.50	7.25	7.50	7.25	7.25	7.75	7.25	8.00
B2	7.75	7.50	8.00	7.75	7.50	7.75	8.00	7.50	7.50	7.50	7.50	7.50	7.75	7.50	7.50	7.50	7.75	7.50	7.75	7.50	7.00
B3	7.75	7.50	7.50	7.25	7.25	7.75	7.50	7.00	7.00	7.50	7.50	7.25	7.50	7.50	7.00	7.25	7.50	7.75	7.50	7.50	7.50
Mean	7.75	7.58	7.67	7.50	7.42	7.75	7.92	7.25	7.33	7.42	7.50	7.42	7.67	7.50	7.25	7.42	7.50	7.50	7.67	7.42	7.50
SD	0.00	0.14	0.29	0.25	0.14	0.00	0.38	0.25	0.29	0.14	0.00	0.14	0.14	0.00	0.25	0.14	0.25	0.25	0.14	0.14	0.50
C1	7.25	7.50	7.50	7.50	7.25	8.00	7.75	7.50	7.50	7.50	7.75	7.50	7.25	7.50	7.25	7.50	7.50	7.50	7.50	7.25	7.25
C2	8.00	8.25	8.00	7.50	7.25	8.00	8.00	7.50	7.50	7.50	7.50	7.50	7.75	7.75	7.75	7.75	7.50	7.50	7.50	7.75	7.75
C3	7.75	7.50	7.75	7.25	7.25	7.75	7.50	7.50	7.50	7.75	7.25	7.50	7.25	7.50	7.75	7.25	7.50	7.00	7.50	7.25	7.25
Mean	7.67	7.75	7.75	7.42	7.25	7.92	7.75	7.50	7.50	7.58	7.50	7.50	7.42	7.58	7.58	7.50	7.50	7.33	7.50	7.42	7.42
SD	0.38	0.43	0.25	0.14	0.00	0.14	0.25	0.00	0.00	0.14	0.25	0.00	0.29	0.14	0.29	0.25	0.00	0.29	0.00	0.29	0.29
D1	8.00	7.50	7.75	7.25	7.00	8.00	7.50	7.00	7.00	7.25	8.00	7.75	7.50	7.25	7.50	7.50	7.75	7.75	7.75	7.75	8.00
D2	8.00	7.75	7.75	7.75	7.50	8.25	8.00	7.75	7.50	7.50	7.75	7.50	7.25	7.50	7.25	7.50	7.00	7.25	7.75	7.50	7.75
D3	7.75	8.00	7.75	7.75	7.25	7.75	7.75	7.75	7.75	7.25	7.50	8.00	7.75	7.75	7.75	7.75	7.75	8.00	8.00	8.00	7.50
Mean	7.92	7.75	7.75	7.58	7.25	8.00	7.75	7.50	7.42	7.33	7.75	7.75	7.50	7.50	7.50	7.58	7.50	7.67	7.83	7.75	7.75
SD	0.14	0.25	0.00	0.29	0.25	0.25	0.25	0.43	0.38	0.14	0.25	0.25	0.25	0.25	0.25	0.14	0.43	0.38	0.14	0.25	0.25

A: control (0% of *L. cubeba* plant powder enriched-diets), B: 2% w/w of *L. cubeba* plant powder enriched-diets; C: 4% w/w of *L. cubeba* plant powder enriched-diets, D: 8% w/w of *L. cubeba* plant powder enriched-diets. SD: Standard Deviation.

Table 4: Water temperature during feeding experiment

Treatment	Day																				
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**	**D13**	**D14**	**D15**	**D16**	**D17**	**D18**	**D19**	**D20**	**D21**
A1	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	28.0	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
A2	27.5	27.5	27.5	28.0	28.0	26.5	25.5	26.0	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
A3	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.0	28.5	27.5	28.5	28.0	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
Mean	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.8	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B1	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	28.0	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
B2	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
B3	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
Mean	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.7	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C1	27.5	27.5	27.5	28.0	28.0	26.5	25.5	26.0	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
C2	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
C3	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.0	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
Mean	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D1	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
D2	27.5	27.5	27.5	28.0	28.0	26.5	25.5	26.0	28.5	27.5	28.5	27.5	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
D3	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.0	28.5	27.5	28.5	28.0	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
Mean	27.5	27.5	27.5	28.0	28.0	26.5	25.5	25.5	28.5	27.5	28.5	27.7	26.0	27.5	28.5	26.5	26.0	24.5	25.5	26.5	26.5
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

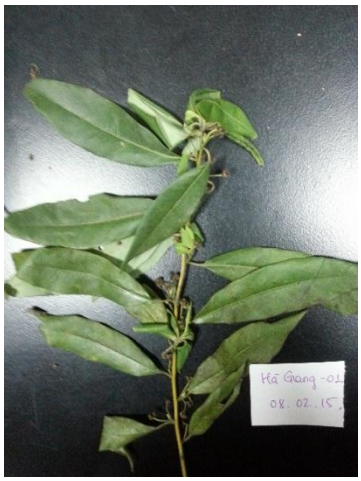
A: control (0% of *L. cubeba* plant powder enriched-diets), B: 2% w/w of *L. cubeba* plant powder enriched-diets; C: 4% w/w of *L. cubeba* plant powder enriched-diets, D: 8% w/w of *L. cubeba* plant powder enriched-diets. SD: Standard Deviation.

Table 5: NH_3, NH_4 of water aquarium during feeding experiment

	D1			D4			D7			D10			D13			D17			D19			D22		
	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3
A1	0.5	8.5	0.08	0.5	7.5	0.009	0.5	8	0.03	1	7	0.006	0.5	8	0.03	0.5	7	0.003	1	8	0.05	0.5	7.5	0.009
A2	0.5	8.5	0.08	0.5	7.5	0.009	0.5	8.5	0.08	1	7	0.006	0.5	8	0.03	0.5	8	0.03	1	7	0.006	0.5	7	0.003
A3	0.5	8	0.03	0.5	7	0.003	0.5	7	0.003	0.5	7.5	0.009	0.5	7.5	0.009	1	7.5	0.02	0.5	7.5	0.009	0.5	7.5	0.009
Mean			0.063			0.007			0.038			0.007			0.023			0.018			0.022			0.007
SD			0.029			0.003			0.039			0.002			0.012			0.014			0.025			0.003
B1	0.5	8	0.03	1	7.5	0.02	0.5	8.5	0.08	0.5	7	0.003	0.5	7.5	0.009	0.5	7	0.003	1	8	0.05	0.5	7.5	0.009
B2	0.5	8.5	0.08	1	7.5	0.02	0.5	8	0.03	0.5	7.5	0.009	0.5	7.5	0.009	0.5	7	0.003	0.5	7.5	0.009	0.5	7.5	0.009
B3	1	8	0.05	0.5	7.5	0.009	0.5	7.5	0.009	0.5	7.5	0.009	0.5	7.5	0.009	1	7.5	0.02	0.5	7.5	0.009	0.5	8	0.03
Mean			0.053			0.016			0.040			0.007			0.009			0.009			0.023			0.016
SD			0.025			0.006			0.036			0.003			0			0.010			0.024			0.012
C1	1	8.5	0.15	1	7.5	0.02	0.5	8	0.03	1	7.5	0.02	0.5	7	0.003	1	7	0.006	0.5	7.5	0.009	0.5	7.5	0.009
C2	0.5	8	0.03	0.5	7.5	0.009	0.5	8	0.03	0.5	7.5	0.009	0.5	7.5	0.009	0.5	8	0.03	1	7.5	0.02	0.5	7.5	0.009
C3	0.5	7.5	0.009	0.5	7.5	0.009	0.5	7.5	0.009	0.5	7.5	0.009	1	7	0.006	0.5	7.5	0.009	0.5	7.5	0.009	0.5	7.5	0.009
Mean			0.063			0.013			0.023			0.013			0.006			0.015			0.013			0.009
SD			0.076			0.006			0.012			0.006			0.003			0.013			0.006			0
D1	0.5	8.5	0.08	1	7	0.006	0.5	7.5	0.009	0.5	7	0.003	0.5	8	0.03	0.5	7	0.003	0.5	8	0.03	0.5	8	0.03
D2	0.5	8	0.03	1	7.5	0.02	0.5	8	0.03	0.5	7.5	0.009	0.5	7.5	0.009	1	8	0.05	0.5	8	0.03	1	7.5	0.02
D3	0.5	8	0.03	1	7.5	0.02	0.5	8	0.03	0.5	7	0.003	0.5	8	0.03	1	8	0.05	0.5	8	0.03	0.5	8	0.03
Mean			0.047			0.015			0.023			0.005			0.023			0.034			0.03			0.027
SD			0.029			0.008			0.012			0.003			0.012			0.027			0			0.006

A: control (0% of *L. cubeba* plant powder enriched-diets), B: 2% w/w of *L. cubeba* plant powder enriched-diets; C: 4% w/w of *L. cubeba* plant powder enriched-diets, D: 8% w/w of *L. cubeba* plant powder enriched-diets. SD: Standard Deviation.

Figure

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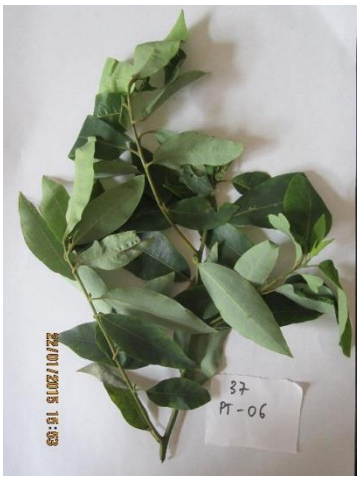
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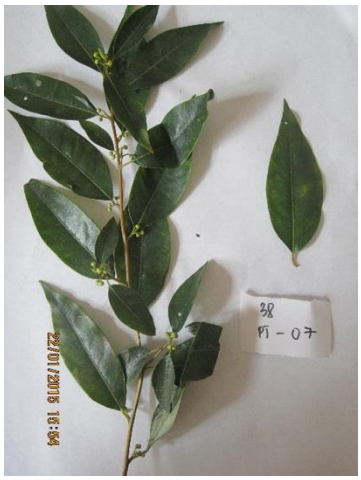
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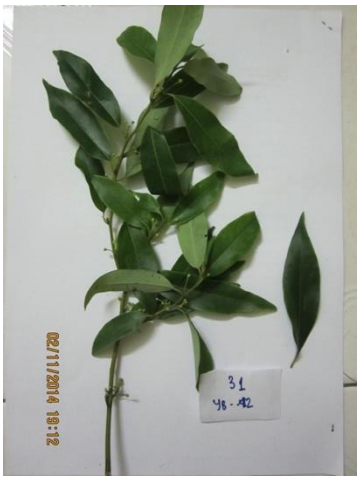
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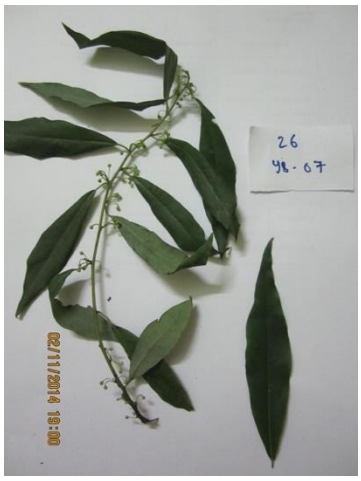
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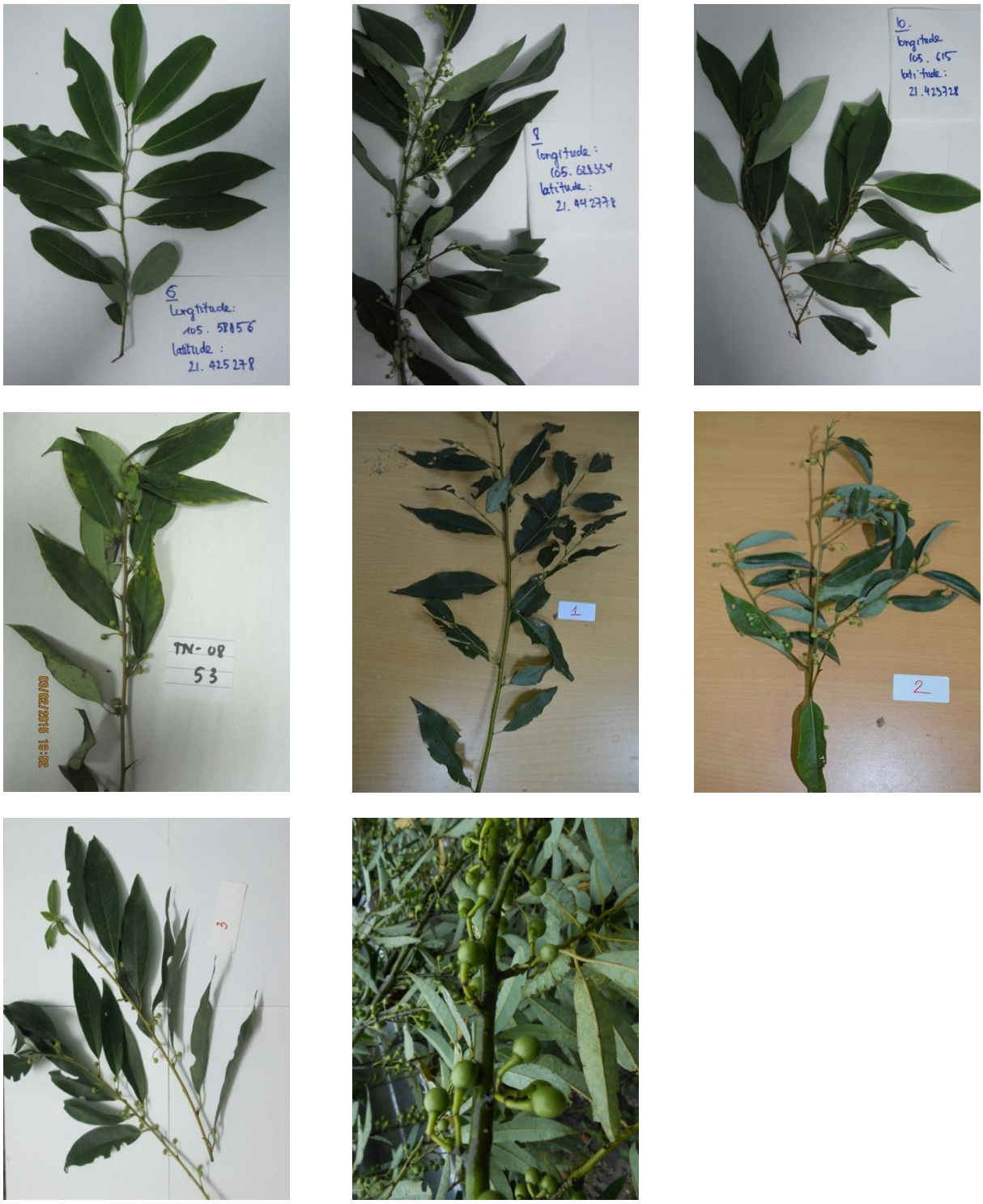


Figure 1: *Litsea cubeba* leaf and fruit samples

Figure 2: Extraction essential oils by *Clevenger-type* apparatus

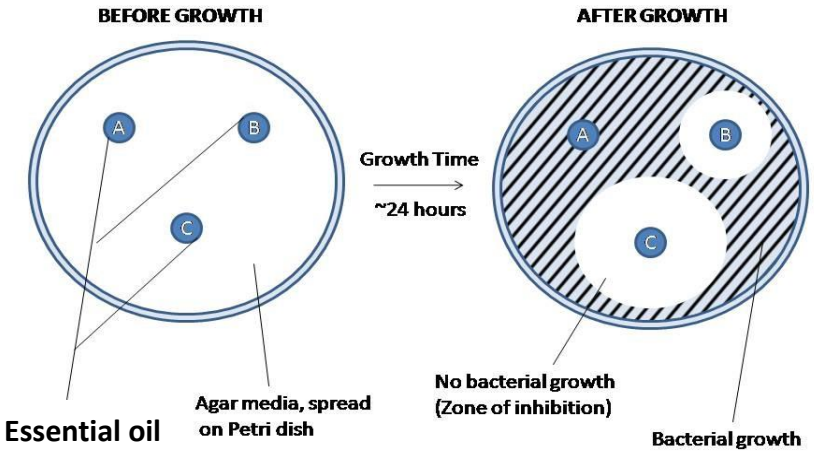


Figure 3: Principe of diffusion method

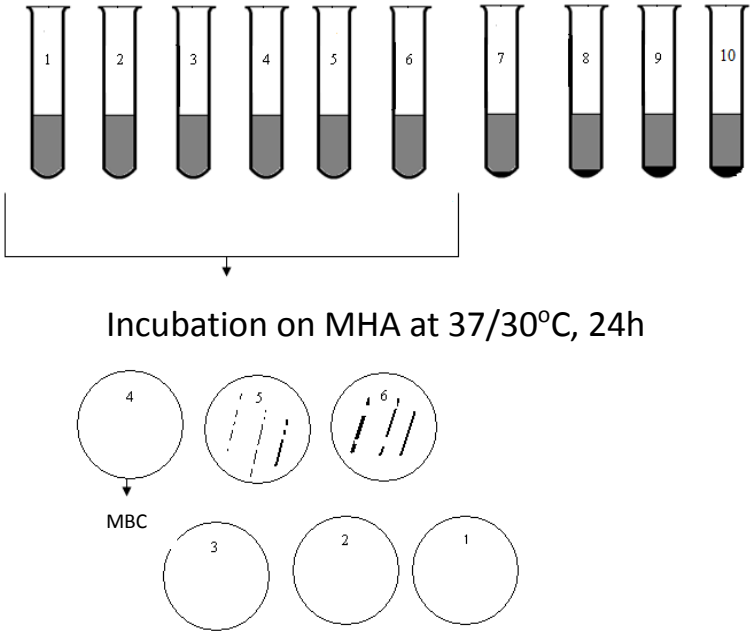


Figure 4: Principe of MIC and MBC method

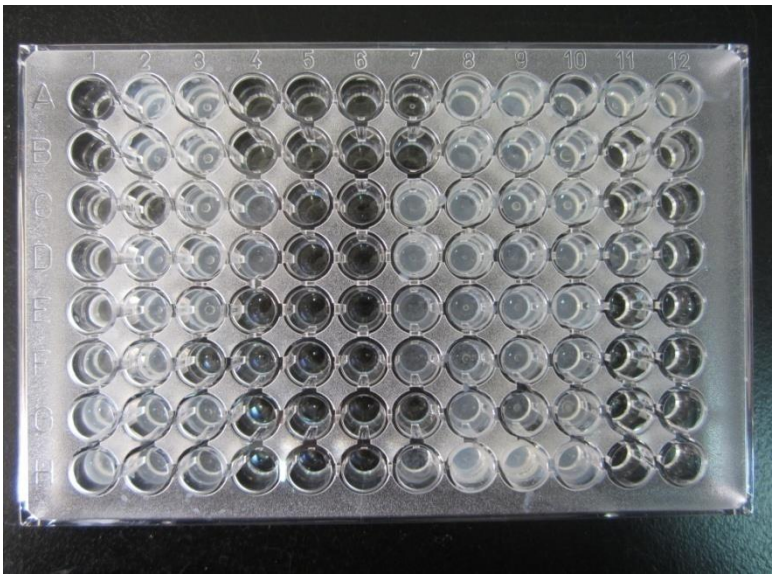


Figure 5: 96-well microplates

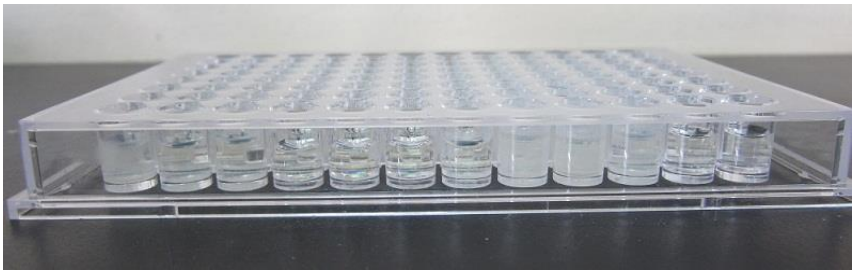
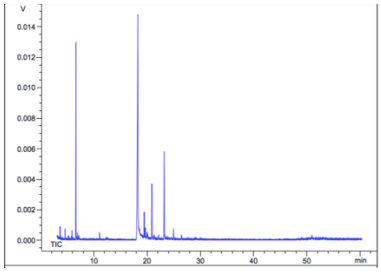
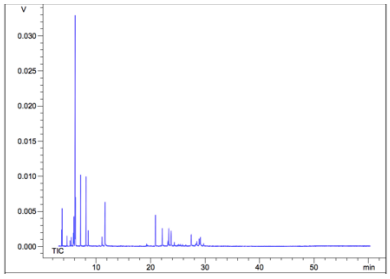


Figure 5: Differences in absorbance of suspension after incubation



Ocimum gratissimum



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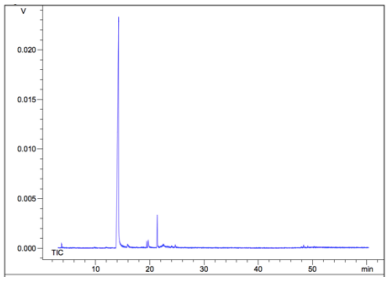
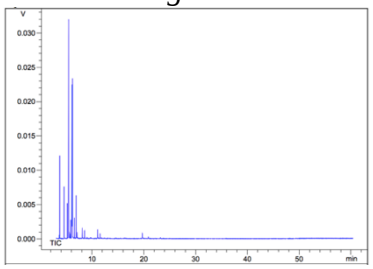
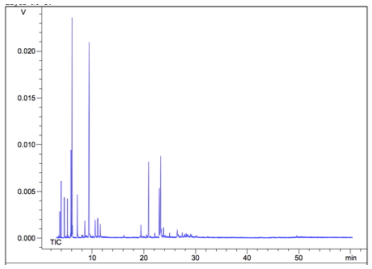


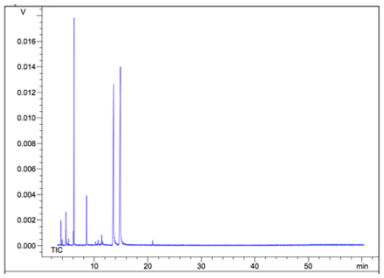
Cinnamomum cassia



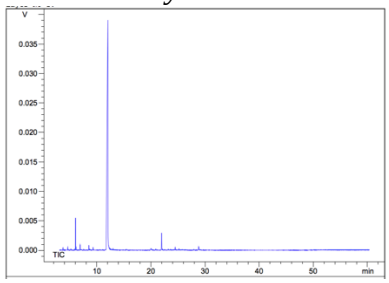
Zanthoxylum rhesta



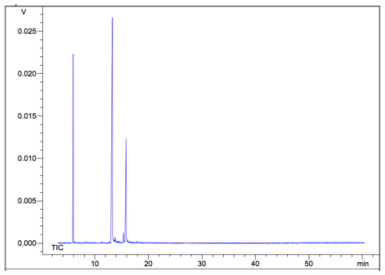
Artemisia annua



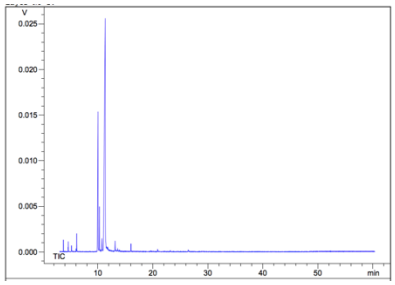
Litsea cubeba



Ocimum basilicum

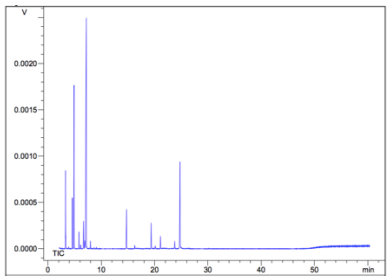


Chenopodium ambrosioides

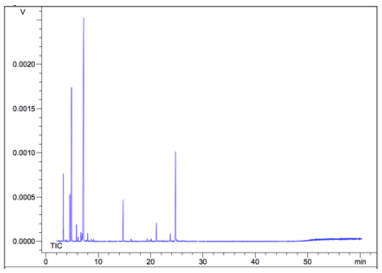


Mentha arvensis

Figure 5: Chromatogram of 9 essential oils used in this study



Litsea cubeba HG01



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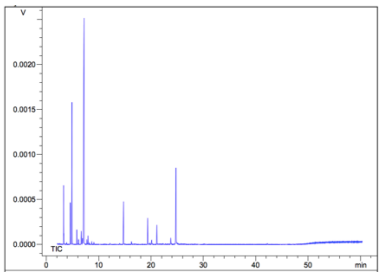

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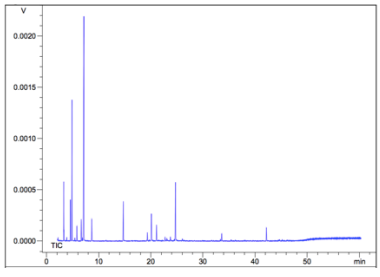

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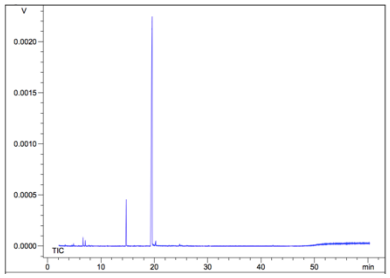
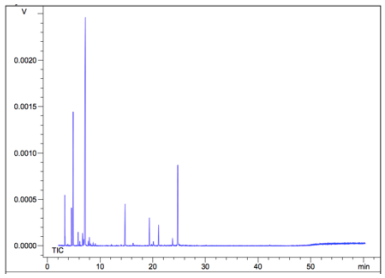


Litsea cubeba BV06



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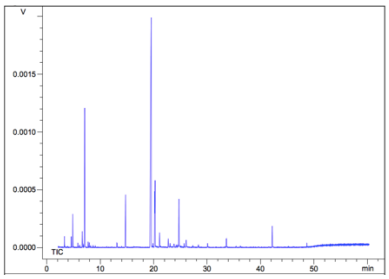
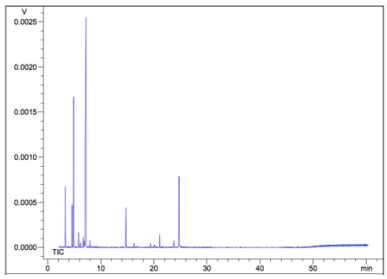


Litsea cubeba TN08



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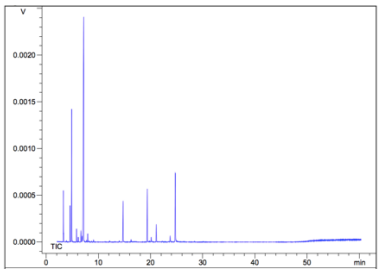

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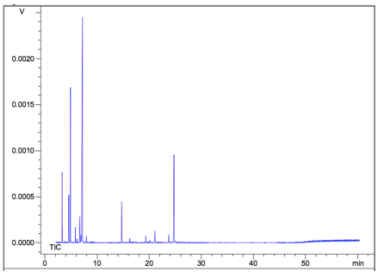

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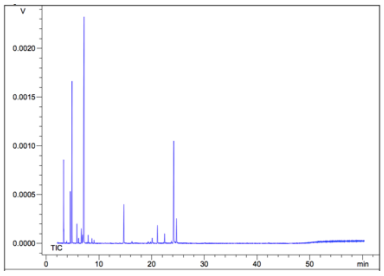

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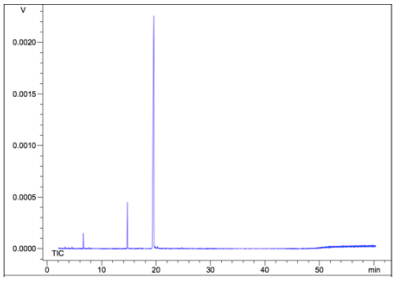
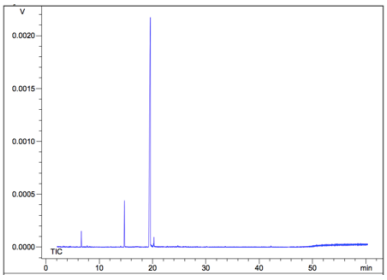


Litsea cubeba PT13



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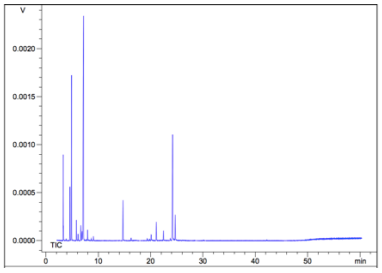

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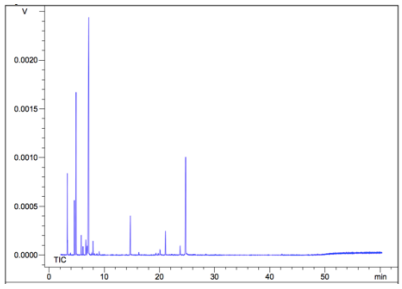

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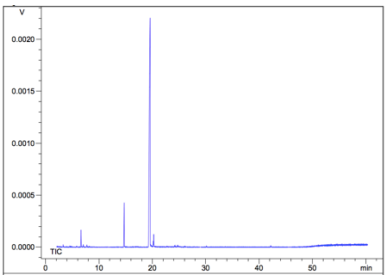

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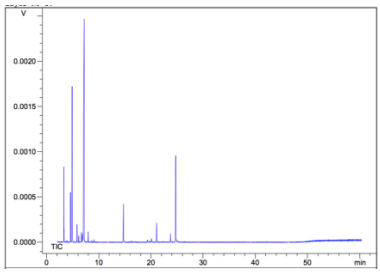

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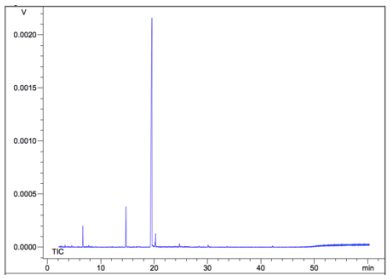
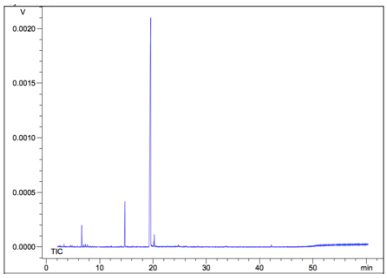


Litsea cubeba PT20



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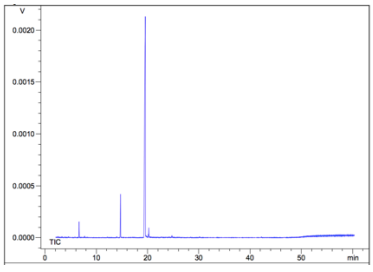

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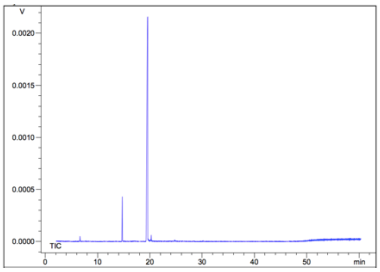

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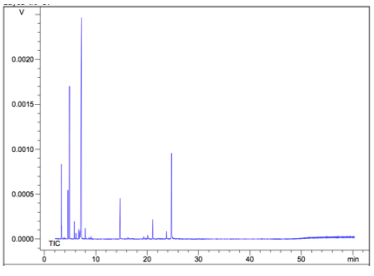
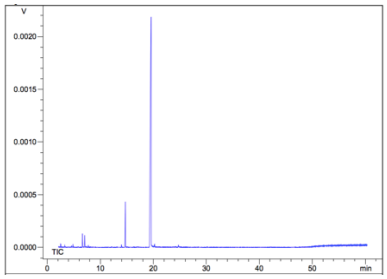
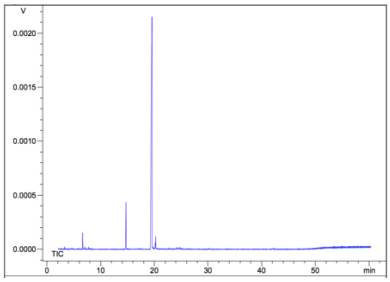


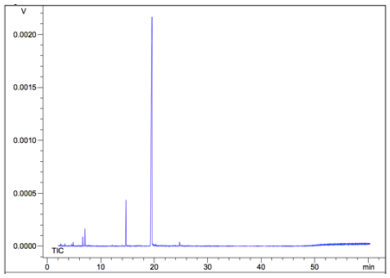
Litsea cubeba LC24



Litsea cubeba BV25

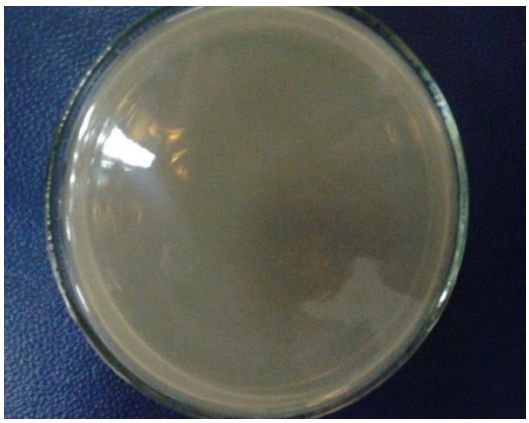


Litsea cubeba TD26

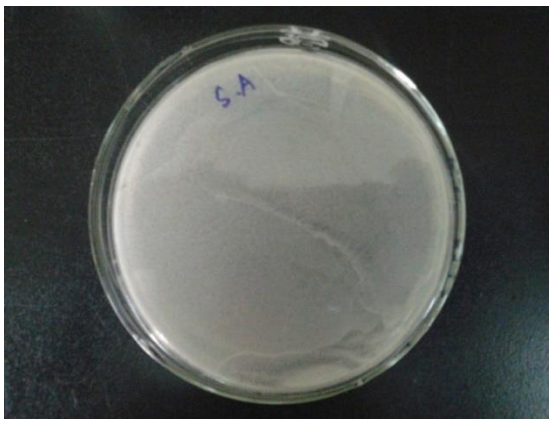


Litsea cubeba BV27

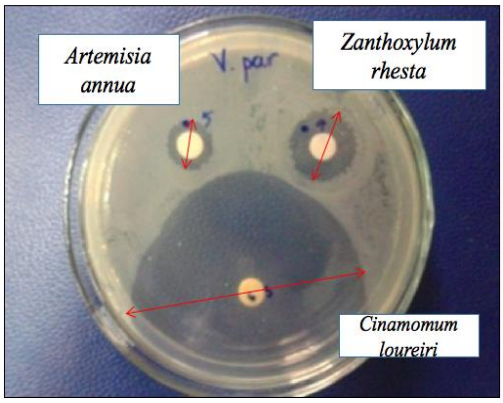
Figure 6: Chromatogram of 25 *Litsea cubeba* leaf essential oils



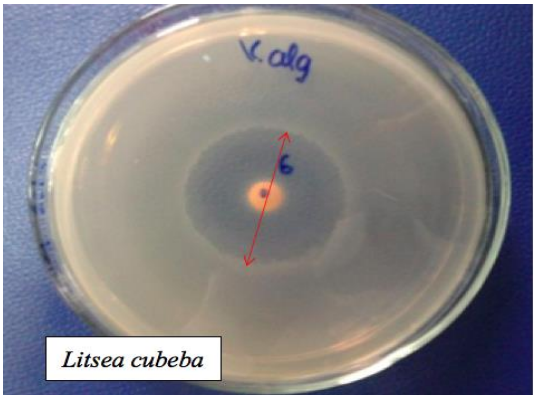
Control negative



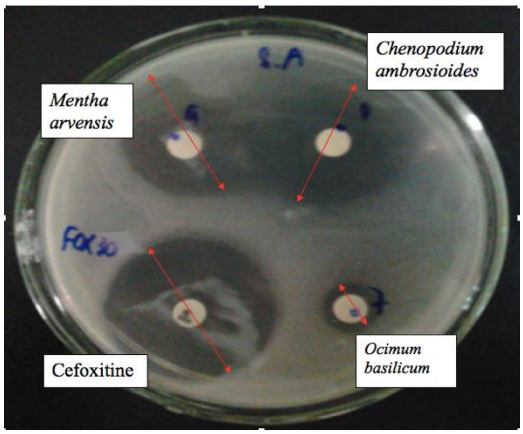
Control positive



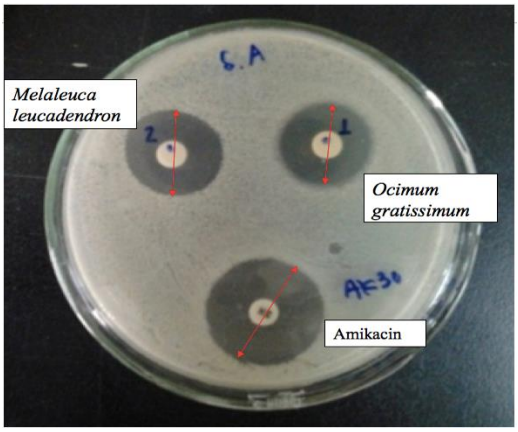
Zone inhibition of cinnamon *C. loureiri*, Indian prickly ash *Z. rhesta*, Sweet wormwood *A. annua* against *V. parahaemolyticus*



Zone inhibition of May chang *L. cubeba* against *V.algenolyticus*



Zone inhibition of basil *O. basilicum*, Mexican tea *C. ambrosioides*, corn mint *M. arvensis* and cefoxitine against *S. aureus.*



Zone inhibition of clove basil *O.gratissimum*, cajeput *M. leucadendron* and amikacine against *S. aureus*

Figure 7: Zone inhibition of essential oil and antibiotic against pathogenic strains

Figure 8: Microscopy LEICA DM6000B

Figure 9: Water quality test kit

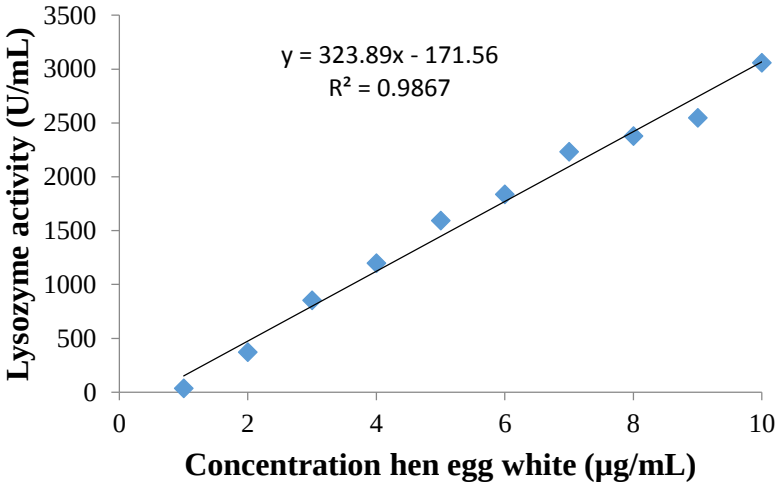


Figure 10: Lysozyme hen egg white standard

Figure 11: Preparation of carp feed