國立屏東科技大學生物科技系
Department of Biological Science and Technology
National Pingtung University of Science and Technology

碩士學位論文
Master Thesis

微脂囊包覆肉桂醛對水產病原菌之抗菌研究

Studies on the Antimicrobial Effect of Liposome-Emulsified Cinnamaldehyde Against to Aquatic Pathogens

指導教授：胡紹揚 博士
Adviser：Shao-Yang Hu, Ph.D
Prof. Ir. Marsoedi, Ph.D

研究生：艾寧
Graduate student：Elok Ning Faikoh

中華民國102年7月25日
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摘要

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Prof. Ir. Marsoedi, Ph.D

論文摘要內容:

廣泛分布於世界各處的水生病原菌是導致水產養殖損失的一大主因，革蘭氏陰性菌是引起魚類疾病的主要細菌。為了治療魚類疾病，抗生素普遍地應用於水產養殖領域，然而持續性地使用抗生素化學藥劑，不僅增加生產成本也使問題更為嚴峻，水產上抗生素的使用不但有環境污染的疑慮，藥物的殘留也影響人們的健康，且病原菌演化為具有抗藥性問題菌株。肉桂醛是由肉桂樹皮上所提煉的精油經體外實驗證實具有多種生物活性，本研究將探討微脂囊包覆肉桂醛(LEC)的抗病原菌活性與魚、蝦類免疫相關基因的表現。藉由體內試驗與即時定量聚合酶鏈式反應驗證本研究的抗菌活性。實驗結果顯示，微脂囊包覆肉桂醛可抑制 *Aeromonas hydrophila, Streptococcus agalactiae, Vibrio vulnificus, Vibrio alginolyticus* 與 *Vibrio parahaemolyticus* 的生長，LEC 也提高了注射病原菌的斑馬魚與泰國蝦的存活率，將感染 *V. vulnificus* 的斑馬魚使用了 LEC 提高了 60%的存活率，與此同時，感染了 *S. agalactiae* 與 *A. hydrophila* 的斑馬魚持續使用了 LEC 給藥八天後，存活率分別提高了 30%與 38%；注射 *V. alginolticus* 的泰國蝦藉由 LEC 治療，因而提高至 40%的存活率。另一方面，LEC 抑制了斑馬魚 ILs, TNFα 與 TLRs 的基因表現。以上結果推測，LEC 可用來當作水產養殖的免疫抑制與抗微生物試劑。

關鍵字：微脂囊包覆肉桂醛、抗菌活性、水產病原菌、基因表現

Abstract

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Name of Institute: National Pingtung University of Science and Technology

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Name of Student: Elok Ning Faikoh Advisor: Shao-Yang Hu, Ph.D

Prof. Ir. Marsoedi, Ph.D

The contents of abstract in this thesis:

Worldwide bacteria cause significant stock losses in aquaculture. Most of the bacterial fish diseases are caused by Gram negative bacteria. To treat the disease in aquaculture, antibiotics are generally applied. Uses continually of chemicals, especially antibiotics, not only increase production costs but also intensify adverse consequences. The use of antibiotics for aquatic animals may not only initiate environmental pollution problems but also can affect human health due to drug residues and the development of resistant strains of pathogens. Cinnamaldehyde is the essential oil from cinnamon bark that exhibits various biological activities *in vitro*. This study focus on the immuno-modulatory, effects of liposome-emulsified cinnamaldehyde (LEC) on antibacterial activity of a several pathogens and expression of immune-related gene in fish and crustacean. Assay of antibacterial activity, *in vivo* challenge test, and quantitative real time PCR to determaine the level

expressions of immune-related gene were carried out in this study. The results have shown liposome-emulsified cinnamaldehyde can inhibit the growth of *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Vibrio vulnificus*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus.* LEC also can increase the survival rate of zebrafish and giant fresh water prawn (*Macrobrachium rosenbergii*) after pathogen injection. LEC can increase up to 60% survival rate of zebrafish infected *V.vulnificus*. Meanwhile, survival rate of zebrafish infected by *S. agalacticiae* and *A. hydrophila* then treated with LEC consecutively was increase up to 30% and 38 % after 8 days treatment. LEC can increase survival rate of *M. rosenbergii* up to 40% after injected by *V. alginolyticus*. In other hand, LEC can suppress the expression of ILs, TNFα, and TLRs in zebrafish that immersed with LEC. It is assumed that LEC is a potential immunosuppression and antimicrobial agent for against bacterial infection in aquaculture.

Keywords: Liposome-emulsified cinnamaldehyde, Antibacterial activity, Aquatic pathogens, Gene expression

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I must express thankfulness to NPUST scholarship, Department of Biological Science and Technology, and Double Degree Program University of Brawijaya Indonesia and also Faculty of Fisheries and Marine Science Indonesia for their support during my master degree education and improving my knowledge. It is a pleasure to thank those who made this thesis possible my advisor, Dr. Shao-Yang Hu and Prof. Ir. Marsoedi, PhD whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. And I am indebted to my many of my Lab. mates to support me and their assistance to teach me how handles the experiment correctly.

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I. Introduction

1.1 Background

Worldwide bacteria cause significant stock losses in aquaculture. Most of the bacterial fish diseases are caused by Gram negative bacteria however there are a few Gram positive bacteria that cause disease. *Vibrio* spp., have been implicated as the main bacterial pathogens of shrimps. The number of reported *Vibrio* species has increased rapidly in the last decade. Thompson et al. (2004) have reported 63 environmental species comprising the genus *Vibrio*, *Aeromonas* and *Salmonella* are also very common species of bacteria found in fresh, brackish and coastal water that can infect a wide range of fish species.

To treat the disease in aquaculture, antibiotics are generally applied orally by mixing with feed or injection. Uses continually of chemicals, especially antibiotics, not only increase production costs but also intensify adverse consequences. The use of antibiotics for aquatic animals may not only initiate environmental pollution problems but also can affect human health due to drug residues and the development of resistant strains of pathogens (Tonguthai, 2000). Therefore, it is necessary to exploit non chemotherapeutics methods instead of the chemotherapeutic methods, such as in the use of the vaccine, probiotic, immunostimulants and natural therapeutics from plants.

Cinnamaldehyde is the organic compound that gives cinnamon its flavor and odor. This pale yellow, viscous liquid occurs naturally in bark of cinnamon trees and other species of *Cinnamomun*. Several report have documented that cinnamaldehyde exhibit various biological activities *in vitro*,

such as inhibit the growth of all the 30 *H. pylori* strain tested, at a concentration of 2 µg/ml, in the 9th and 12th hours of incubation respectively (Ali et al., 2005). Zhou et al. (2007) also reported that the lowest concentrations of cinnamaldehyde, thymol and carvacrol inhibiting the growth of *Salmonella typhimurium* significantly. Other investigation reported that cinnamaldehyde can inhibit the growth of adenovirus (Liu et al., 2009).

However, the applied aspects of cinnamaldehyde in aquatic animals were restricted due to the property of poor water soluble. Liposome is an artificial microscopic vesicle consisting of an aqueous core enclosed in one or more phospholipids layers, used to convey vaccines, drugs, enzymes, or other substances to target cells or organs. It is a well-established matter that offers a dynamic and adaptable technology for improving substances solubility due to their biphasic characteristic.

In present study, the essential oil from the bark of cinnamon trees was used to evaluate potential as well as therapeutic agent and immunostimulant in aquaculture. The liposome-emulsified cinnamaldehyde (LEC) were used to test antibacterial activity against pathogens in aquaculture. The immuno-modulatory effects of liposome-emulsified cinnamaldehyde on the antibacterial activity, expression of immune-related gene and survival rate in giant fresh water prawn and zebra fish was investigated.

1.2 Objectives of Study

Antibacterial research using plant extract has gained momentum since a long time ago. Several medicine herbs have already been tested and used with good result on the control of bacterial diseases in aquaculture. This study focus on the immuno-modulatory, effects of LEC on antimicrobial activity of several aquatic pathogens and their expression of immune-related genes.

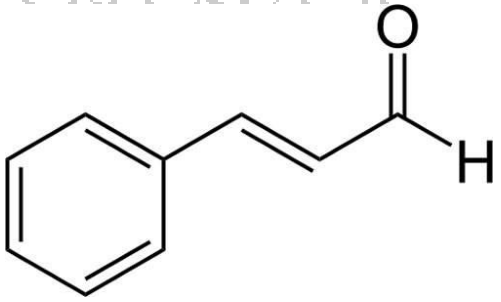
II. Literature Review

2.1 Cinnamaldehyde

Cinnamaldehyde is an aromatic aldehyde and main component of bark extract of cinnamon (*Cinnamomum verum*) (approximately 65%) (Holley and Patel, 2005 and Lens-Lisbonneetal et al., 1987). Cinnamaldehyde, or 3-phenylprop-2-enal to use its IUPAC name (Khan and Ahmad, 2012), is an oily yellow liquid at room temperature, Miscible with alcohol, ether, chloroform, oils (O'Neil, 2006) with boiling point 253˚C at 760 MM HG (Lide, 1991).

2.1.1 Structure and Synthesis

The natural cinnamaldehyde product from cinnamon is trans-form (Subash Babu et al., 2007). Egawa et al. (2008) was described the molecular structure of trans-cinnamaldehyde ((E)-3-phenyl-2-propenal) which determined by means of gas electron diffraction. It was found that this molecule has two stable conformers, s-cis and s-trans, which differ in the orientation of the —CH=O group. The C1, C2 and C6 atoms are on the ring with the C1 attached to the —CH=CH—CHO group, and the C2 and C6 are on the cis and trans sides to the C=C bond, respectively. Cinnamaldehyde has a molecule formula C9H8O (see below).



Chemical structure of cinnamaldehyde (Chang et al., 2001)

Cinnamaldehyde has been efficiently isolated in high purity by fractional distillation from cassia and cinnamon bark essential oils (Kirk-Othmer, 1991). For the synthesis, only the base-catalyzed condensation of benzaldehyde with acetaldehyde has been adopted on an industrial scale (Ullmann, 2003).

2.1.2 Application

Several reports have documented that cinnamaldehyde can used as an antimicrobial. Ali et al. (2005) report that Eugenol and cinnamaldehyde inhibited the growth of all the 30 *H. pylori* strains tested, at the concentration of 2 μg/ml. Both oil and pure cinnamaldehyde of *C. cassia* were equally effective in inhibiting the growth of various isolated of bacteria including Gram positive (*Staphylococcus aureus*), and Gram negative (*E. coli, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholera, Vibrio parahaemolyticus, and Salmonella typhymurium*). The MICs of both oil and cinnamaldehyde for bacteria ranged from 75 μg/ml to 600 μg/ml (Ooi et al., 2006).

Another use of cinnamaldehyde is an antiviral. Several reports have documented that cinnamaldehyde exhibit various biological activities *in vitro*. Such as can inhibit the growth of influenza A/PR/8 virus *in vitro* and *in vivo*. Cinnamaldehyde inhibited the virus growth in a dose-dependent manner (20-200 μM), and at 200 mM, the virus yield was reduced to an undetectable level (Hayashi et al., 2007).

However, the use of cinnamaldehyde against bacterial pathogens in aquaculture still lack because of their poor solubility.

2.2 Bacterial Pathogens in Aquaculture

The intensification of the aquaculture industry and the *trans*fer of aquatic organisms worldwide have been accompanied over the last two decades by an increased incidence of infectious pathogens. Pathogens in fish and crustacean can be caused by viruses, bacteria, fungi, and parasites.

Among the infectious diseases, bacterial fish diseases are reported to infest to most of the cultivable as well as wild fish species. There are 40 - 60 bacterial fish pathogens found to be involved in fish diseases. Generally, fishes are prone to various microbial diseases because they live in potentially hostile world filled with bewilder array of infectious microbes which would very happily use fish as aliment and render them to a variety of ailments.

2.2.1 *Aeromonas hydrophila*

Aeromonas hydrophila is an oxides-positive, glucose-fermenting, Gram negative rod. It is found in fresh and brackish water, sewage, soil, and foodstuffs (Snower et al., 1989).

Aeromonas hydrophila causes disease in fish known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia," "Ulcer Disease," or "Red-Sore Disease." The many synonyms of this disease relate to the lesions caused by this bacterium which include septicemia where the bacteria or bacterial toxins are present within numerous organs of the fish, and ulcers of the fish's skin. The disease caused by this bacterium primarily affects freshwater fish such as catfish, several species of bass, and many species of tropical or ornamental fish. Unsatisfactory water quality such as high nitrite levels, low levels of dissolved oxygen, or high levels of carbon dioxide are more susceptible to infection by *Aeromonas hydrophila* (Swann and White, 1991).

2.2.2 *Streptococcus agalactiae*

Streptococcus agalactiae (also known as goup-Bstreptococcus or beta-strep) is a Gram positive coccus, non-motile, non-spore-forming, catalase-negative, spherical or avoid, and less than 2 µm in diameter. It is usually β-hemolytic and can grow in pairs or short chains (Rajagopal, 2009).

The last few years saw numerous *S. agalactiae* infection outbreaks and were documented in many fish farms especially tilapia farms in Asia including Malaysia (Suanyuk et al., 2005). Eldar and Ghittino (1999) reported marked clinical signs such erratic swimming, decrease in feeding, lethargy, exophthalmia with intraocular hemorrhage and corneal opacity and as cites in the fishes infected by *Streptococcus* sp.. Salvador et al. (2005) observed a high morbidity and mortality in cultivated tilapia infected with *S. agalactiae* which presented erratic swimming, loss appetite, exophthalmia and visceral cavity distension as main clinical signs.

2.2.3 *Vibrio vulnificus*

Vibrio vulnificus is a species of Gram negative, motile, curved, rod-shape bacteria of the genus *Vibrio*. Present in marine environments such as estuaries, brackish ponds, or coastal areas (Jones and Oliver, 2009). *V. vulnificus* can be found free-living, and may associate with zooplankton and other aquatic biological flora. It is taken up by filter-feeding mollusks such as oysters, clams, mussels, and scallops, and becomes concentrated in the gut and other tissues. Under certain conditions this bacterium has the ability to cause serious and often-fatal infections. These include an invasive septicemia usually contracted through the consumption of raw or undercooked shellfish, as well as wound infections acquired through contact with shellfish or marine waters where the organism is present (Strom and Paranjpye, 2000).

2.2.4 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a curved, rod-shaped, Gram negative bacterium found in brackish saltwater. *V. parahaemolyticus* is oxidative positive, facultative aerobic, and does not form spores. Like other members of the genus *Vibrio*, this species is motile, with a single, polar flagellum (Ryan and Ray, 2010).

Numerous *Vibrio* species have been reported as pathogenic to various penaeid shrimps. Among them, *V. parahaemolyticus* has emerged as an important shrimp pathogen. Vibriosis causes mortality in larvae, post larvae, juveniles, sub adults and also adults.

2.2.5 *Vibrio algynolyticus*

Vibrio alginolyticus is a hemophilic (salt-tolerant) Gram negative bacterium found naturally in temperate marine and estuarine environments. *Vibrio alginolyticus* was isolated from diseased *Litopenaeus vannamei* (also called *Penaeus vannamei*) in Taiwanese culture ponds. The diseased shrimp displayed poor growth, anorexia, inactivity, reddish pleural borders of antennae, uropods and telson, opaque and whitish musculature, and mortality (Liu et al., 2009).

2.3 Antibacterial Activity of Cinnamaldehyde and Its Mechanism of Action

Several studies report that cinnamaldehyde has an antimicrobial activity against several pathogens including Gram positive (*Staphylococcus aureus*), and Gram negative (*Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholera, Vibrio parahaemolyticus, and Salmonella typhymurium*).

Brackman et al. (2008), determine the mechanism of action of cinnamaldehyde and evaluate its effect on virulence of *Vibrio* spp. *in vivo* and *in vitro*. The results indicate that cinnamaldehyde and several substituted derivatives interfere with AI-2 based QS without inhibiting bacterial growth. The active compounds neither interfered with the bioluminescence system as such, nor with the production of AI-2. Study of the effect in various mutants suggested that the target protein is LuxR. Mobility shift assays revealed a decreased DNA-binding ability of LuxR. The compounds were further shown to (i) inhibit biofilm formation in several *Vibrio* spp., (ii) result in a reduced ability to survive starvation and antibiotic treatment, (iii) reduce pigment and protease production in *Vibrio anguillarum* and (iv) protect gnotobiotic *Artemia* shrimp against virulent *Vibrio harveyi* BB120.

Another investigation reported that antibacterial activity is caused by aldehyde group in cinnamaldehyde. According to the Wendakoon and Sakaguchi (1995), the carbonyl group on cinnamaldehyde was thought to be binded to protein, preventing the action of enzyme in *Enterobacter aerogenes*.

2.3 Antibiotic Use in Aquaculture and Development of Antibiotic Resistance

Common antibiotic used in aquaculture are: penicillin, cephalosporin, tetracycline, chloramphenicol, amino glycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones. The application of antibiotics at subtherapeutical levels for increased growth and feed efficiencies is highly controversial. The following antibiotics are approved for those two purposes in the US: ampicillin, arsenilic acid, bacitracin, bambermycin, chlortetracycline, dihydrostreptomycin, efrotomycin, lacalocid, monensin, oleandomycin,

penicillin, roxarsone, spectinomycin, tylosin and virginiamycin (Prescott and Baggot, 1993).

The use of antibiotics for aquatic animals may not only initiate environmental pollution problems but also can affect human health due to drug residues and the development of resistant strains of pathogens (Tonguthai, 2000). *Vibrio* spp. isolated from shrimp hatcheries in Indonesia has demonstrated multiple antibiotic resistances to antimicrobials such as ampicillin, tetracycline, amoxycillin and streptomycin (Tjahjadi et al. 1994). Miranda and Zemelman (2002) reported that bacteria resistant to six to ten antibacterial were common. Studies on the antibiotic resistance in bacteria from shrimp ponds. Tendencia and de la Peña (2001), demonstrated a correlation between multiple bacterial antibiotic resistance levels and use of particular drugs.

Ko et al. (1996) also reported that *Aeromonas* strain in Taiwan, primarily *A. hydrophila* were found to be susceptible to moxalactam, ceftazidime, cefepime, aztreonam, imipenem, amikacin, and fluoroquinolones, but they were more resistant to tetracycline, trimethoprim-sulfamethoxazole, some extended-spectrum cephalosporins, and aminoglycosides than strains from the United States and Australia. Kim et al. (2004), report the onother tetracycline resistance was detected in 34 Japanese and Korean isolates, which included *Vibrio* sp., *Lactococcus garvieae*, *Photobacterium damsela* subsp. *piscicida*, and unidentified Gram positive bacteria.

2.4 Fish Cytokines and Immune Response

2.4.1 Innate and Adaptive Immune Response

The immune system of fish is physiologically similar to that of higher vertebrates, despite certain differences. In contrast to higher vertebrates, fish

are free-living organisms from early embryonic stages of life and depend on their innate immune system for survival (Rombout et al., 2005). Nonspecific immunity is a fundamental defense mechanism in fish. In addition, it plays a key role in the acquired immune response and homeostasis through a system of receptor proteins. These receptor proteins identify molecular patterns that are typical of pathogenic microorganisms, including polysaccharides, lipopolysaccharide (LPS), peptidoglycan bacterial DNA, viral RNA and other molecules that are not normally on the surface of multicellular organisms. This response is divided into physical barriers and cellular and humoral immune response. These immunological parameters include growth inhibitors, lytic enzymes, the classic complement pathways, the alternative and lectin pathway, agglutinins and precipitins (opsonins and primary lectins), antibodies, cytokines, chemokines and antibacterial peptides.

Various internal and external factors can influence innate immune response parameters. Temperature changes, stress management and density may have suppressive effects on this type of response, while several food additives and immunostimulants can enhance their efficiency (Magnadottir, 2010).

The adaptive system recognizes foreign structures by means of two cellular receptors, the B cell receptor (BCR) and the T cell receptor (TCR). Adaptive immunity is highly regulated by several mechanisms. It increases with antigen exposure and produces immunological memory, which is the basis of vaccine development and the preventive function of vaccines (McHeyzer-Williams and McHeyzer-Williams, 2005). The adaptive response generally starts days after infection and is capable of recognizing specific protein motives of peptides, which leads to a response that increases in both speed and magnitude with each successive exposure (Dixon and Stet, 2001).

The main effectors cells of the adaptive immune response are the lymphocytes, specifically B cells and T cells. When B cells are activated, they are capable of differentiating into plasma cells that can secrete antibodies. Upon activation T cells differentiate into either helper T cells or cytotoxic T cells. Helper T cells are capable of activating other cells of 4 New Advances and Contributions to Fish Biology the adaptive immune response such as B cells and macrophages, while cytotoxic T cells upon activation are able to kill cells that have been infected (Abbas, 2007).

2.4.2 Fish cytokines

Cytokines are secreted proteins with growth, differentiation, and activation functions that regulate the nature of immune responses. Cytokines are involved in several steps of the immune response, from induction of the innate response to the generation of cytotoxic T cells and the production of antibodies. In higher vertebrates, the combination of cytokines that are secreted in response to an immune stimulation induces the expression of immune-related genes through multiple signaling pathways, which contributes to the initiation of the immune response. Cytokines can modulate immune responses through an anticrime or paracrine manner upon binding to their corresponding receptors (Wang et al., 2011).

- Tumor necrosis factor (TNF)

Several studies in fish have provided direct evidence suggesting that TNF-α and -β are important activators of macrophages. Studies in rainbow trout, turbot, sea bream (*Sparus aurata*), goldfish (*Carassius auratus*) and catfish have shown that TNF causes the activation of macrophages, leading to increased respiratory activity, phagocytosis and nitric oxide production (Reyes-Cerpa et al., 2012).

- Interleukins

IL-1 in mammals is comprised of 10 ligands and 10 protein receptor molecules and plays an important role in inflammation and host defense (Dinarello, 1997). IL-1β has been detected in 13 teleost fish species and is involved in the regulation of immunity through the stimulation of T cells. The function of IL-1β in these fish species is analogous to mammalian IL-1β. In teleost fish, IL-1 receptors have been cloned and sequenced from the rainbow trout and Atlantic salmon. The expression of the IL-1 receptor in salmon appears to be constitutive in all tissues tested and is regulated in the anterior kidney, spleen, liver and gills after stimulation with LPS and TNF-α, suggesting a role for the IL-1 receptor in regulating IL-1β during the inflammatory response (Reyes-Cerpa et al., 2012).

- Toll Like Receptor

Receptors that recognize conserved pathogen molecules are the first line of cellular innate immunity defense. Toll-like receptors (TLRs) are the best understood of the innate immune receptors that detect infections in mammals. Key features of the fish TLRs and the factors involved in their signaling cascade have high structural similarity to the mammalian TLR system. However, the fish TLRs also exhibit very distinct features and large diversity which is likely derived from their diverse evolutionary history and the distinct environments that they occupy. Six non-mammalian TLRs were identified in fish. TLR14 shares sequence and structural similarity with TLR1 and 2, and the other five (TLR19, 20, 21, 22 and 23) form a cluster of novel TLRs. TLR4 was lost from the genomes of most fishes, and the TLR4 genes found in zebra fish do not recognize the mammalian agonist LPS and are likely paralogous and not orthologous to mammalian TLR4 genes. TLR6 and 10 are also absent from all fish genomes sequenced to date. Of the at least 16 TLR types

identified in fish, direct evidence of ligand specificity has only been shown for TLR2, TLR3, TLR5M, TLR5S and TLR22 (Reyes-Cerpa et al., 2012)

III. Material and Methods

3.1 Materials

3.1.1 Animals

a. Zebrafish

Zebrafish (*Danio rerio*), AB strain is wild type zebrafish were purchased from the Zebrafish International Resource Center. The fish were raised and maintained in a freshwater recirculating tank with a controlled light cycle of 14 h light/10 h dark at 28˚C. They were fed with commercial feed for three times a day.

b. *M. rosenbergii*

Giant fresh water prawn (*M. rosenbergii*) were obtained from a local aquaculture farm in southern Taiwan and were acclimated in glass aquarium tank for seven days with aerated freshwater at 26-29˚C before experiments started. During the acclimatization period, prawn fed with commercial pellets two times a day. Only prawns in intermoult stage were used in this study. The molt stage was determined by examination of the uropoda in which partial extent of epidermis retraction could be distinguished (Peebles, 1978).

3.1.2 Pathogens

- *Streptococcus agalactiae*

These strain bacteria were provided by Prof. Jyh-Yih Chen, Marine Research Station, Institution of Cellular and Organismic Biology, Academica Sinica, Taiwan.

- *Aeromonas hydrophila*
- *Vibrio vulnificus*
- *Vibrio parahaemolyticus*

- *Vibrio alginolyticus*

Aeromonas and *Vibrio* strain were provided by Prof. Chun-Hung Liu. Department of Aquaculture, National Pingtung of Science and Technology, Taiwan.

3.1.3 Culture Medium

a. Tryptic Soy Broth (TSB)

- Add 7.5 g of Bacto™ Tryptic Soy Broth (Becton, Dickinson and Company, USA) into flask 250 ml
- Add ddH_2O to 250 ml
- Autoclave at 121˚C for 15 min

b.TSB plate

- Add 7.5 g of Bacto™ Tryptic Soy Broth (Becton, Dickinson and Company, USA)
- Add 3.75 g NaCl (Merck)
- Add 3.75 g Agar A (Bio Basic Inc., Canada)
- Add ddH_2O to 250 ml
- Autoclave at 121˚C for 15 min
- Pour into petri dish (±25 ml/10 cm dish)
- Allow the agar to set into the dishes by leaving them on a flat, clean surface to dry in the room temperature. Secure lids once set and store upside down.

c. Tryptic Soy Broth + 1.5% NaCl

- Add 7.5 g of Bacto™ Tryptic Soy Broth (Becton, Dickinson and Company, USA)
- Add 3.75 g NaCl (Merck)
- Add ddH_2O to 250 ml

- Autoclave at 121˚C for 15 min

d. TSB plate + 1.5% NaCl

- Add 7.5 g of Bacto™ Tryptic Soy Broth (Becton, Dickinson and Company, USA)
- Add 3.75 g NaCl (Merck)
- Add 3.75 g Agar A (Bio Basic Inc., Canada)
- Add ddH_2O to 250 ml
- Autoclave at 121˚C for 15 min
- Pour into Petri dish (±25 ml/10 cm dish)
- Allow the agar to set into the dishes by leaving them on a flat, clean surface to dry in the room temperature. Secure lids once set and store upside down.

3.1.4 Chemicals

- iScript reverse *trans*criptase (Bio-Rad, USA) cat. No. #170-8891
- SYBR gene PCR kit (Qiagen) cat. No. #170-8882AP
- Bacto™ Tryptic Soy Broth (Becton, Dickinson and Company, USA) cat. no. 211825
- NaCl (Merk)
- Agar A (Bio Basic Inc., Canada) cat. No. G1212
- Tetracycline hydrochloride (Sigma-Aldrich,Inc.,Germany) cat. No. T7660-5G
- Kanamycin sulfate (Sigma-Aldrich, Inc., Germany) cat. No. K1377-5G
- Ampicillin (BioShop Canada, Inc.) cat. No. AMP201.25
- Liposome-emulsified cinnamaldehyde (6% cinnamaldehyde), provide by Medisome Enterprise Ltd. Company

- Resazurin solution : dissolving a 0.1g Resazurin sodium salt (Sigma, USA) in 100 ml of sterile distilled water. Homogenized with vortex mixer. cat. No. R7017.5G
- TriPure Isolation Reagent (Roche, Germany) cat. No. EP055435
- Diethyl pyrocarbonate, DEPC (Sigma, USA) cat. No. D5758-25ML
- Chloroform (J.T. Baker, USA) cat. no. 9180-01
- Isopropanol alcohol (Sigma, USA) cat. No. I9516-500ML
- Ethanol (Sigma, USA) cat. no. 32221-1L
- TAE solution 25X (Zeju, USA) cat. No. R2003-25
- EtBr (Alfa Caesar, UK) cat. No. J62282 ea
- Agarose (SeaKem, USA) cat. no. 50004
- Hydrogen peroxide (Sigma, USA) cat. No. H3410-1L

3.1.5 Facility and Equipment

- qPCR machine (Siegen)
- PCR machine (Bio-Rad, USA)
- Vortex mixer and Shaker Vortex Genie® 2 (Scientific Industries, USA)
- Low temperature centrifuge (GOYZEN, 1730MR)
- Homogenizer T10 basic (IKA, Germany)
- NanoDrop MicroSpectrofotometer (Clubio CB2800, Taiwan)
- 4˚C Refrigerator (King Cool)
- -20˚C Refrigerator (King Cool)
- -80˚C (Innova, U725)
- Microwave (Panasonic, NN-ST557)
- Orbital Shaker Incubator (JSL-530)
- Freshwater resirculating tank (Taikong Corp. CAP-5000)
- Pipet (Eppendorf; Rainn)

3.2 Methodologies

3.2.1 Preparation of Liposome-emulsified Cinnamaldehyde

Cinnamaldehyde (2E-3-phenylprop-2-enal) (CAS RN.: 104-55-2) was purchased from Panreac Quimica (S.A., Spain). The nano-emulsified cinnamaldehyde was prepared by high pressure homogenization (HPH) procedure. A 5 (v/v) % cinnamaldehyde, 10 (v/v) % lecithin and 0.5 (v/v) % α-tocopherol was gently mixed in 5% ethanol which supplemented with potassium dihydrogen phosphate to maintain the pH of mixed solution in 6.7. The mixed solution was processed into nano- emulsified cinnamaldehyde by HPH M-110P Microfludizer (Microfludics, Newton, MA, USA) with the set pressure around 5,000-30,000 psi and 50-100 ml/min of flow rate. Cinnamaldehyde in nano-emulsified cinnamaldehyde was replaced with equal volume of 5 % ethanol, and named as nano-emulsified ethanol for using as a control.

3.2.2 Determination of MIC and MBC of LEC against Aquatic Pathogens

A. hydrophila, V. vulnificus, S. agalactiae, V. parahaemolyticus and *V. algynolyticus* pathogenic organisms were stored in 40% glycerol at -20°C and used in this study. Frozen glycerol cell stock (3µl) was inoculated into a 3 ml tryptic soy broth (TSB medium with 1.5% NaCl for *Vibrio* strains and without NaCl for *A. hydrophila* and *S. agalactiae*) in a shaking incubator at 27˚C at 125 rpm. After 12 hr cultivation, 3 µl of culture was *trans*ferred to a 3 ml the same medium and incubated for 9 hr under the same conditions as the seed culture.

Based on the Sarker et al., (2007), minimum inhibition concentrations (MICs) were determined using Microtitre plate with Resazurin solution as indicator bacterial growth. The resazurin solution was prepared by dissolving

a 270 mg tablet in 40 ml of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

Plates were prepared under aseptic conditions. A sterile 96 well plate was labeled. Approximately, 67 µl of sterile TSB + 1.5% NaCl were added to all wells in 96 microtitre plates. Resazurin solution were added in each well (13 µl per well). Dilutions of LEC in different concentration were added 10 µl in each well. Finally, 10 µl of bacterial suspension (1 x 10^6 CFU/ml) was added to each well and incubated overnight at 38˚C. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value.

Then to determine Minimum bactericidal concentration (MBC), from each MIC well without visible growth, a 100 µl volume of the broth were aliquot onto TSB with 5% NaCl agar and spread across the entire surface of the plate. The dilution of the sub cultured MIC well on each plate was recorded and incubates at 28˚C for 12-14 h. Following overnight incubation, examine the MBC plates for colony growth or lack of growth for each dilution sub cultured. No growth indicates that the antibiotic was bactericidal at that dilution. Growth indicates that the antibiotic was bacteriostatic but not bactericidal at that dilution (Ali *et al.*, 2005).

In this study, we also determine the MIC and MBC of some antibiotics such as kanamycin ampicillin, and tetracycline used as a comparison.

3.2.3 Susceptibility Test of LEC against Aquatic Pathogens by disc diffusion methods

The bacterium *V. alginolyticus, V. vulvunicus, and V. parahaemolyticus* stocks were cultured on TSB with 1.5% NaCl, for 12 hour at 28˚C. After

incubation, 3 µl of innoculum was *trans*ferred to 3 ml tryptic soy broth (TSB supplemented with 1.5% NaCl) and incubated for 9 hour at 28˚C . For *A. hydrophila* and *S. agalactiae*, all the culture conditions are the same with Vibrio except the TBS medium without 1.5% NaCl.

The disc diffusion method was used to carry out the antibacterial sensitivity assay for antibiotic standard and bioactive compound (LEC). A 0.1% (v/v) of seed culture were took respectively and added into TSB that contains 1.5% (w/v) agar, and mixed well then 20 ml of the liquid agar plate was poured into a Petri dish. To determine the diameter of the inhibition zone, the 15 µl of liposome with and without emulsified cinnamaldehyde were added on a 0.7 cm diameter filter paper on the TSB plate containing tested bacteria and incubated for 12 h at 27˚C. The antibacterial efficacy of LEC was calculated on the basis of the diameters of inhibition zones obtained from antibiotic standard like ampicillin, kanamycin and tetracycline on the same plate.

2.3.4 *In vivo* challenge analysis

a. Determination Tolerable Concentration of LEC for Zebrafish

Adult AB zebrafish were incubated at 28˚C in different concentration of LEC. The LEC concentration are 25 µl/L LEC (equal to 1.5 mg/L cinnmaldehyde), 50 µl/L LEC (equal to 3 mg/L cinnmaldehyde), 75 µl/L LEC (equal to 4.5 mg/L cinnmaldehyde), 100 µl/L LEC (equal to 6 mg/L cinnmaldehyde), and 125 µl/L LEC (equal to 7.5 mg/L cinnmaldehyde). Water was renewed everyday. Each group consists of 10 zebrafish. The survival rate of each group was recorded daily .

b. *In vivo* challenge analysis in Zebrafish

The pathogenic bacterium *V.vulnificus, A. hydrophila* and *S. agalactiae* at 10^3 colony-forming unit (CFU)/zebrafish in 10 µl of PBS buffer was intraperitoneal (i. p.) injected into the adult zebrafish (about 4.12±0.3cm in body length; 0.85±0.12 g in body weight) respectively to carry out challenge test. Four different experimental groups for each pathogen were designed. In the first group, zebrafish injected with PBS buffer without pathogen was used as control. The second group, zebrafish injected with PBS containing pathogen was used to evaluate survival rate. The third group, zebrafish was injected with PBS containing pathogen and then cultured in 1L of water which containing 75 µl liposome. The four groups, zebrafish were injected with PBS containing pathogen and then cultured in 1L of water which containing 75 µl of LEC. Each group consists of 10 zebrafish, and each experiment was performed in triplicate. The survival rate of each group was recorded daily after injection for 7 days.

b. *In vivo* challenge analysis in *Macrobrachium rosenbergii*

Three studies were conducted for the experiment of susceptibility of giant fresh water prawn (*M.rosenbergii)* against *V.alginolyticus.* The preparation of pathogen was based on procedure of Cheng et al. (2005). The bacterial was prepared in PBS buffer at 1.6 x 10^6 CFU/ ml as a stock bacterial suspension. In the first group, giant fresh water prawn injected with PBS buffer without pathogen was used as control. The second group, giant fresh water prawn injected with 20 µl PBS containing pathogen into the ventral sinus of the cephalothorax was used to evaluate survival rate. The third group, giant fresh water prawn was injected with PBS containing pathogen and then cultured in 1L water containing LEC. Experimental and control prawn were kept in glass aquaria containing fresh water. Each treatment was conducted

with 10 prawns. Water was renewed daily, and cumulative mortality for each group was observed for seven days.

3.2.5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) for gene expression analysis

Zebrafish RNA was isolated from the whole zebrafish body. RNA was isolated at different times (1D, 3D, and 5D) during immersion with LEC. Tissue was homogenized TriPure Isolation Reagent (Roche, Germany) (1ml/50 mg tissue) following the manufacturer's instructions. Per 1 ml of TRIzol isolation reagent was added 0.2 ml chloroform, cap the tube and shake vigorously for 15-30 second. The sample was store on ice (or 4˚C) for 5 min. The homogenate was centrifuge at 12,000 for 15 minutes at 4˚C. Following centrifugation, the sample forms the lower blue phenol-chloroform phase, interphase, and the upper colorless aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and protein are in the interphase and organic phase.

Aqueous phase was *trans*ferred to a clean tube, 0.5 ml isopropanol was added and stored the sample for 5-10 minutes at room temperature. Centrifuge at 12,000g for 10 minutes at 4˚C-25˚C. RNA precipitate forms a white-yellow pellet at the bottom of the tube. Supernatant was removed and RNA pellet was wash once with 75% ethanol, shake to dislodge the pellet from the side of the tube. Centrifuged for 5 minutes at 7,500g at 4˚C-25˚C. At the end of the procedure, DNA pellet was briefly air-dry (5-10 minutes). After that, RNA was dissolve in water, 0.5 % SDS or buffer by passing the solution through a pipette tip and/or incubating for 10-15 minutes at 55˚-60˚C. Total RNA was quantified through UV spectrophotometry.

cDNA synthesis was carried out by iScript cDNA synthesis kit following the manufacturer's instructions. RNA templete was mixed with 5x iScript

reaction mix (4µl), iScript reverse *trans*criptase (1µl), and nuclease-free water to obtain final volume 20µl. Complete reaction mix was incubated at 25˚C for 5 minutes, 42˚C for 30 minutes, and 85˚C for 5 minutes.

mRNA expression level of IL-1β, -10, -15, and -22, TRL-1, -3, and -4a, TNF-a, and Ef1-α (as an internal control) was measured by quantitative real-time PCR. The specific primers were designed based on published zebrafish cDNAs (Table 1). Each reaction was performed in a total volume of 20 ml using 50µg of cDNA, 0.5 µl of 10 mM of each gene specific primer pair with 10 µl SYBR gene supermix (Qiagen). The PCR reaction condition was set as below: 95˚C for 5 min, step 1, 95˚C for 10 sec, step 2, 60˚C for 30 sec, step 1 and step 2 for 40 cycles. Each reaction was carried out in triplicate and a melting curve analysis was performed to confirm the specificity of the reaction.

3.2.6 Statistical Analysis

Data are expressed as mean ± S.E. A multiple comparison Tukey test was conducted to compare the significant difference among treatments using the SAS computer software (SAS Institute, Cary, North Carolina, USA). For statistically significant differences, *P* value was required ≤ 0.05.

IV. Results

4.1 MIC and MBC of Liposome-emulsified Cinnamaldehyde against Fish Pathogens

The liposome emulsified cinnamaldehyde (LEC) were screened for their antimicrobial activity in compare with standard antibiotic *in vitro* using *A. hydrophila* , *S. agalactiae* and *V. vulnificus*, as test organism. The results of the antimicrobial screening assays are summarized in Table 2. As result shown in Table 2, LEC is effective against Gram negative that commonly attack fish. Minimum inhibitor concentration (MICs) is the lowest concentration of antibiotic that inhibits growth of bacteria. The MIC value against *A. hydrophila* were 1.36 mM, 1.81 mM for *S. agalactiae*, 1.36 mM and for *V. vulnificus*. Meanwhile, the minimum bactericidal concentration (MBCs) of LEC against *S. agalactiae* was 2.72 mM, 3.18 mM for *A. hydrophila*, and 1.81 mM for *V. vulnificus.* Kanamycine and ampicillin are antibiotic that commonly use to treat bacterial pathogens in fish. The results shown that those antibiotics have better bacteriostatic and bactericidal activity against to *A. hydrophila*, *S. agalactiae* and *V. vulnificus*.

4.2 Antimicrobial Potency of LEC against to Fish Pathogens compared with Ampicillin

For further analysis of antimicrobial activity from LEC, we observe the clear zone by paper disc diffusion methods. We use 20 µl of LEC (equal to 1.2 mg) applied in the blank disc. Furthermore, we use ampicillin in the different concentration as a compare. On the basis of the diameters of clear zone derived from the known amount of ampicillin against pathogens on the agar plate, an equation expressed as $y=0.0005x+0.7225$ ($R^2=0.9838$) was used

to calculate the antimicrobial potency against *A. hydrophila* (Figure 1). Therefore, the antimicrobial activity of 1 µg LEC against *A. hydrophila* was equivalent to 0.19 ug of ampicillin. By the similar way, we determine the antimicrobial potency of LEC against *V. vulnificus* and *S. agalactiae*. Results revealed a clear zone of LEC against *V. vulnificus* was 0.82 cm on the agar plate. In accordance with the equation $y=0.0006x+1.086$ ($R^2=0.9693$), the antimicrobial activity of 1 µg of LEC against *V. vulnificus* was equivalent to 0.24 µg of ampicillin (Figure 1). An equation $y=0.0005x+0.7035$ ($R^2=0.9938$) basis on the diameter of clear zone from ampicillin against *S. agalactiae*, reveal that antimicrobial potency of 1 µg LEC against *S. agalactiae* was equivalent to 0.26 ug of ampicillin (Figure 1).

4.3 Survival Rate of Zebrafish Infected Bacterial Pathogens and Treated with LEC

In order to determine the survival rate of zebrafish infected bacterial pathogens which then treated with LEC, the determination tolerable concentration of LEC for zebrafish was conducted. Adult AB strain zebrafish were incubated at 28˚C in a range of LEC (1.5–7.5 mg/L LEC). A phenomena of chest hemorrhage was observed obviously at 1 days post-incubation in 6 mg/L and 7.5 mg/L of LEC, and then fish were dead gradually after consecutive immersion. The concentration of 1.5-4.5 mg/L LEC resulted in 100% survival of zebrafish while increasing concentration to 7.5 mg/L LEC decreased the survival rate to 60% (Figure 2). Therefore, the optimum concentration of LEC is 4.5 mg/L.

4.5 mg/L concentration of LEC then used to immerse zebrafish after injected with bacterial pathogen. Beside immersed with LEC, zebrafish also immersed with only liposome served as a negative control.

Generally, the survival rate of zebrafish after injected with *A. hydrophila*, *S. agalacticiae*, and *V. vulnificus*, in the LEC immersed group was statistically significant higher than in the groups that were immersed with only liposome or just injected with pathogens at several days treatment. The significance of the increase varied with the type of pathogens. Survival rate of zebrafish infected *V.vulnificus* treated with LEC was increased up to 60% after 11 days treatment compared with untreated and negative control (Figure 3). Meanwhile, survival rate of zebrafish infected by *S. agalacticiae* and *A. hydrophila* then treated with LEC consecutively was increase up to 30% (Figure 4) and 38 % (Figure 5) after 8 days treatment compare with untreated and negative control.

4.4 Expression Profiles of Immune-related Genes in Zebrafish Following LEC Immersion

For further explore whether LEC immersion influence immune-related gene expression in zebrafish, quantitative real-time PCR (qPCR) analysis was performed at the different days treatment.

The expression of all pro-inflammatory genes analyzed such as IL 1β, IL 10, IL 15, IL 22, TNF α, TLR 1, TLR 3, and TLR 4a in zebrafish immersed LEC was significantly down-regulated compared with wild type and immersed liposome. For instance, IL1β expression was lower than wild type and zebrafish immersed liposome after 1 day immersion and gradually decrease at 0.6 and 0.5 at 3rd day and 5th day, respectively (Figure 5a). For other interleukin, the expression level of IL 10 was same with wild type at the 1st day's treatment. At the 3rd day and and 5th day, IL 10 expression was significantly down regulated wild type and zebrafish immersed liposome (Figure 5b). This similar trend was also observed in IL 15 (Figure 5c) and IL 22 (Figure 5d).

The TNFα was expressed around 0.5 after 1 day immersion, and it was lower compare with wild type and zebrafish immersed liposome. These patterns were gradually decreased to below 0.5 after 3rd and 5th day of immersion. This was the lowest level of expression observed for any tested gene in this study (Figure 5e).

Dramatic difference was seen in TLR 3 at 1 day after immersion, where the expression level was higher than wild type and zebrafish immersed liposome reach above 1.0. But after 3rd and 5th day of immersion, the expression level of TLR3 was gradually decreased around 0.8 and 0.6 (Figure 5g). For other toll like receptor gene, the expression level of TLR 1 was almost same with wild type at 1st day of immersion which is around 1.0. The TLR 1 expression level slightly decreases at 3rd of immersion with a continuous increase after 5th day immersion (Figure 5f).

TLR 4α has the same trend with almost all expression genes in this study. After 1st, 3rd, and 5th day immersion, the TLR 4α level expression were gradually decrease to around 0.8, 0.5, and 0.25, respectively (Figure 5h).

4.5 MIC and MBC of Liposome-emulsified Cinnamaldehyde against Crustacean Pathogens

Minimum inhibitor concentration (MICs) and minimum bactericidal concentration (MBCs) for *V. alginolyticus* and *V. parahaemolyticus* were determined. MIC and MBC values of LEC against *V. parahaemolyticus* and *V. algynolyticus* were found higher than tetracycline. LEC can inhibit the growth of *Vibrio parahaemolyticus* at 14.1 mM and at 113.5 mM it was bactericidal (table 3). While tetracycline can inhibit the growth of *Vibrio parahaemolyticus* at 1.1 mM and it was bactericidal at 1.7 mM (Table 3).

Vibrio alginolyticus has a high sensitivity against tetracycline. It just needs 0.009 mM to inhibit *Vibrio alginolyticus* and at 0.28 mM it was bactericidal (table 3). Otherwise, LEC can inhibit the growth of *Vibrio alginolyticus* at 28.6 mM and at 113.5 mM it was bactericidal (Table 3).

4.6 Antimicrobial Potency of LEC against to Crustacean Pathogens Compared with Tetracycline

For more confirmation about antimicrobial potency of LEC compared with antibiotic, disc diffusion method was performed. We use 10 µl of LEC (equal to 0.6 mg cinnamaldehyde) applied in the blank disc. On the basis of the diameters of clear zone, we can calculate the antimicrobial potency of LEC compared with tetracycline. Results revealed a clear zone of LEC against *V. parahaemolyticus* was 17.42 mm on the agar plate. In accordance with the equation y=3.725x+11.8 (R^2=0.9919), the antimicrobial activity of 1 µg of LEC against *V. parahaemolyticus* was equivalent to 0.0025 ug of tetracycline(Figure 7).Meanwhile, an equation expressed as y=4.2217x+9.165 (R^2=0.9973) was used to calculate the antimicrobial potency against to *V. alginolyticus*. Therefore, the antimicrobial activity of 1 µg LEC against *V. alginolyticus* was equivalent to 0.0017 ug of tetracycline (Figure 7).

4.7 Survival Rate of *M.rosenbergii* Infected Bacterial Pathogens and Treated with LEC

In order to know the effect of LEC to crustacean survival rate, *M. rosenbergii* was injected with *V. alginolyticus* then immerse with 4.5 mg/L LEC. All unchallenged control (PBS injection) shrimp survived. By contrast, death began to occur after 24 h in challenged shrimp that had been immerse with 4.5 mg/L LEC. After 2nd day of challenge, survival rates of shrimp that had been immerse with 4.5 mg/L LEC were significantly higher than those of

shrimp that had been inject with pathogen without LEC treatment. Survival rate of *M. rosenbergii* injected *V. alginolyticus* then treated with LEC was increased up to 40% after 7 days treatment compared with untreated one and negative control (Figure 7).

V. Discussion

In this study, LEC showed antibacterial activities against aquatic pathogens. Low concentrations of LEC were demonstrated to inhibit the growth of *A. hydrophyla, S. agalactiae, V. vulnificus, V. alginolyticus* and *V. parahaemolyticus* (Figure 2 and Figure 6). These results in agree with study by Shahverdi et al. (2007) who documented that cinnamaldehyde exhibited various biological activities *in vitro.* Antibacterial assay in *Cinnamomunn zeylanicum* showed that both the essential oil and the *trans*-cinnmaldehyde have antimicrobial activity for *Clostridium difficile,* and low concentrations of trans-cinnamaldehyde were demonstrated to enhance the antimicrobial action of antibiotic clindamycin *in vitro.* The cinnamaldehyde from *C. cassia* bark-derived compound was also exhibited antibacterial activity on human intestinal bacteria, *Clostridium.* Another investigation reported that cinnamon extract has been observed to display antimicrobial activity and to be effective against many types of bacteria, such as *Mycobacterium, Staphylococcus, Enterococcus, Pseudomonas* and *Micrococcus* (AĞAOĞLU et al., 2007). However, antibiotic ampicillin and tetracycline show better bacteriostatic and bactericidal activity against to *A. hydrophila, S. agalactiae* and *V. vulnificus* (Table 2). Antibiotics play an important role in the disease management of aquaculture practices, but antibiotic resistance develops readily in pathogens following antibiotic treatment. In consequence, medicinal herbs play an alternative role to tackle this problem.

Another herbal medicine also reported to had antibacterial activity against aquatic pathogens. For instance, studies by Direkbusarakom et al. (1998) reported that *Psidium guajava* and *Momordica charantia* (belong to Thai traditional herb) displayed antibacterial activity again *V. harveyi* and *V. parahaemolyticus.* The MIC value of *P. guajava* against tested bacteria was

found to be 0.625 mg/ml, while the MIC of *M. charantia* was 1.25 mg/ml. Meanwhile, extract of some Indian herb: *Allium sativum* (garlic) and *Myristica fragans* (nutmeg) were shown antibacterial activity again *A. hydrophila* (Indu et al., 2006).

In order to know the effect of LEC *in vivo*, bacterial challenge assay was conducted by using zebrafis and *M. rosenbergii* as animal model. Previously, we need to know the tolerable concentration of LEC to zebrafish and *M.rosenbergii.* Huang and Ho (1998) conduct the experiment to determine cinnamaldehyde toxicity level. Cinnamaldehyde were tested against *Tribolium castaneum* adults and larvae and *Sitophilus zeamais* adults. *T. castaneum* and *S. zeamais* adults showed similar susceptibility to the contact toxicity of cinnamaldehyde, both having an LC_{50} of 0.7 mg cm^{-2} and an LC_{95} of 0.9 mg cm^{-2}. Meanwhile, the tolerable concentration of Zebrafish and *M rosenbergii* to LEC were 75 µl/L. So, we use that concentration for the next assay.

In the challenge test, zebrafish received LEC via immersion showed increase resistance against *V.vulnificus, A. hydrophila,* and *S. agalactiae* compare with untreated and negative control. Survival rate in the *M. rosenbergii* also increase following the LEC immersion. This present study is in conjunction with the study by Alsaid et al. (2010) that feeding the fish with feed supplemented with dry bark powder of cinnamon (18.7%) or extract of *C.verum* (11.5%) significantly helped to reduce cumulative mortality after challenging the fish with *S. agalactiae* and had no toxic influence on the fish.

Similar findings were reported in the Atlantic salmon (*Salmo salar*) fed with the immunostimulant algibind wet feed when challenged with *Aeromonas salmonicida*. The algibind diet fed group got improved survival, growth and reduced bacterial load (Nordmo et al., 1995). The dietary

incorporation of β-1,3 glucan from *Schizophyllum communae* was enhancing the resistance of post-larvae, juveniles and adults of *P. monodon* to white spot syndrome virus (Chang et al., 2003). Song and Sung (1993) also reported that wheat germ agglutinin (WGA), a lectin administered as feed additive, has promoted the bacterial resistance of *Penaeus orientalis*. From the present results, it is evident that the juveniles of *P. indicus* fed with the enriched herbal and seaweed diets displayed better survival and growth in addition to inhibit bacterial load. This may be due to the improved immunostimulant effect in their bodies by enhancing antimicrobial activity of haemolymph and phagocytosis of cells.

To study the effect of LEC immerses treatment of zebrafish to immune-related gene expression, quantitative RT-PCR was performed at the different day's treatment. Generally, the expression of TNF α, TLR and IL was lower compare with wild type and liposome immersion after 1 day immersion and gradually decrease after 3rd day and 5th day treatment. This result suggests that cinnamaldehyde was suppressing the expression of cytokine.

Cytokines, a large goup of soluble extracellular proteins or glycoprotei ns, are key intercellular regulators and mobilizes. Classified into family groups (e.g., interleukins, interferon, and chemokines) based on the structural homologies of their receptors, these are now seen to be crucial to innate and adaptive inflammatory responses, cell growth and differentiation, cell death, angiogenesis, and developmental as well as repair processes. Their secretion, by virtually every nucleated cell type, is usually an inducible response to injurious stimuli (Spelman et al., 2006). Chao et al. (2008) was demonstrated that cinnamaldehyde can suppresses the LPS-induced production of TNFα , IL 6 and IL 1. Those result suggest that cinnamaldehyde could show

suppressive effect on the production of various type of inflammatory mediators.

The suppression of immunity levels as measured through the above parameters, in zebrafish due to the LEC immersion, clearly indicated LEC as a potential immunosuppressor. Its presence should be given due importance in intensive systems of fish farming, to prevent the increase of susceptibility to diseases due to secondary infections.

VI. Conclusions

This present study inform that liposome emulsified cinnamaldehyde compound have positive effect against aquatic pathogens. The results have shown LEC can inhibit the growth of *A. hydrophyla*, *S. agalactiae*, *V. vulnificus, V. alginolyticus and V. parahaemolyticus*. The MIC value against *A. hydrophila* was 1.36 mM, 1.81 mM for *S. agalactiae*, and 1.36 mM for *V. vulnificus*. Meanwhile, the minimum bactericidal concentration (MBCs) of LEC against *S. agalactiae* was 2.72 mM, 3.18 mM for *A. hydrophila*, and 1.81 mM for *V. vulnificus*. LEC can inhibit the growth of *Vibrio parahaemolyticus* at 14.1 mM and at 113.5 mM it was bactericidal. LEC can inhibit the growth of *Vibrio alginolyticus* at 28.6 mM and at 113.5 mM it was bactericidal.

LEC also can increase the survival rate of zebrafish and *M. rosenbergii* after pathogen injection. The significance of the increase varied with the type of pathogens. Survival rate of zebrafish infected *V.vulnificus* treated with LEC was increased up to 60% after 11 days treatment compared with untreated and negative control. Meanwhile, survival rate of zebrafish infected by *S. agalacticiae* and *A. hydrophila* then treated with LEC consecutively was increase up to 30% and 38 % after 8 days treatment compare with untreated and negative control. Survival rate of *M. rosenbergii* injected *V. alginolyticus* then treated with LEC was increase up to 40% after 7 days treatment compared with untreated one and negative control.

In other hand, LEC can suppress the expression of ILs, TNFα, and TLRs in zebrafish that immersed with LEC. It is assumed that LEC is a potential immunosuppressor and antimicrobial agent for against bacterial infection in Aquaculture.

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Appendix

Table 1. Primer sequence and gene names listed in this study

Gene name	Primer sequence(5'→3')	PCR size (bp)	Accession number
Interleukin-1β (IL 1β)	TGGACTTCGCAGCACAAAATG (f) CACTTCACGCTCTTGGATGA (r)	147	AY340959
Interleukin-10 (IL 10)	TCACGTCATGAACGAGATCC (f) CCTCTTGCATTTCACCATATCC (r)	151	BC163031
Interleukin-15	ATGTCATTGGAACTCAGAGGTTTG (f) CTGTTCTGGATGTCCTGCTTGA (r)	95	BC162843
Interleukin-22	CATCGAGGAACAACGGTGTACA (f) CACGAGCACAGCAAAGCAAT (r)	100	BC163192
Toll-like receptor-1 (TLR1)	CAGAGCGAATGGTGCCACTAT (f) GTGGCAGAGGCTCCAGAAGA (r)	100	AY389444
Toll-like receptor-3 (TLR3)	TGGAGCATCACAGGGATAAAGA (f) TGATGCCCATGCCTGTAAGA (r)	100	AY616582
Toll-like receptor-4a (TLR4a)	TTTCAGATGCCACATCAGA (f) TCCACAAGAACAAGCCTTTG (r)	150	EU551724
Tumor necrosis factor-α (TNF-α)	AAGGAGAGTTGCCTTTACCG (f) ATTGCCCTGGGTCTTATGC (r)	152	BC165066
Elongation factor 1α (EF-1α)	AACAGCTGATCGTTGGAGTCAA (f) TTGATGTATGCGCTGACTTCCT (r)	100	EJ915061

Table 2. MIC and MBC value of LEC against fish pathogens

Pathogen	Liposome-emulsified cinnamaldehyde (mM)		Kanamycin (μM)		Ampicillin (μM)	
	MIC	MBC	MIC	MBC	MIC	MBC
A. hydrophila	1.36	3.18	0.86	3.43	3	5
S. agalactiae	1.81	2.72	3.43	6.86	3	5
V. vulnificus	1.36	1.81	5.15	34.33	3	7

Table 3. MIC and MBC value of LEC against crustacean pathogens

Pathogen	Liposome-emulsified cinnamaldehyde (mM)		Tetracycline (mM)	
	MIC	MBC	MIC	MBC
V. parahaemolyticus	14.07	113.5	1.1	1.7
V. algynolytius	28.6	113.5	0.009	0.28

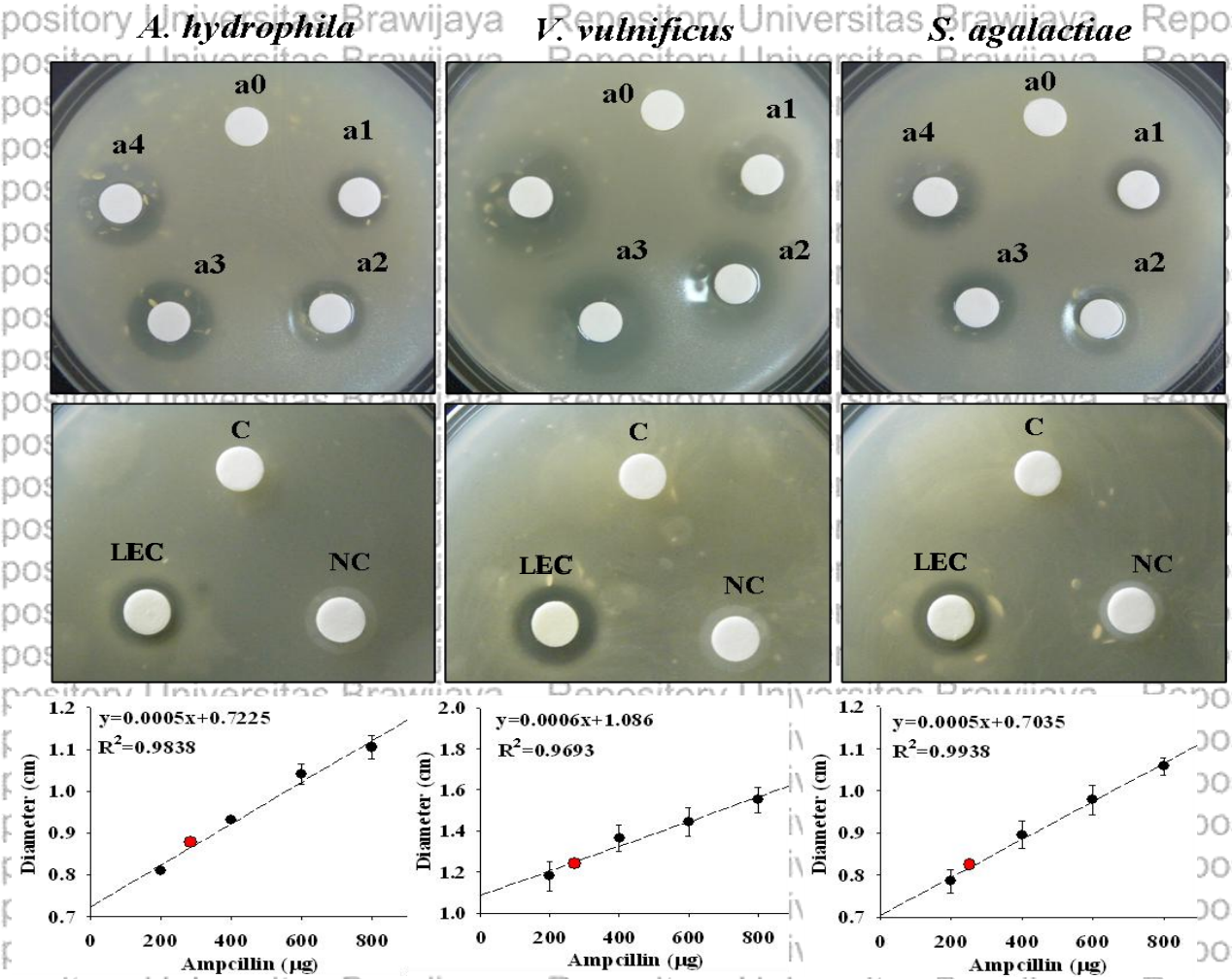


Figure 1. Inhibition zone of liposome-emulsified cinnamaldehyde (LEC) against *A. hydrophila, V.vulnivicus,* and *S. agalactiae* in compare with ampicillin in the different concentration (a0, a1, a2, a3, and a4). Water (C) served as negative control 1 and liposome (NC) served as a negative control 2. Linear regession of inhibition zone of ampicillin used to determine antimicrobial potency of LEC.

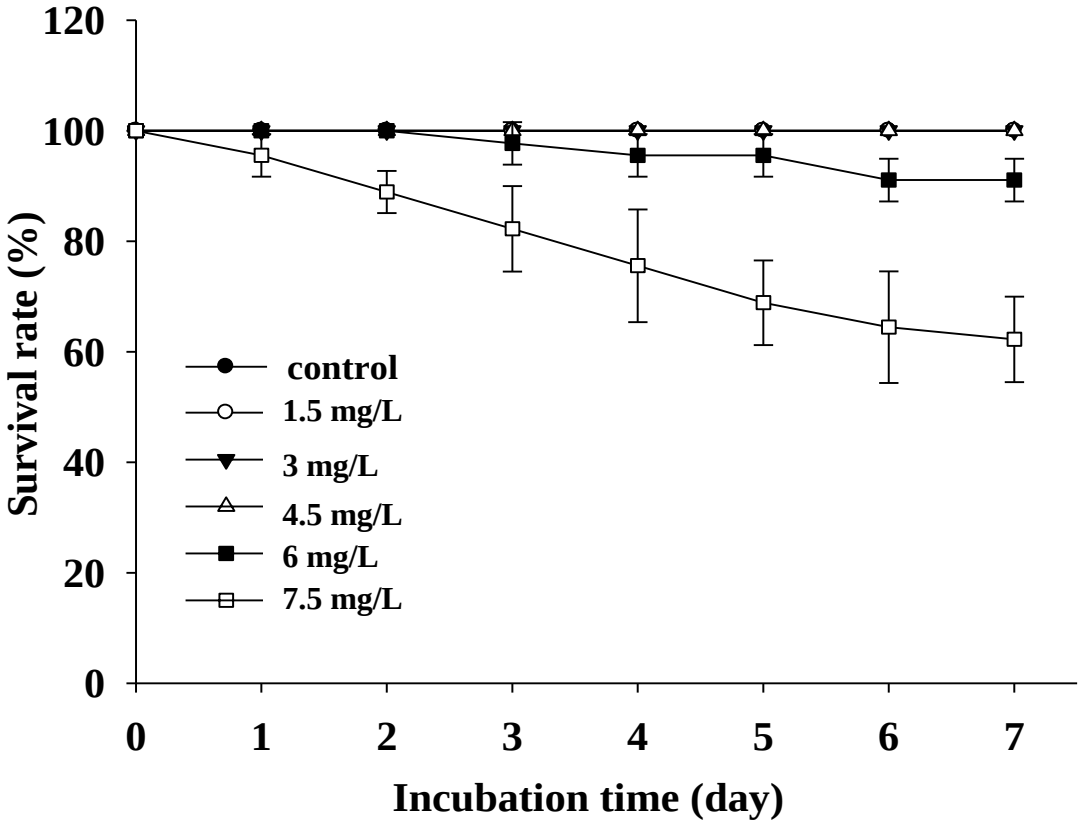


Figure 2. Tolerable concentration of LEC for wild type zebrafish. Different concentration of LEC used to immerse zebrafish and maintained in a fresh water recirculating tank at 28˚C for 7 day.

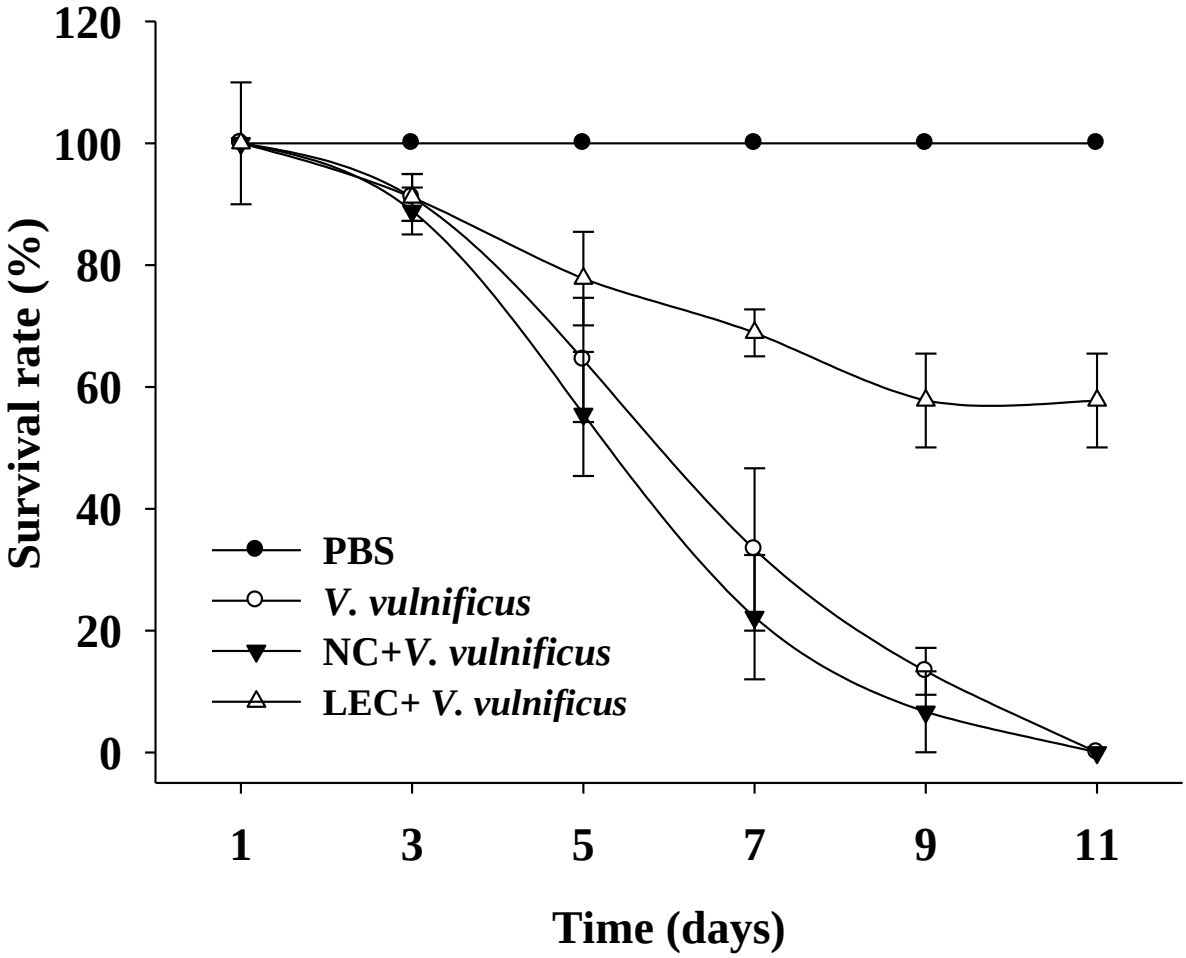


Figure 3. Survival rate of AB zebrafish over 11 days after injection with *V. vulnivicus* following by LEC immersion (4.5 mg/L).

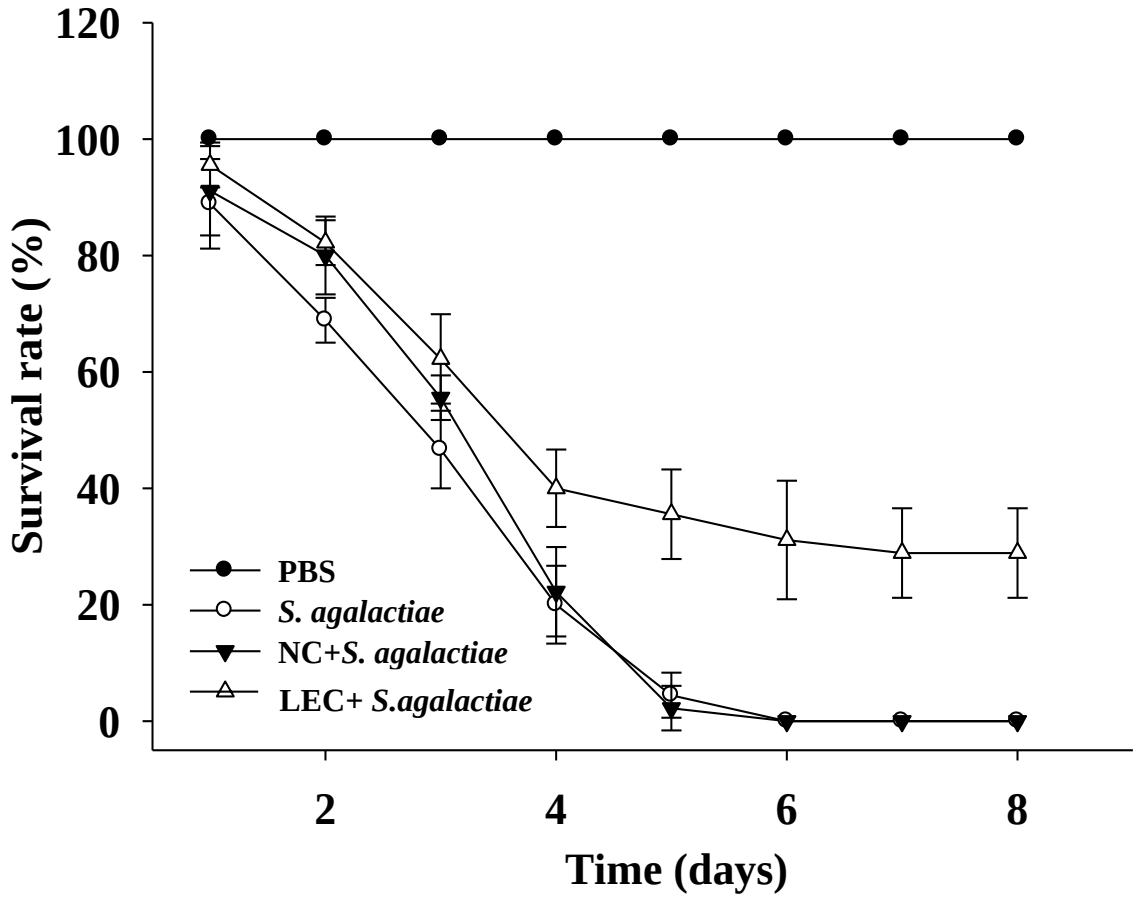


Figure 4. Survival rate of AB zebrafish over 8 days after injection with *S. agalactie* following by LEC immersion (4.5 mg/L).

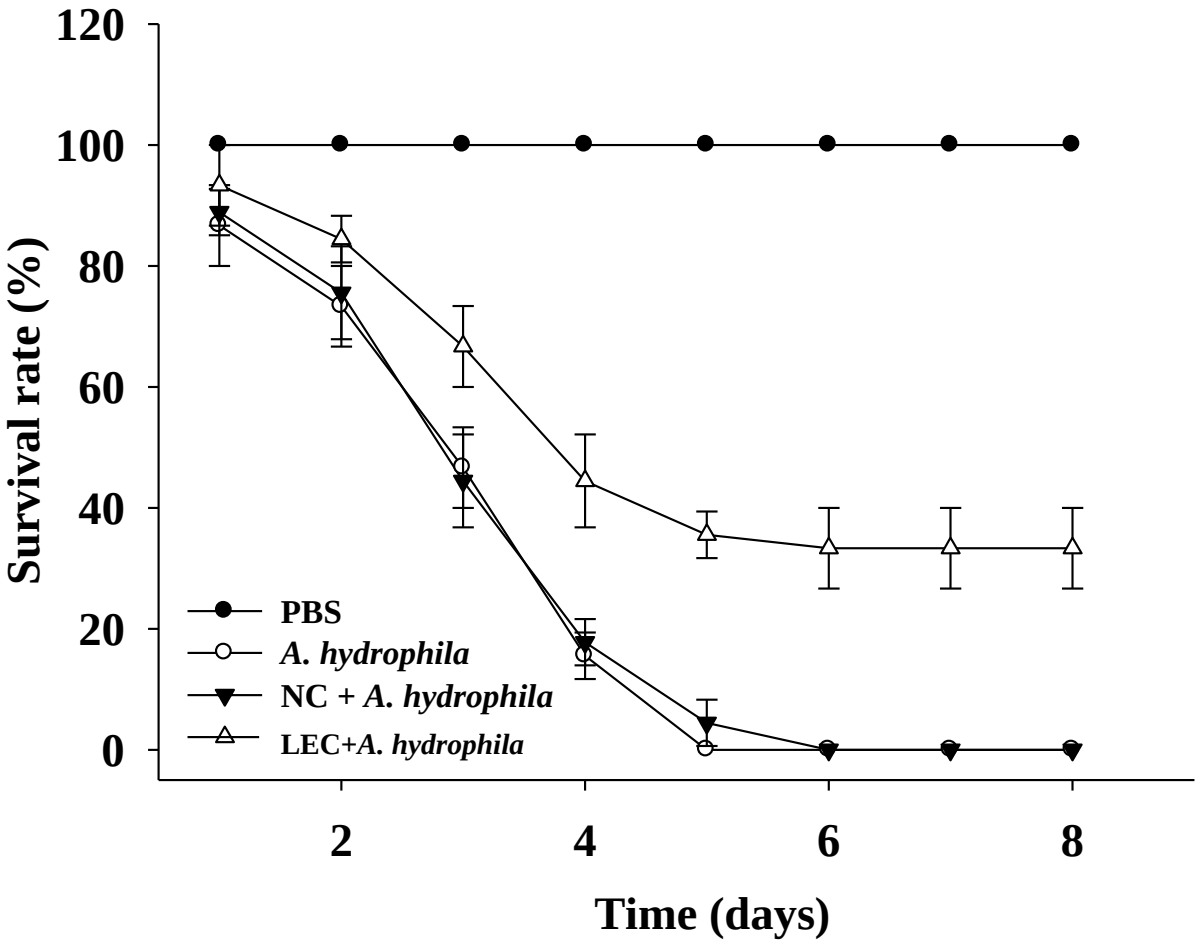
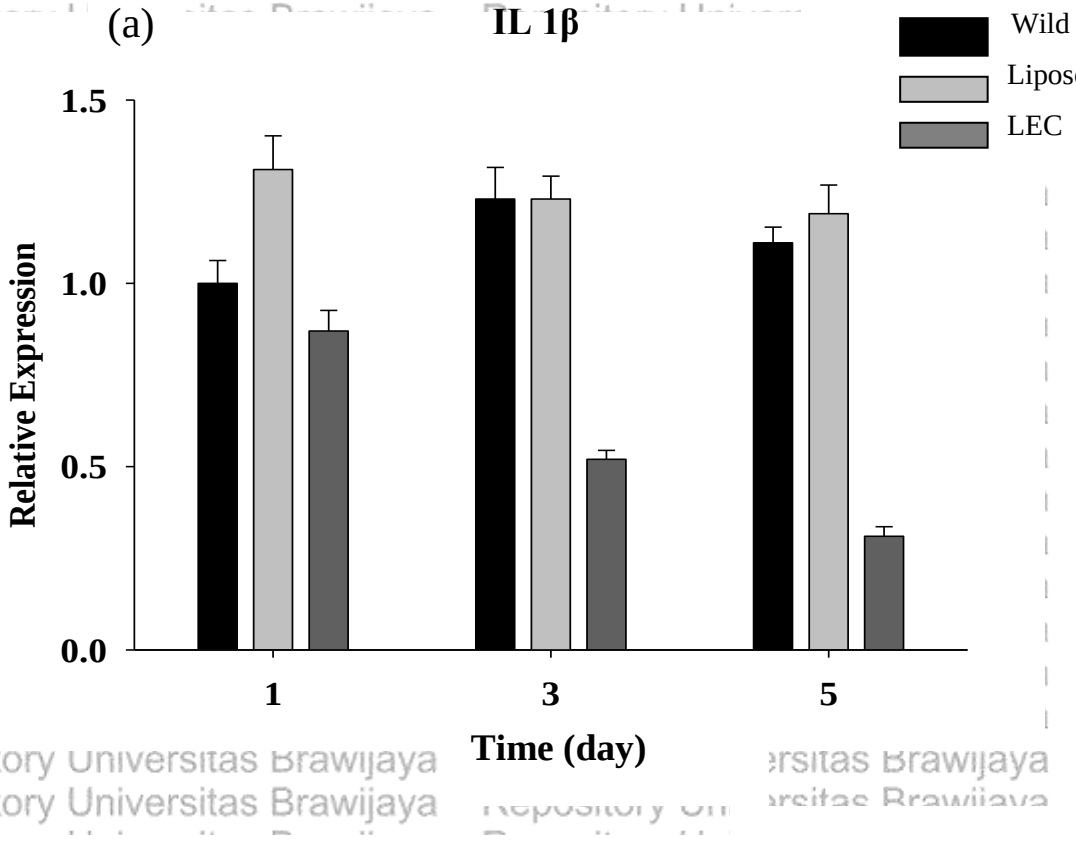
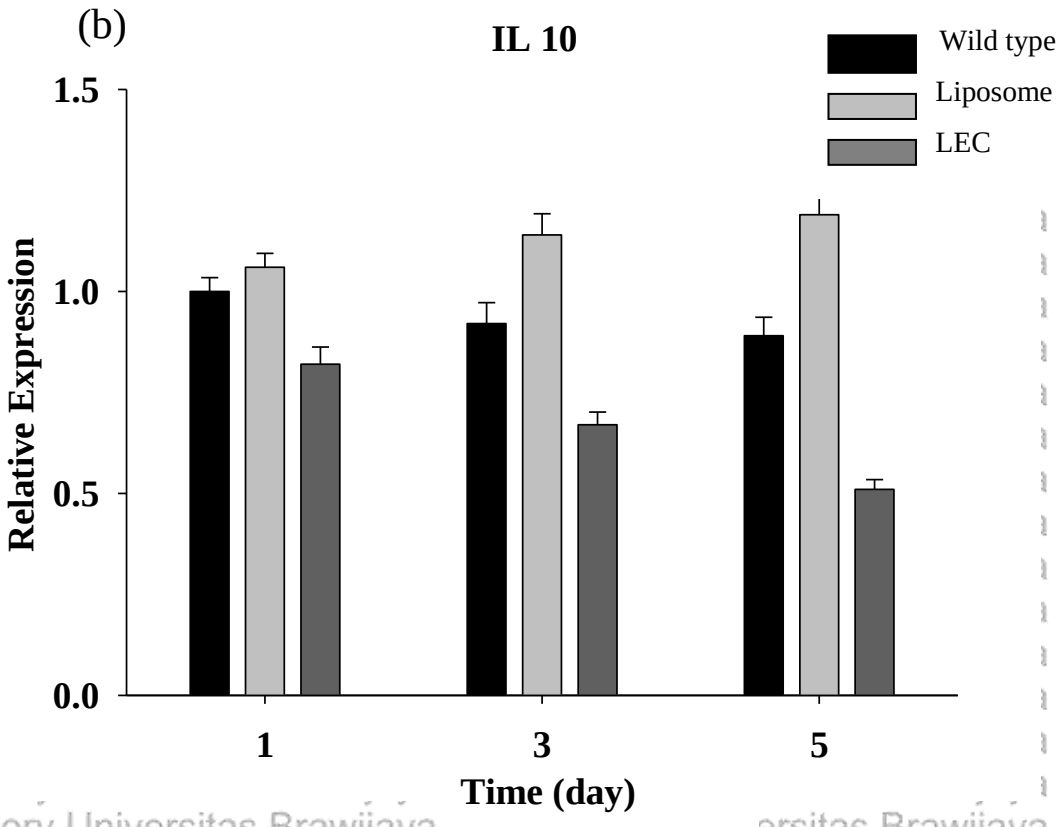
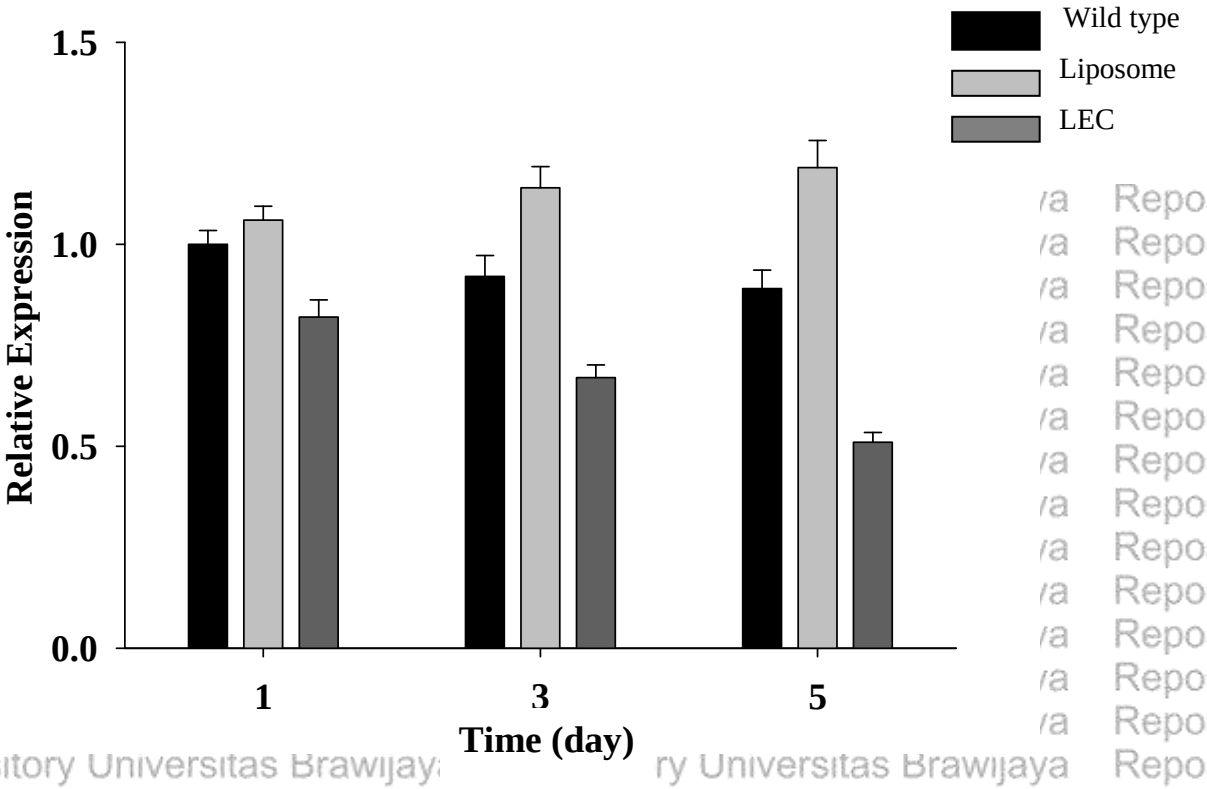


Figure 5. Survival rate of AB zebrafish over 8 days after injection with *A. hydrophila* following by LEC immersion (4.5 mg/L).

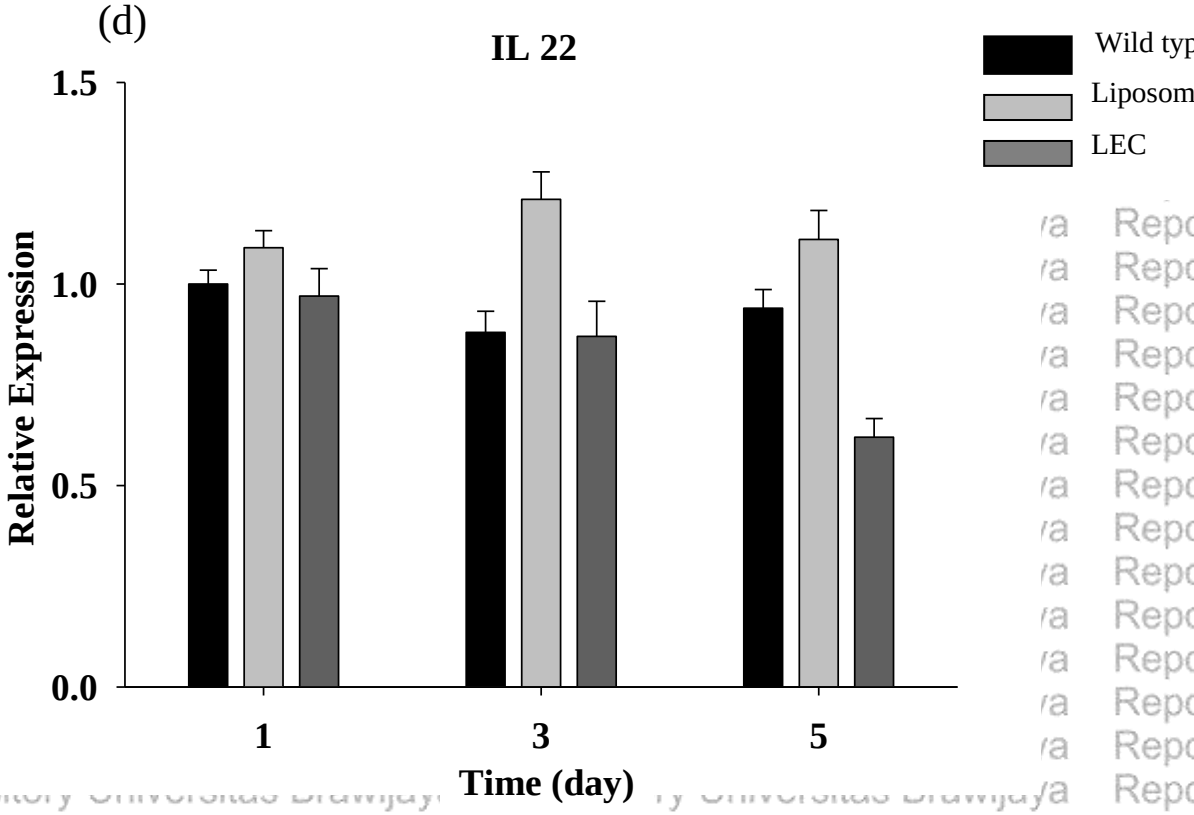

(a)
IL 1β
Wild type
Liposome
LEC
Relative Expression
1.5
1.0
0.5
0.0
1
3
5
Time (day)


(b)
IL 10
Wild type
Liposome
LEC
Relative Expression
1.5
1.0
0.5
0.0
1
3
5
Time (day)

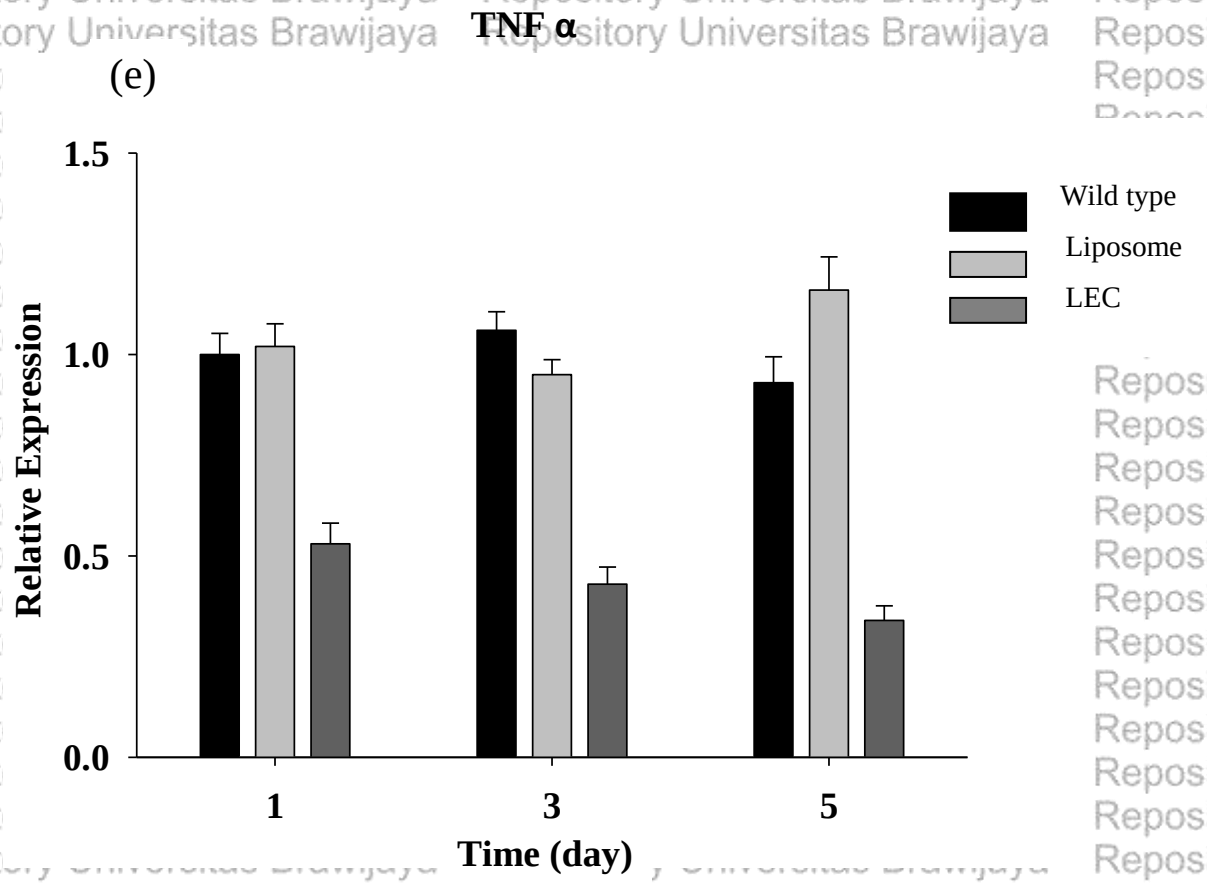
(c)



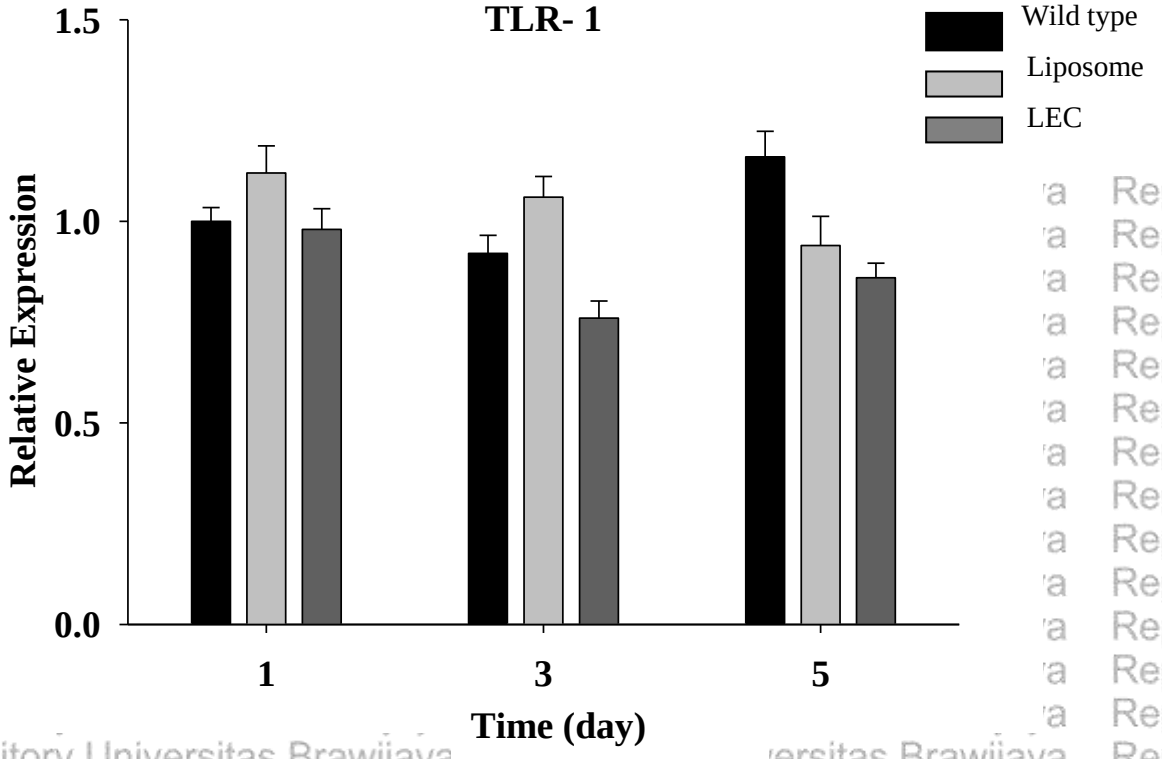
(d)



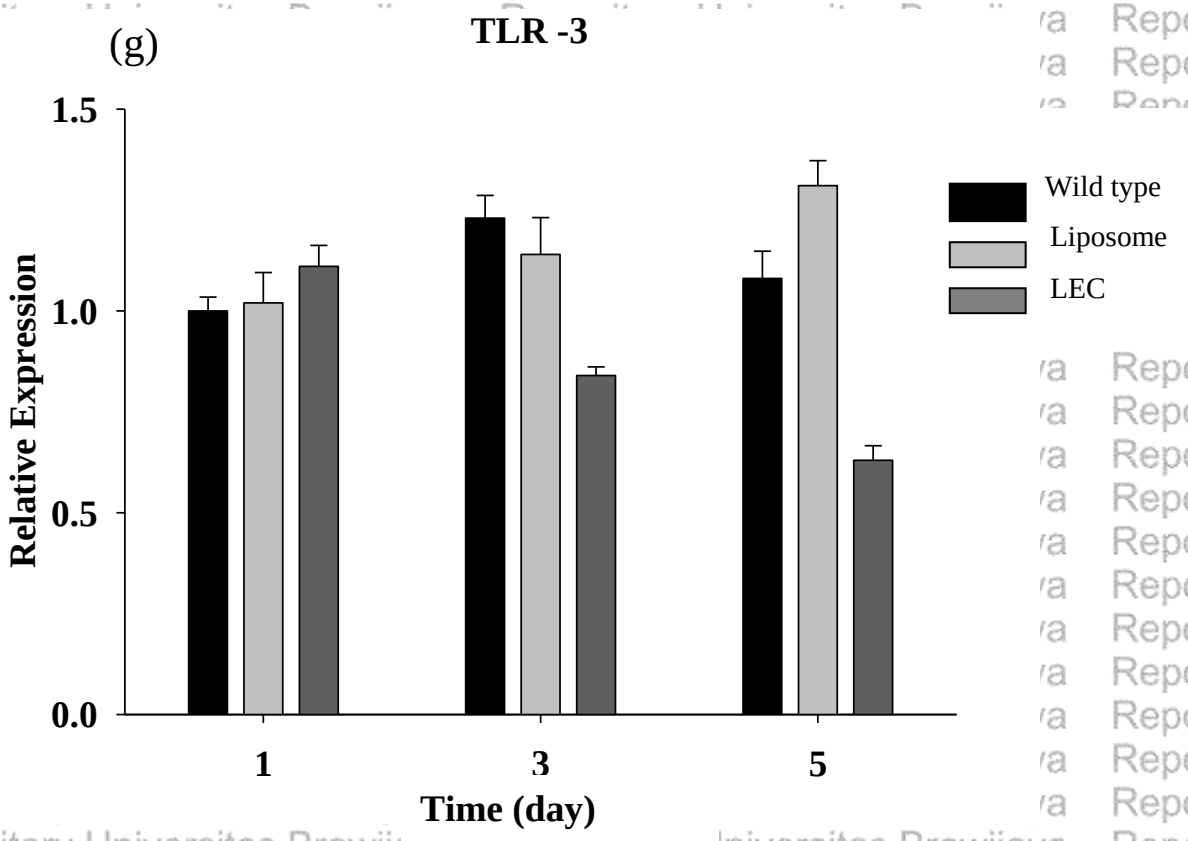
(e)



(f)



(g)



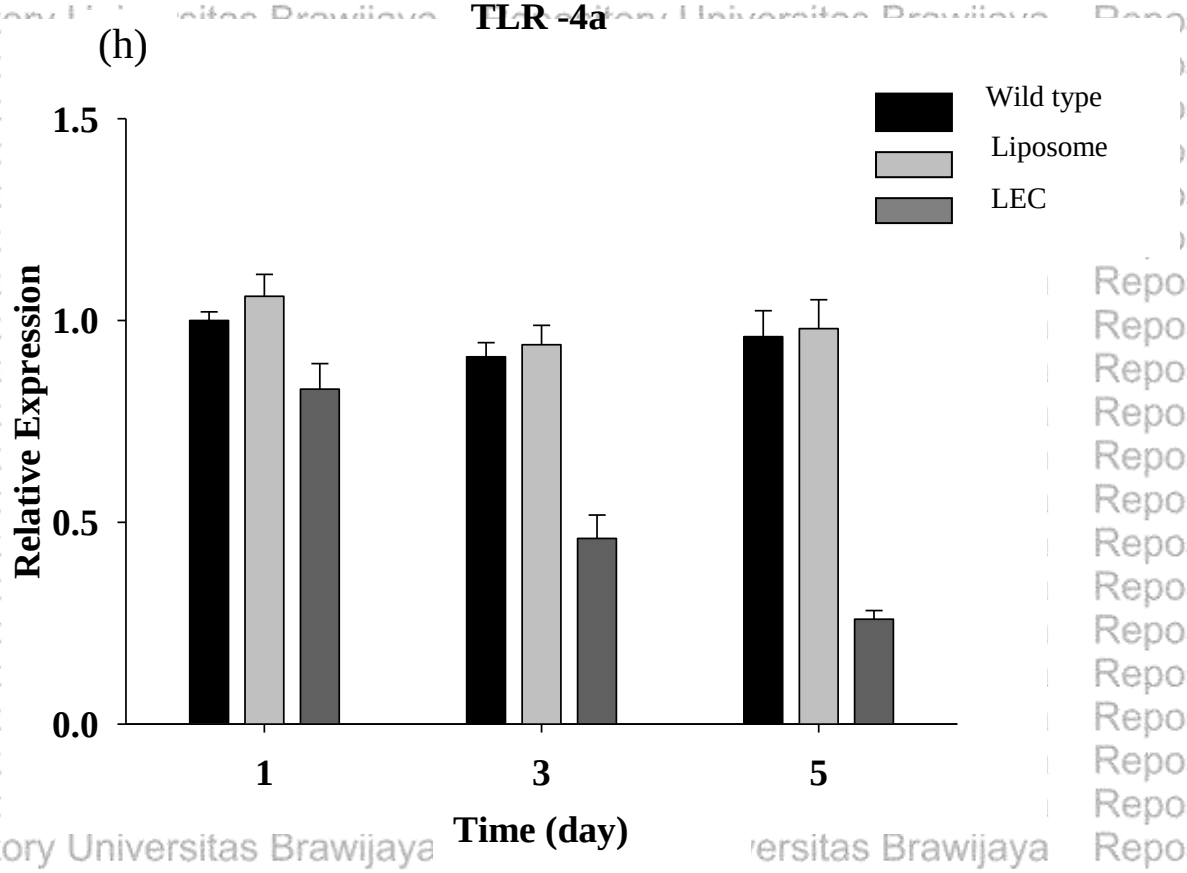
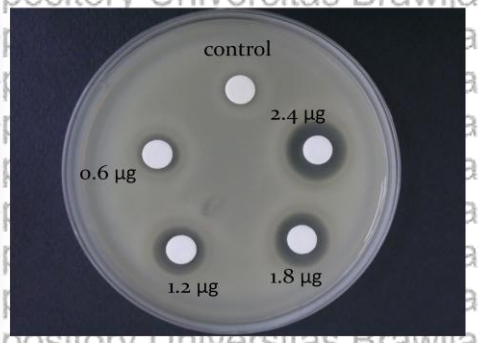
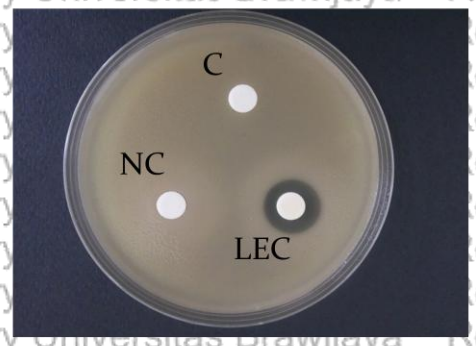


Figure 6. Quantitative Real Time PCR analysis of immune-related gene expression: (a) IL1β, (b) IL 10, (c) IL 15, (d) IL 22, (e) TNF α, (f) TLR- 1, (g) TLR- 3, and (h) TLR- 4a were examined in whole body of AB zebrafish at 1, 3, and 5 days of LEC immersion. The expression level of EF1α served as an internal control and each expression data were normalized to non-treated wild type zebrafish. Each bar represented the mean value from triplicate treatment with the S.D.

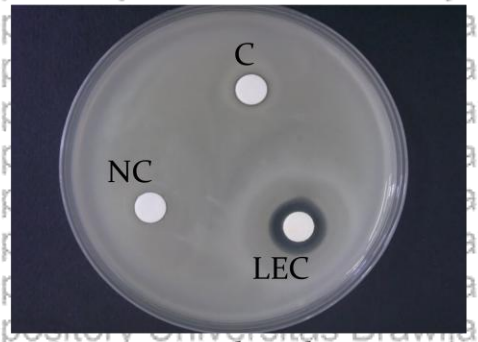
Tertacycline against *V. parahaemolyticus*



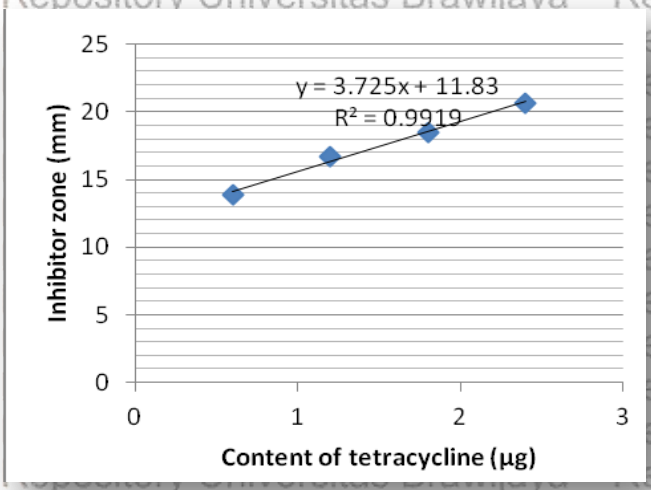
Tertacycline against *V. alginolitycus*



LEC *against V. parahaemolyticus*



LEC against *V. alginolitycus*



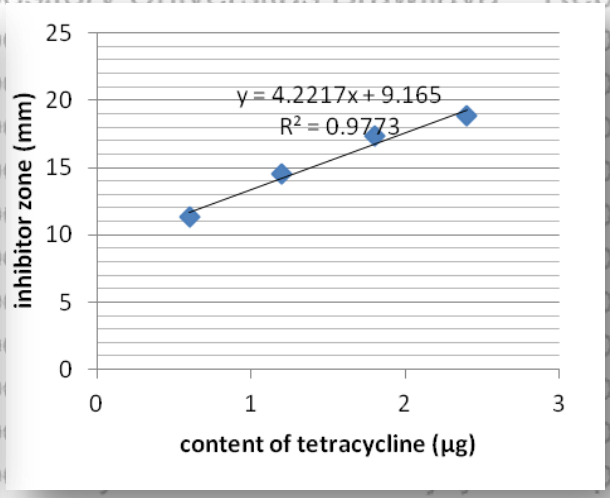


Figure 7. Inhibition zone of 0.6 mg liposome-emulsified cinnamaldehyde (LEC) against *V. parahaemolyticus* and *V. alginolyticus* in compare with tetracycline in the different concentration. Water (C) served as negative control 1 and liposome (NC) served as a negative control 2. Linear regression of inhibition zone of tetracycline was used to determine antimicrobial potency of LEC.

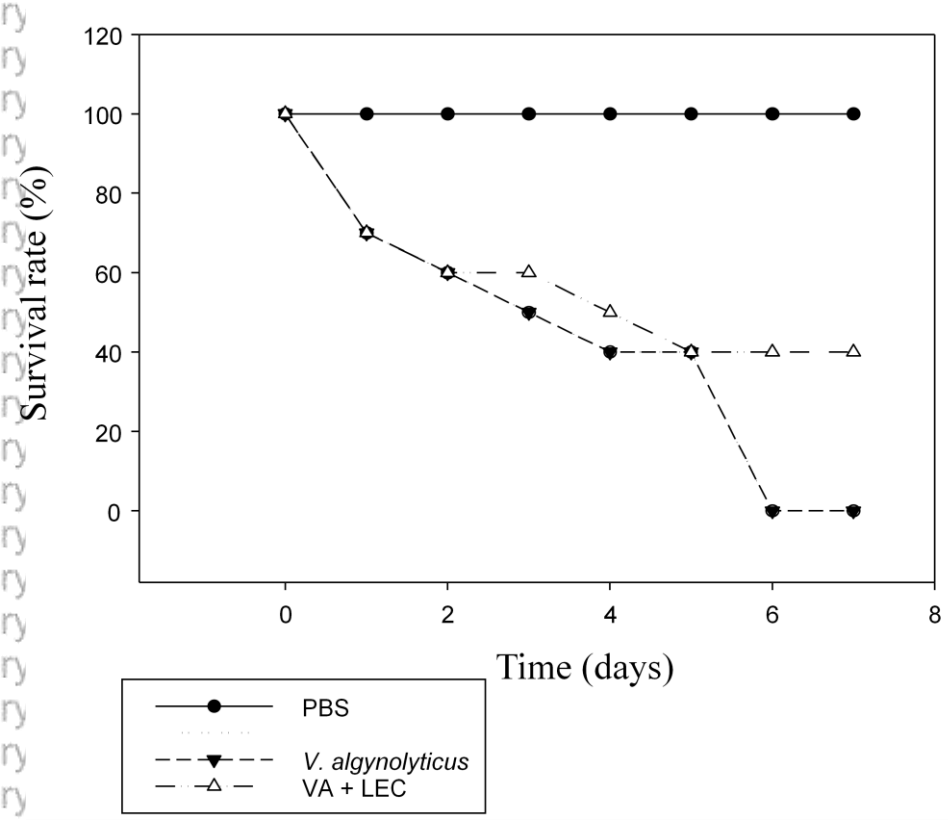


Figure 8. Survival rate of *M. rosenbergii* over 7 days after injection with *V. alginolyticus* following by LEC immersion (4.5 mg/L).