Study on Effect of Essential Oils from *Houttuynia cordata* Thunb Supplemented Diets on Growth Performance and Immune Response of Hybrid Red Tilapia (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.)

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ABSTRACT

Houttuynia cordata Thunb (dokudami) is a flowering plant native to Japan, Southern China and Southeast Asia. It contains volatile oil, fatty acids, sterols, flavonoid and alkaloids. Moreover, it possesses a variety of pharmacological functions. This study investigated the effects of essential oils (EOs) from *H. cordata* Thunb as diet supplements on growth performance and immune responses of Hybrid red tilapia (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.). *H. cordata* Thunb were extracted using hydrodistillation produced a clear colorless to pale yellow oil with a yield of 0.057%v/w and analyzed by gas chromatograph-mass spectrometer (GC-MS). Beta-myrcene (44.220%) and 2-undecadone (21.985%) were the major components out of the 28 components present. The *in vitro* antibacterial activity of *H. cordata* Thunb essential oils (HEO) showed minimal inhibitory concentration (MIC) against *Aeromonas hydrophila*, *Streptococcus* sp. and *Flavobacterium columnare* at 250, 250 and 500 ppm, respectively (Ampicillin sodium exhibited similar MIC values against the bacteria used). On the other hand, minimum bactericidal concentration (MBC) was not determined for all concentration. The *in vivo* analysis on growth performance of Hybrid red tilapia fed with three diet containing HEO 0, 1.5 and 3.0 g/kg showed no mortality after the 60 days experiment period. Also, weight gain and average daily growth of Hybrid red tilapia fed with HEO 1.5 g/kg were the highest among the other setups ($p<0.05$). Feed conversion ratio of HEO 1.5 g/kg was significantly ($p<0.05$) lower than the control and HEO 3.0 g/kg. The proximate composition of Hybrid red tilapia fillet showed that the protein content of fish fed with HEO 1.5 g/kg was significantly ($p<0.05$) higher than HEO 3.0 g/kg and control. In contrast, fish fed with HEO 3.0 g/kg yielded to a lipid content that is significantly ($p<0.05$) higher than HEO 1.5 g/kg and control. It was also found out that the lysozyme activity of Hybrid red tilapia was improved in fish fed with HEO 1.5 g/kg ($p<0.05$), while the lowest values were obtained in the HEO 3.0 g/kg. However, no significant difference was observed in hematocrits, red blood cells and white blood cells values among all treatments (p>0.05). Based on the results of this paper, it can be concluded that HEO can be applied in aquaculture as supplement to commercial diets because these can improve the growth performance and enhance the fish's immune response against pathogens of Hybrid red tilapia.

CHAPTER 1

General Introduction

1.1 Concepts

Tilapia is one of the most important freshwater fish in aquaculture. In Thailand, commercial culture of tilapia has focused on Hybrid red tilapia and Nile tilapia. Hybrid red tilapia is now becoming very popular for freshwater aquaculture, especially for cage culture. Thai red tilapia is the hybrid between (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.). It has been available for culture in Thailand since the mid-1980s when the Department of fisheries (DOF) developed a strain at the Ubonratchathani Freshwater Fisheries Station. The annual report stated that, more than 188,000 tons of tilapia were produced in 2014 (Food and Agriculture Organization of the United Nation, 2016). Hybrid red tilapia is cultured in ponds and floating cages in rivers or reservoirs where infectious diseases are common. This is the major cause of huge economic loss in commercial aquaculture. Thus, the aquaculture industry began to focus on the prevention of diseases rather than treating them with chemotherapeutics and antibiotics (the use of which has been criticized for their negative side effects).

In avoiding chemotherapeutics and antibiotics, natural products are perceived as the best alternative to control infectious diseases. Currently, Thai herbs have been widely used in commercial aquaculture as growth-promoting substances, antibiotics, antimicrobial agents, nutrient source etc. There are a lot of herbal medicines found to be effective against fish pathogens. Essential oils are plant-derived products reported to have anti-microbial activity against fish pathogens. An example is the *Cinnamomun verum* oil (0.4%) that has a protective effect against *Streptococcus iniae,* which is a pathogen of tilapia (Pongsak and Parichat, 2010). Moreover, thyme oil (0.25%) was also reported to have an antibacterial effect against pathogens of Nile tilapia (Shehata et al., 2013).

The whole plant of *Houttuynia cordata* Thunb (*Khao-tong* or *Plu-khao* in Thailand) can be used as a herbal medicine. It contains volatile oil, sterols, fatty acids, flavonoid and alkaloids (Bauer et al., 1996). Moreover, it possesses a variety of pharmacological functions including anti-platelet aggregation, anti-bacterial, anti-microbial, anti-tumor, anti-inflammatory, anti-leukemic and immunomodulatory effects (Chen et al., 2003; Hayashi et al., 1995; Lu et al., 2006a, b). Decanoyl acetaldehyde (houttuynin or 2-undecanone) is one of the main components of *H. cordata* Thunb essential oils. It was reported that houttuynin has various medicinal effects and well known for its antibacterial effects (Lu et al., 2013). Essential oils from this plant have antibacterial activity against *Staphylococcus aureus* and *Sarcina ureae* (Zhang et al., 2008). Thus, the current investigation was conducted to identify the chemical composition of *H. cordata* Thunb and determine the potential effects of its essential oils on growth performance and immune response of Hybrid red tilapia.

1.2 Objective and outline of the thesis

- To identify the chemical composition of *Houttuynia cordata* Thunb essential oils by gas chromatography-mass spectrometry (GC-MS)
- To determine the potential of *H. cordata* Thunb essential oils as antibacterial agent against fish pathogen
- To evaluate *H. cordata* Thunb essential oils in terms of stimulation of immune function and growth promoter of Hybrid red tilapia (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.) as followings:

- To conduct a challenge test with Hybrid red tilapia using concentration of *H. cordata* Thunb essential oils which show good performance
- To analyze the lysozyme activity of blood cells treated with *H. cordata* Thunb essential oils
- To monitor hemocyte profile of Hybrid red tilapia blood after treating with *H. cordata* Thunb essential oils

(In CAHPETR 1) The concept studying essential oil from *Houttuynia cordata* Thunb was discussed. This study aims to investigate and identify the chemical composition of *H. cordata* Thunb and determine the potential effects of its essential oils on growth performance and immune of Hybrid red tilapia. A literature review that gives an overview of Hybrid red tilapia, herbs in aquaculture, *H. cordata* Thunb and activity of essential oils was presented. A main component in the essential oils of *H. cordata* Thunb, decanoyl acetaldehyde (2-undecanone), has various medicinal effects. However, the properties of the metabolites found in essential oil from *H. cordata* Thunb in North of Thailand have rarely been investigated. **(In CAHPETR 2)** Because of we have to take into account the fact that it is the composition of *H. cordata* Thunb essential oils that provides their medical effect, an analysis of these is really important. Gas chromatography-mass spectrometry (GC-MS) is the best method due to its rapidity and efficiency for the identification and quantification of essential oils components and composition variations. The chemical composition of *H. cordata* Thunb essential oils was extracted by distillation and examined by GC-MS. **(CAHPETR 3)** Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism. MIC is used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antibacterial substances. It was attempted to examine the potential of *H. cordata* Thunb essential oils as an antibacterial agent against fish pathogen by MIC. In addition, the advancement of knowledge about *H. cordata* Thunb essential oils can be applied in the field of aquaculture. **(CAHPETR 4)** Considering chemical composition and antimicrobial activity against fish pathogen, the use of essential oils could be an adequate tool because it is a natural component. The challenge test with adult Hybrid red tilapia showed good performance and immune response. **(CAHPETR 5)** Based on the discussion of the results obtained, it was concluded that the use of *H. cordata* Thunb essential oils in aquaculture could be economically and practically important. Finally, a general discussion **(CAHPETR 6)** relates all data. Data are viewed in the perspective of these essential oils as alternatives for the aquaculture and future prospects are discussed

1.3 Overview of Hybrid red tilapia

Tilapia (all species) is a commercially important fish in Thailand. Thai red tilapia is the hybrid between (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.). Department of Fisheries in (DOF) Thai government established a strain for commercial farming around mid-1980s at the Freshwater Fisheries Station in Ubonratchathani province. Since then, aquaculture of Thai red tilapia has been conducted widely. This fish has gained increasing preference of commercial farmers because of their reddish color liked by consumers and their resemblance to premium marine species. In Thailand, commercial culture of tilapia has focus on Hybrid red tilapia and Nile tilapia. The annual tilapia production reported in 2014 was over 188,000 tons (Food and Agriculture Organization of the United Nation, 2016); this is over 10 times more than the production level reported in 1986 **(Fig. 1)**.

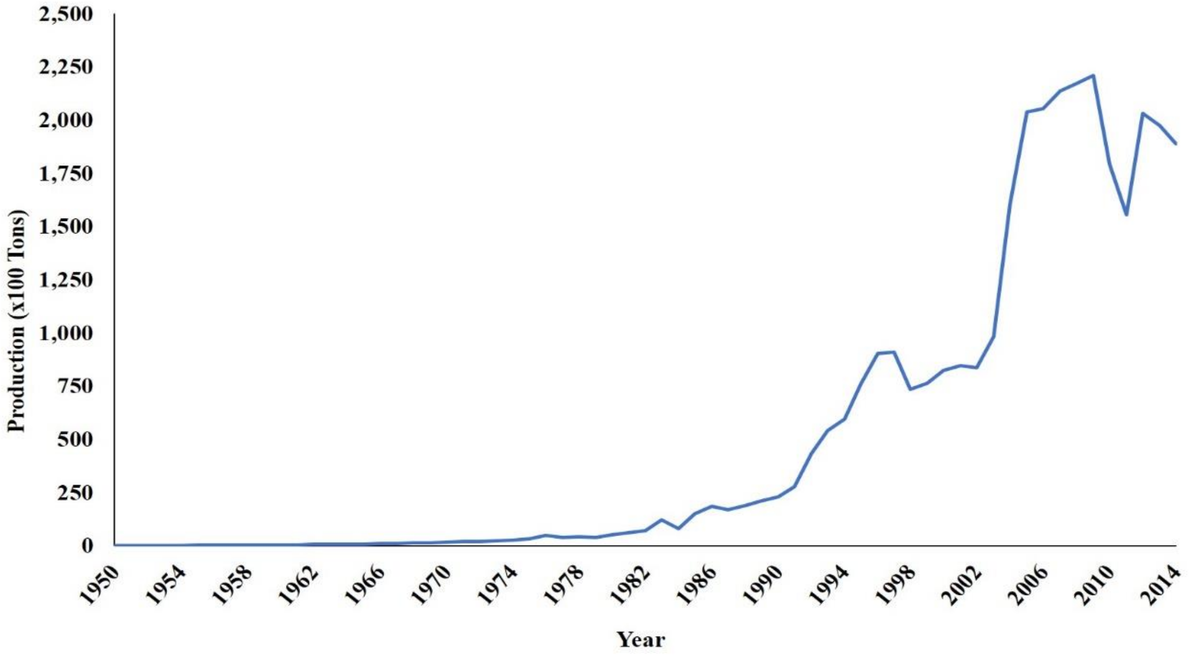


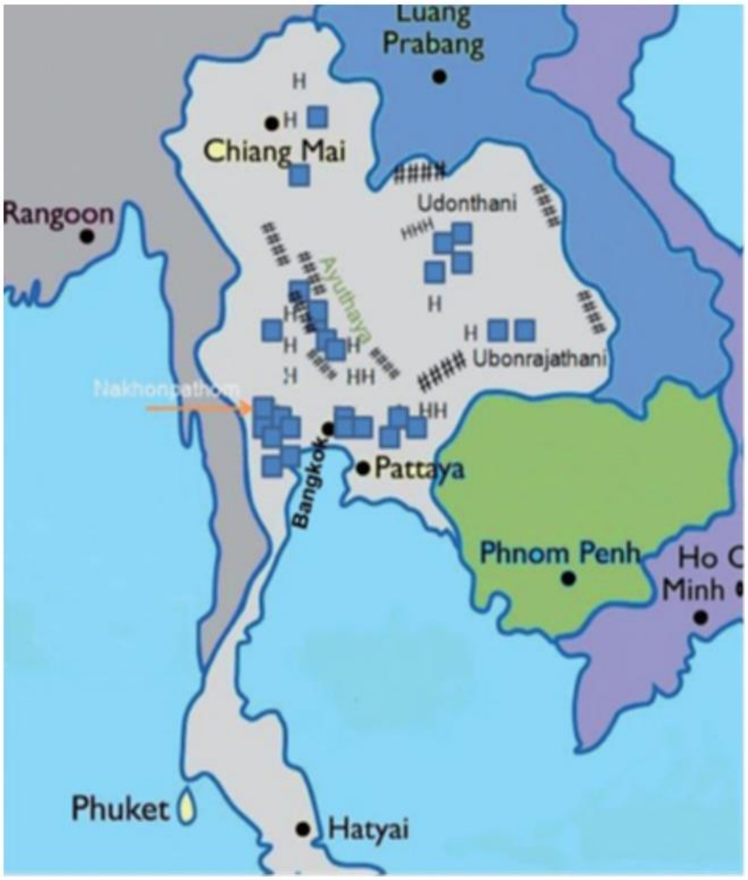
Figure 1 Total production of tilapia in Thailand (1950-2014); FAO, 2016

1.3.1 Hybrid red tilapia

Red tilapias are obtained through genetic crossing of at least two different species of tilapia. The main purpose of breeding for red tilapia is to obtain their bright red and orange hues which is very attractive for market as “red snappers” and “orange snappers”. Red tilapia is one of the most successful tilapia strains in terms of commercial production and research. Red tilapia has also advantages in terms of stronger and faster growing than their original strains. More importantly, the bright-red color makes them more appealing and attractive to the consumer than their wild-caught relatives. They also have the most commercial potential (Belton et al., 2006).

Hybrid red tilapia is now becoming very popular for freshwater aquaculture in Thailand, especially for cage culture. In Thailand, majority of pond farmers are using polyculture systems. Also, almost all of the subsistence tilapia farms use polyculture systems, whereas most of the commercial farms produce single species all-male tilapia. On the other hand, all cage farmers practice monoculture, either of Red or Nile tilapia (Bhujel, 2013) **(Fig. 2)**. Culture of tilapia in cages has become quite popular over the last few years. Red tilapia is the main species in Central, North and South Thailand, but Nile tilapia is more commonly cultured in the Northeast region. Most Hybrid red tilapia cage farms are located on rivers, large canals and reservoirs where infectious diseases are common. Water flow and quality are hugely different in each of the different site types.

The major diseases in floating cage cultured tilapia farms are probably caused by pathogens, which resulted to high mortality rates and huge economic loss in commercial aquaculture. Fortunately, tilapia is a very disease-resistant fish, but they are not immune. External parasites are common in young fish and *Streptococcus* (a bacterial pathogen) infection is very



Note: tilapia hatcheries (H), ponds (🟩) and cages (##) contacted/visited

Figure 2 Map of Thailand showing the major sites of tilapia hatcheries, pond and cages contacted/visited for the collection of information; Bhujel, 2013 common during the hot season and early rainy season. Previously, several important pathogens of tilapia in Thailand were reported, such as *Flavobacterium columnare*, *Aeromonas veronii*, *Vibrio cholera*, *Plesiomonas shigelloides*, *Streptococcus agalactiae* (Dong et al., 2015) and *S. iniae* (Yuasa et al., 2008). Infectious diseases are always a hazard, may cause significant stock losses and problems with animal welfare. Intensive tilapia farming has led to growing problems with bacterial diseases, thus, nowadays the treatment of which requires the intensive use of antimicrobials. To solve this problem, farmers frequently use chemotherapeutics and antibiotic compounds to treat bacterial diseases (Cabello, 2006). Therefore, researchers have intensified efforts to take advantage of natural products such as herbs in developing alternative dietary supplements that can enhance growth performance and immune system.

1.3.2 Fish pathogen of tilapia

High stocking densities in aquaculture farms can lead to outbreaks of parasites and diseases if the management is not optimal. In practice, diseases develop when a certain combination of sub-optimal environmental factors meet causing the stress level of the culture population to reach a point that is detrimental to the animals' immune systems. For example, water temperature increases or decreases drastically depending on the season which can induce fish stress.

From the different pathogens found to be affecting tilapia, *Streptococcus* is the most common, widespread and pathogenic. On the other hand, *F. columnare* infection is a common pathogen in early stages. For the viruses, only the group of Iridovirus has been documented to cause diseases in tilapia **(Fig. 3)**.

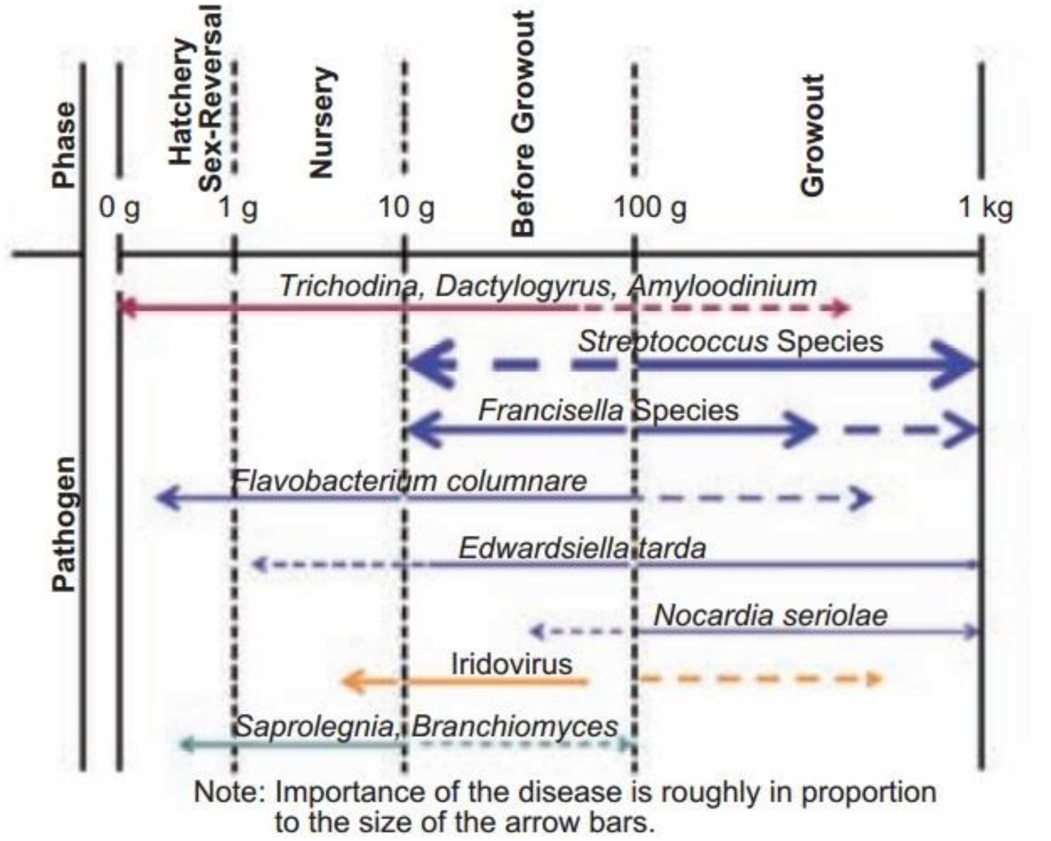


Figure 3 Major diseases affecting tilapia; Komar, 2008

Aeromonas hydrophila

A. hydrophila is one of the most important agents of outbreaks in fresh water fish. In the acute form of disease, a fatal septicemia may occur so rapidly that the fish dies before it develops a few gross signs of the disease. When clinical signs of infection are present, the affected fish may show exophthalmia, reddening of the skin and an accumulation of fluid in the scale pockets (Faktorovich, 1969). The abdomen may become distended as a result of an edema and the scales may bristle out from the skin to give a “washboard” appearance. The gills may form hemorrhage and ulcers may develop on the dermis. The results of the study conducted by Deen et al. (2014) on *A. hydrophila* from culture tilapia showed that the clinical examination of diseased fish exhibited: loss of equilibrium, ascites, skin darkness, exophthalmia and ulcers varied in their degrees. Congestion and enlargement in internal organs also appeared in postmortem examination **(Fig. 4)**.

The presence of these bacteria, by itself is not indicative of disease and consequently, stress is often considered to be a contributing factor in outbreaks of disease caused by these bacteria. Such stressors are most commonly associated with environmental and physiological parameters that adversely affect fish under intensive culture (Cipriano, 2001). Elevated water temperature (Esch and Hazen, 1980), a decrease in dissolved oxygen concentration or increases in carbon dioxide and ammonia concentrations have been shown to promote trigger motile aeromonad infections and stress in fish (Walters and Plumb, 1980). In Malaysia, the predominant bacterial species found in tissues of hybrid red tilapia reared in cage culture system from Kenyir Lake and Semantan River was *A. hydrophila*. This is because the temperature of water in Kenyir Lake was observed to be higher than in Semantan River. Resulting to lower cooling, that could account for higher numbers of bacteria isolated since dissolved oxygen (DO) in the water column is one of the most important factor for maintenance of life of cultured organisms (Marcel et al., 2013).

***Streptococcus* sp.**

S. agalactiae is the major cause of streptococcosis in farmed tilapia. *S. iniae* also causes mortality but to a lesser extent. *Streptococcus* spp. are Gram positive, non-motile, non-acid fast, catalase-negative cocci and oxidase-positive. Infection by *Streptococcus* leads to various clinical signs, which include hemorrhages at the gill plate, loss of appetite, spine displacement, hemorrhages in the eye, corneal opacity and hemorrhages at the base of the fins and in the opercula. The most prominent signs are unilateral or bilateral exophthalmia **(Fig. 5)**, also known as “popeye” and distended abdomen (Amal and Zamri-Saad, 2011).

The presence of the pathogen in the fish environment is inadequate to cause a disease outbreak. Other factors usually come into play, such that either the pathogen has an advantage over the host or the immune system of the host is compromised in some ways, increasing its susceptibility to the pathogen. This phenomenon is often precipitated by “stress” (Yanong and Floyd, 2002). Therefore, stress often plays a significant role in the outbreaks of infectious disease in fish populations. Some stressors that have been associated with the streptococcal outbreaks include high and low water temperatures, high salinity and alkalinity (pH>8), low dissolved oxygen concentration, poor water quality (such as high ammonia or nitrite concentrations), high stocking densities and as well as harvesting and handling effects. Bunch and Bejerano (1997) indicated that certain water quality parameters affected tilapia’s susceptibility to streptococcal infection.

Flavobacterium columnare

F. columnare is a Gram negative and rod-shaped bacterium. These bacteria have a characteristic rhizoid pattern of growth on a low nutrient agar medium. Columnaris, caused by *F. columnare* is a contagious disease that can be transmitted horizontally through direct contact, skin wounds as well as through or fecal route (Bullock et al., 1986; Welker et al., 2005). Dong et al. (2015) studied Nile tilapia with concurrent infections from floating cage cultured farms along Mekong River in Nong Khai provice, Thailand. They found out that fishes exposed to *A. veronii* or *F. columnare* mimicked major internal and external clinical signs of naturally infected fish, respectively **(Fig. 6)**. These two main pathogens are co-responsible for the death of fishes in cultured farms in contrast with other opportunistic pathogens causing outbreaks.

Due to the ubiquitous nature of *F. columnare* in the freshwater, may result in injury to the skin or gills of fish related to elevation of water temperature may quickly initiate the columnaris infection. The seasonal resurgence of mass mortalities due to *F. columnare* infection in the polycultured Nile tilapia and catfish during the early summer with some environmental stresses (abrupt temperature rise and polluted pond water) and faulty management (high ammonia and pH and low dissolved oxygen) are the potential initiating factors for such outbreaks (Eissa et al., 2013).

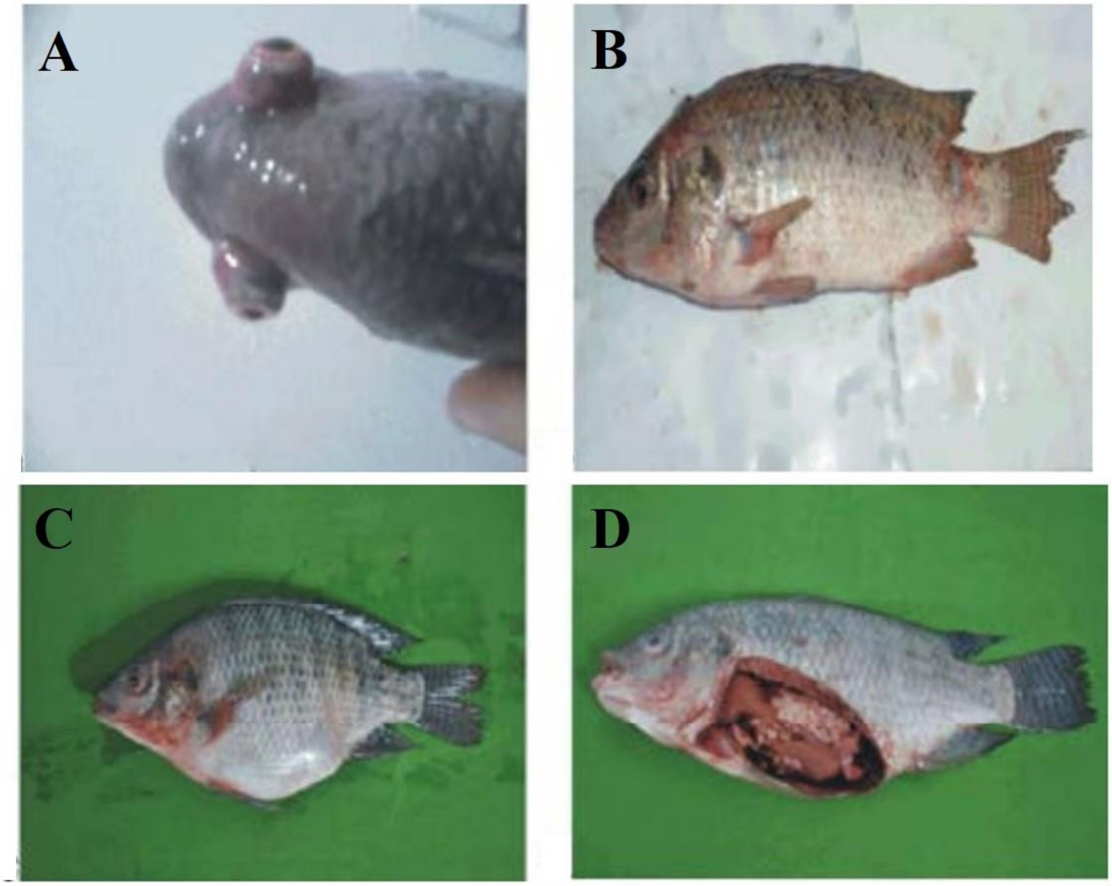


Figure 4 In *Oreochromis niloticus* fish bilateral showing exophthalmia (A), ulcer on skin and erosion on fins and tail (B), severe abdominal ascites (C) and congestion and hemorrhages in all internal organs (D); El Deen et al., 2014

Figure 5 Bilateral exophthalmia in infected *Streptococcus* sp. tilapia; Siti-Zahrah et al., 2008

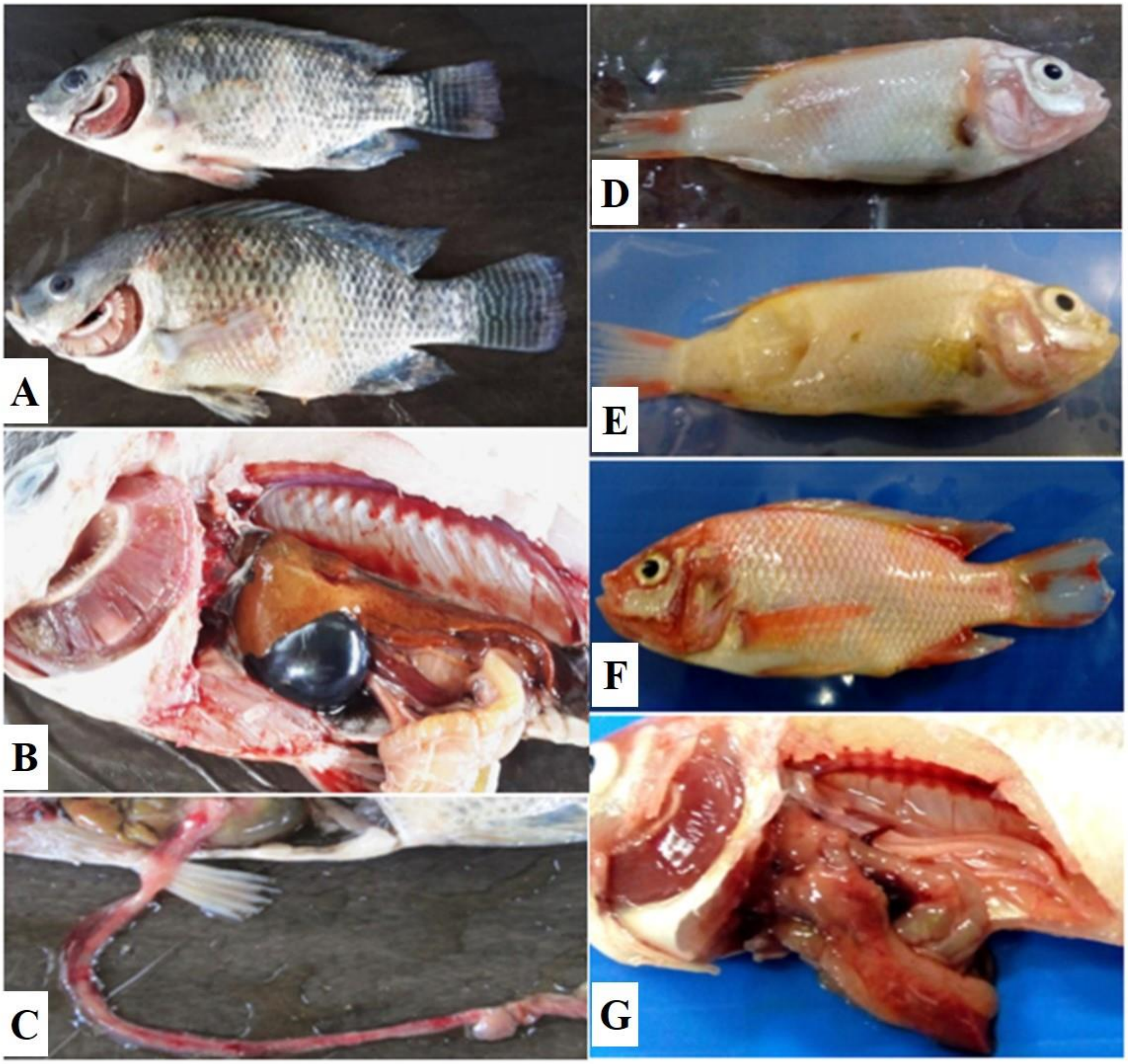


Figure 6 Naturally diseased Nile tilapia exhibited discoloration and hemorrhage on body surface (A), necrotic gills, swollen gall bladder, hemorrhagic liver (B), and enteritis (C). Red tilapia challenging to *F. columnare* NK-Fc01 exhibited typical skin lesion (D) and severe epidermis ulcer (E). Red tilapia exposed to *A. veronii* NK01 or NK06 showing hemorrhage at basal fins (F) and liver (G); Dong et al., 2015

1.4 Herb in aquaculture

The aquaculture industry began to focus on the prevention of diseases rather than treating them with chemotherapeutics and antibiotics. This is because the improper and continuous use of antibiotics may lead to potential development of antibiotic resistant bacteria, environmental pollution and the accumulation of residues in fish. Reverter et al. (2014) assembled database searches with the keywords “plant extracts” with results clearly showing an increasing number of published papers about the potential application of natural products and plant extracts in aquaculture **(Fig. 7)**.

Herbs have advantages when used as natural sources of safer and cheaper chemicals. Thai herbal medicines seem to have potential immunostimulation against pathogenic bacteria. In general, herbs have a variety of advantages in aquaculture, which are as follows (Ramudu and Dash, 2013; Syahidah et al., 2015; Citarasu, 2010):

- Herbs play the part of growth promoters (appetite stimulator)
- Herbs play the part of immunostimulants (anti-stress and antioxidant)
- Herbs play the part of antimicrobial agents (anti-parasitic, antibacterial, antiviral and antifungal)

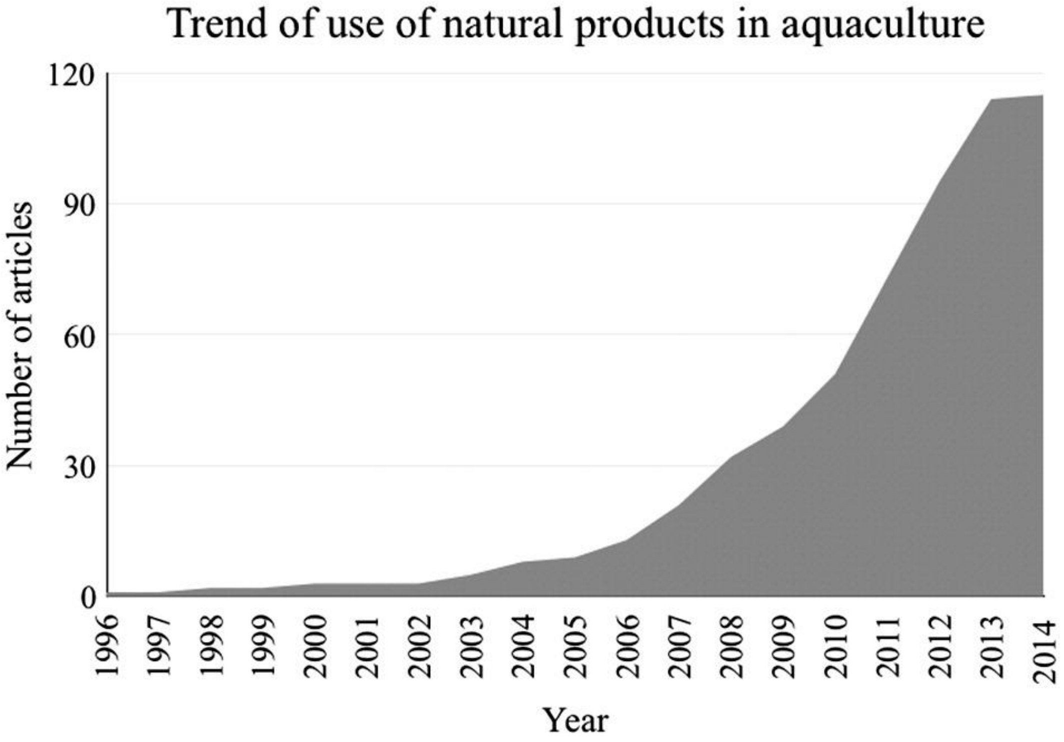


Figure 7 Number of published papers about the use of plant or natural products in aquaculture; Reverter et al., 2014

1.4.1 Herbs as growth promoters

Ramudu and Dash (2013) and Citarasu (2010) reviewed the ability of herbal extract and spices in feed to improve animal performance by stimulating action on gut secretions or by having a direct bactericidal effect on gut microflora and stimulate salivation (amylase production). They found out that these substances stimulated the appetite and increased food consumption and efficiencies of the animal. In addition, the herbal growth promoters induce the transcription rate. This process lead to increased RNA production, total amino acid and high proteins synthesis. Shalaby et al. (2006) found out that food intake, specific growth rate and final weight of Nile tilapia increased when garlic was mixed in the diet. Moreover, Putra et al. (2013) discovered that diet supplemented with 1% ethanolic katuk extract (*Sauropus androgynous*) stimulated appetite and growth and improved food utilization (lower feed conversion ratio) in grouper *Ephinephelus coioides*. However, *E. coioides* fed with enriched diets with higher percentages of katuk extract (2.5 and 5%) presented lower growth. These results also indicate the importance of suitable dosage of feed supplement to obtain the desired effects.

1.4.2 Herbs as immunostimulants

Immunostimulants are substances that enhance non-specific defense mechanism and provide resistance against pathogenic organisms. Herbs are rich sources of immune-enhancing substances and immunostimulants in contrast to vaccines which the effect of herbs on animal's immune response is known to be non-specific whereas, vaccines target specific bacteria by the time these are injected to animals. Moreover, immunostimulants not only stimulate the acquired immune response by increasing the diseases resistance, but also enhance innate, humoral and cellular defense mechanisms (Galindo-Villegas and Hosokawa, 2004). Lysozymes play an important role in the defense of fish by inducing antibacterial activity in the presence of a complement (Harikrishnan et al., 2012b). Other studies analyzed the hematological parameters which provide information on fish health status and they found out that erythrocytes, lymphocytes, monocytes, hemoglobin and hematocrit levels significantly increased in fish treated with plant extracts compared to control fish (Harikrishnan et al., 2012a; Innocent et al., 2011; Shalaby et al., 2006). Several studies have monitored the immunological parameters after intraperitoneal orally or injection administered plant extracts on distinct fish species and they found out that treated fish showed increased lysozyme activity, complement activity, phagocytic activity, increased respiratory burst activity and increased plasma protein (globulin and albumin) **(Table 2)**.

Various chemical compounds found in herbs possess antioxidants such as lignans, tannins, coumarins, stilbenes, quinones, xanthones, catechins, anthocyanins, phenolic acids, flavonols and proanthocyanins which help organisms deal with oxidative stress caused by free radical damage, hence, improve the general physiological condition of fish (Ali et al., 2008; Chakraborty and Hancz, 2011; Marwah et al., 2007). In addition, immunostimulants have additional positive effects such as enhancing the growth and leading to increase in immune factors, which indirectly raises fish resistance to various stresses (Chakraborty and Hancz, 2011; Syahidah et al., 2015).

1.4.3 Herbs as antimicrobial agents

Citarasu (2010) reviewed the compounds in herb such as phenolics, polysaccharides, flavonoids and proteoglycans which play a major role in preventing or controlling infectious microbes. The antibacterial active principles of the herbals may inhibit the enzyme secretions, block the protein synthesis and DNA synthesis, lyse the cell wall and interfere with the signaling mechanism of quorum sensing pathway. The antiviral herbal active compounds may inhibit or block the transcription of the virus to reduce the replication in the host cells, hence enhancing the innate immunity of the host. The antifungal activity of herbal extracts involve the cell wall lysis, altering the permeability, affecting the metabolism and RNA and protein synthesis which leads to death.

Bacterial diseases present a major limiting factor for fish culture and several *Aeromonas* groups have been shown to cause major problems in aquaculture. Disease resistance against *A. hydrophila* was enhanced in *Labeo rohita* (Rohu) fed with 0.5% of *Achyranthes aspera* root extract containing various triterpenoid saponins (Rao et al., 2006). Furthermore, the genus *Streptococcus* is the main etiological agent of streptococcosis in a variety of fresh fish species. *Cratoxylum formosum* has been shown to elevate the innate immune response and enhance disease resistance against *S. agalactiae* in tilapia (Rattanachaikunsopon and Phumkhachorn, 2010). Columnaris disease caused mainly by *Flavobacterium columnare* is another common bacterial disease affecting freshwater fish. No mortality was observed in Nile tilapia fed with diet supplemented with Chinese chive (*Allium tuberosum*) oil (800 mg/kg diet) after infection with *F. columnare* (Rattanachaikunsopon and Phumkhachorn, 2009).

Table 1 Herbs or herbal extracts that improve aquatic animal performance

Herbal/Extract	**Activity**	**References**
Ginseng	Enhanced the growth performance, diet utilization efficiency and haematological indices	Goda, 2008
Quillaja saponin	Increase growth in culture fish species and reduce their metabolic rate	Francis et al., 2005
Zingiber officinale	Improvement of digestive enzyme activity, high FCR, feed intake and conversion and production efficiencies	Venketramalingam et al., 2007
Garlic	Induce fish to ingest and increase feed intake	Harada, 1990; Lee and Gao, 2012; Zeng et al., 1996
Green tea	Enhancing the growth, survival rate, feed utilization and protein content in fish body	Hwang et al., 2013

Table 2 Herbs or herbal extracts that improve aquatic animal immunostimulants

Herbal/Extract	Activity	References
Glycyrrhizin	Enhanced the respiratory burst activity of macrophages and the proliferative responses of lymphocytes	Kim et al., 1998
Emblica officinalis, Cynodon dactylon and *Adhatoda vasica*	Improved the immune system and reduced microbial infection	Minomol, 2005; Magdelin, 2005
Ocimum sanctum, Withania somnifera and *Myristica fragrans*	Improved phagocytic activity, serum bactericidal activity, albumin–globulin (A/G) ratio and leukocrit	Sivaram et al., 2004
Picrorhiza kurroa	Antistress	Citarasu et al., 1998
Toona sinensis (Rutin)	Strong antioxidant and antistress activity	Hsieh et al., 2008
Astragalus membranaceus, Portulaca oleracea, Flavescent sophora and *A. paniculata*	Antistress and induce the immunological parameters	Wu et al., 2007
A. membranaceus and *Lonicera japonica*	Improved the non-specific immune response	Ardo´ et al., 2008
Solanum trilobatum	Enhanced the production of reactive oxygen species (ROS) and serum lysozyme activity	Divyagnaneswari et al., 2007
Toona sinensis	Increased respiratory burst, lysozyme activity and the phagocytic cell activity	Wu et al., 2010
Garlic	Increased the activity of antioxidant enzymes	Metwally, 2009; Li et al., 2008; Shahsavani et al., 2010; Chakraborty and Hancz, 2011

Table 3 Herbs or herbal extracts that improve antimicrobial (fish and shrimp pathogen)

Herbal/Extract	Activity	References
Ricinus communis	Reduced *Vibrio parahaemolyticus*	Immanuel et al., 2004
Ashwagandha (butanolic)	Controlled the shrimp pathogens	Praseetha, 2005
Guava (*Psidium guajava*)	Against bacteria pathogenic for shrimp	Direkbusarakom, 2004
Indian almond (*Terminalia catappa*)	Against tilapia ecto parasites and bacterial pathogen *Aeromonas hydrophila*	Chitmanat et al., 2005
Cynodon dactylon	Antiviral activity against White spot syndrome virus (WSSV)	Balasubramanian et al., 2007
Olive tree leaf (*Olea europaea*)	Controlled salmonid rhabdovirus, viral haemorrhagic septicaemia virus (VHSV)	Micol et al., 2005
Datura metel L.	Anti-Aspergillus and a few marine fungi	Dabur, 2004
Ocimum basilicum	Controlled the pathogens (*Aspergillus flavus* and *Fusarium oxysporum*)	Adigu¨zel et al., 2005
Rosemary (*Rosmarinus officinalis*)	Inhibited a common tilapia pathogen, *Streptococcus iniae*	Zilberg et al., 2010
Chamomile	Antibacterial agent against *Streptococcus agalactiae*	Abdelhadi et al., 2012
Green tea (*Camellia sinensis*)	Against *A. hydrophila* infection	Abdel-Tawwab et al., 2010
Black tyme (*Thymbra spicata* L.) Oregano (*Origanu monites* L.) Savory (*Satureja tymbra* L.)	Controlled the fungal pathogen (*Saprolegnia parasitica*)	Gormez and Diler, 2012
Cinnamon (*Cinnamomum verum*)	Protective effect on *Streptococcus iniae* in tilapia	Pongsak and Parichat, 2010
Garlic	Effectively control or delay *Ichthyopthirius multifiliis* and Trichodiniasis infection	Madsen et al., 2000; Bartolome et al., 2010

1.5 *Houttuynia cordata* Thunb (dokudami)

Houttuynia cordata Thunb (*Khao-tong* or *Plu-khao* in Thailand) is a flowering plant **(Fig. 8)** native to Japan, Southern China and Southeast Asia and is commonly known as Chinese lizard tail. It contains volatile oil, sterols, fatty acids, flavonoid and alkaloids (Bauer et al., 1996). Moreover, it possesses a variety of pharmacological functions including anti-platelet aggregation, anti-bacterial, anti-microbial, anti-tumor, anti-inflammatory, anti-leukemic and immunomodulatory effects (Chen et al., 2003; Hayashi et al., 1995; Lu et al., 2006a, b).

In the recent years, *H. cordata* Thunb became increasingly popular for health promotion in human because of its various pharmacological functions. In 2003, severe acute respiratory syndrome (SARS) affected many countries. The SARS complications are associated with severe inflammatory tissue destruction. Some medicinal herbs were assessed *in vitro,* including *H. cordata* Thunb luteolin, that could be potentially used in the prevention of SARS (Zhang and Chen, 2008; Lu et al., 2006b). Furthermore, this herb is suspected to have an activity against leukemia, a hematopoietic cancer which treatment has many side effects (Pawinwongchai and Chanprasert, 2011). As a result, *H. cordata* Thunb is investigated as an alternative medicinal herb in human. It was also previously suggested that it can be beneficial for the treatment of animals by enhanced immune and growth performance **(Table 4)**.

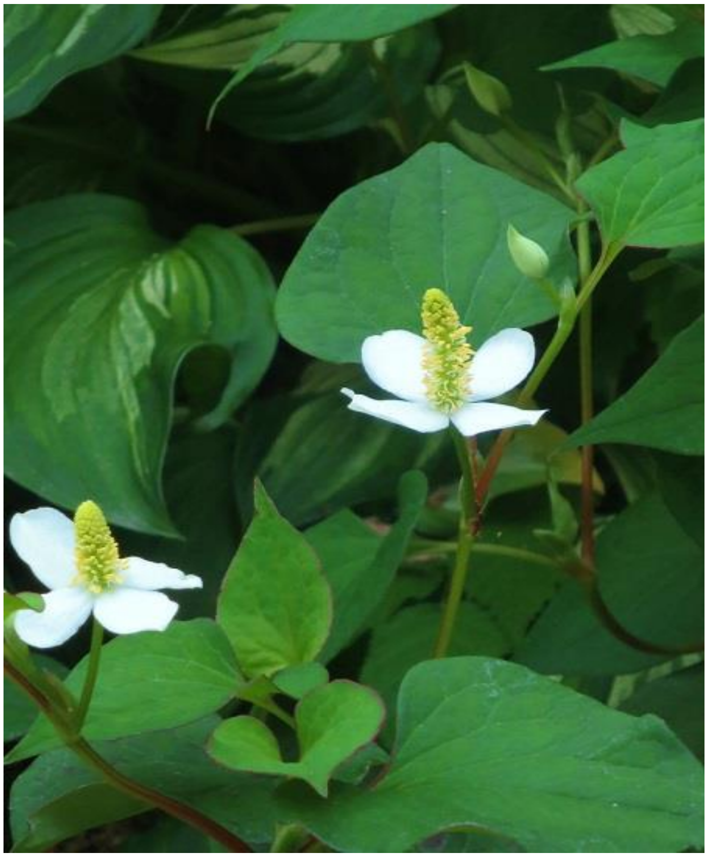


Figure 8 *Houttuynia cordata* Thunb

Table 4 Pharmacology of *Houttuynia cordata* Thunb applied to animals

Animals	Apply	Activity	References
Rat	Essential oil	Anti-inflammatory activity	Lu et al., 2006b
Rat	Leaf extract	Anti-diarrhoeal	Basak et al., 2011
Rat	Houttuynin	Antifibroticrole	Gao et al., 2014
Mice	Power extract	Anti-obesity	Miyata et al., 2010
Mice	Power extract	Against the intracellular bacteria pathogen *Salmonnella*	Kim et al., 2008
Pig	Power extract	Increased growth performance and white blood cell concentration	Yan et al., 2011
Chicken	EOs	Against avian infectious bronchitis virus (IBV) infection	Jiechao et al., 2011
Broiler	Powder	Increased growth performance	Sarker and Yang, 2011
Olive flounder	Powder	Not affect on growth performance	Lee et al., 2015

1.5.1 Antibacterial effects

In aquaculture, people have understood the bad effects of chemotherapeutics and antibiotics. Hence, medicinals herbs are being developed as alternative dietary supplements due to their potential as growth promoters, immunostimulants and antimicrobial agents. *H. cordata* Thunb has a variety of pharmacological activities but the present study focuses mainly on its antibacterial properties.

The main component of *H. cordata* Thunb is decanoyl acetaldehyde (houttuynin), which is an essential oil **(Fig. 9)**. It was reported that houttuynin has various medicinal effect. It is also well known for its antibacterial effects (Lu et al., 2013). Moreover, essential oils from this plant have antibacterial activity against *Staphylococcuc aureus* and *Sarcina ureae* (Zhang et al., 2008), *Bacillus cereus*, *B. subtilis*, *Vibrio cholerae* and *V. parahaemolyticus* (Kwon et al., 1996), *Streptococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Zhang et al., 2008).

1.6 Activity of essential oils (EOs) against bacteria

Essential oils (EOs) extract from herbs have been widely tested as an alternative to conventional drugs. EOs are made from a very complex mixture of volatile molecules that are produced by the secondary metabolism of aromatic and medicinal herbs. The antibacterial activity of EOs has three highlighted effects which are additive, antagonist and synergetic (Faleiro, 2011) **(Fig. 10)**. The antibacterial action of EOs is linked to one of its most important characteristics. It is hydrophobic which results to increased cell permeability and consequent leaking of cell constituents. It is important to comprehend that a disturbed cell structure may affect others cellular structures in a cascade type of action (Carson et al., 2002).

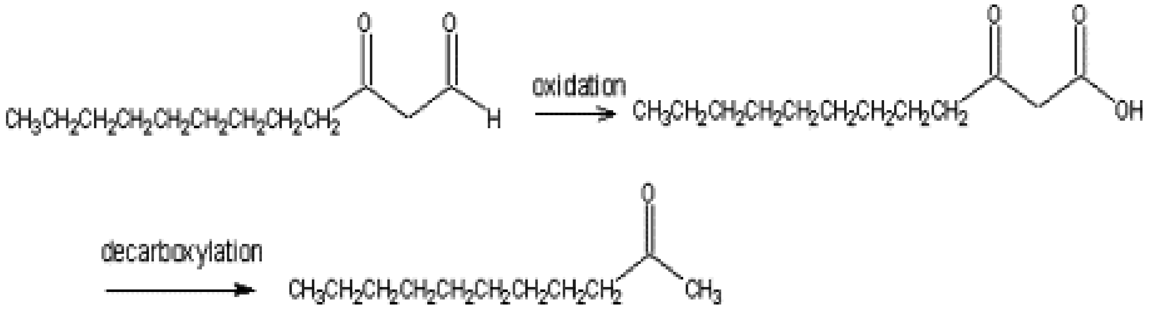


Figure 9 The decanoyl acetaldehyde (houttuynin); Liang et al., 2005

The mechanisms of EOs antibacterial activity are as follows (Faleiro, 2011; Nazzaro et al., 2013):

- Effect on cell wall and membrane
- Action on protein synthesis
- Effect on ATP production
- Anti-quorum sensing activity
- Other; intracytoplasmic changes, pH disturbance and DNA mutation

1.6.1 Effect on cell wall and membrane

Generally, Gram-negative bacteria are more resistant to EOs than Gram-positive bacteria because of the difference in the structures of their cell walls (Nazzaro et al., 2013) **(Fig. 11)**. The lipid biosynthesis pathway is an important target for the development of novel antimicrobials (Caccioni et al., 1998; Doran et al., 2009). Due to their hydrophobic nature, EOs or their components can affect the percentage of unsaturated fatty acids (UFAs) and alter their structure (Warnke et al., 2009). The evaluation of the loss of cell constituents contributes to elucidate the severity of the cell membrane damage and a significant number of studies have used this approach to clarify the antibacterial action of EOs (Bouhdid et al., 2009; Cox et al., 2000; Carson et al., 2002) and those indicate that the tested EOs affect the bacterial cell on the same target, the cytoplasmic membrane.

1.6.2 Action on protein synthesis

The first reported action of EOs components was on protein synthesis. The EOs components, *p*-cymene and carvacrol, was found to induce the synthesis of heat shock proteins

(HSPs) when bacterial cells were treated with these two (Burt et al., 2007). HSPs are molecular chaperones involved in the different processes of assembly and release of newly synthesized polypeptides that, in general, increase when bacterial cells come in contact with toxic substances or other stress conditions (Faleiro, 2011).

1.6.3 Effect on ATP production

The disruption of cell membrane by any antimicrobial agent, including EOs will compromise a series of vital functions (synthesis of structural macromolecules, energy for conversion processes, secretion of many growth key enzymes and nutrient processing). The production of ATP in prokaryotes occurs both in the cell wall and cytosol by glycolysis. Thus, it is expected that there will be alterations on intracellular and external ATP balance due to the action of the EOs on the cell membrane. The correlation between the intracellular and extracellular ATP concentration has been reported (Turgis et al., 2009; Helander et al., 1998; Oussalah et al., 2006).

1.6.4 Anti-quorum sensing activity

Bacteria coordinate both bacterium-bacterium associations and interactions with higher organisms through intercellular communication systems known as quorum sensing (QS) systems **(Fig. 12)**. Researchers have been continuously investigating herbal products in the quest for new anti-pathogenic and therapeutic agents that might act as nontoxic inhibitors of QS, thus controlling infections without encouraging the appearance of resistant bacterial strains (Carson et al., 2002). EOs may represent the richest available reservoir of novel therapeutics (Carson et al., 2002; Nguefack et al., 2004; Oussalah et al., 2006).

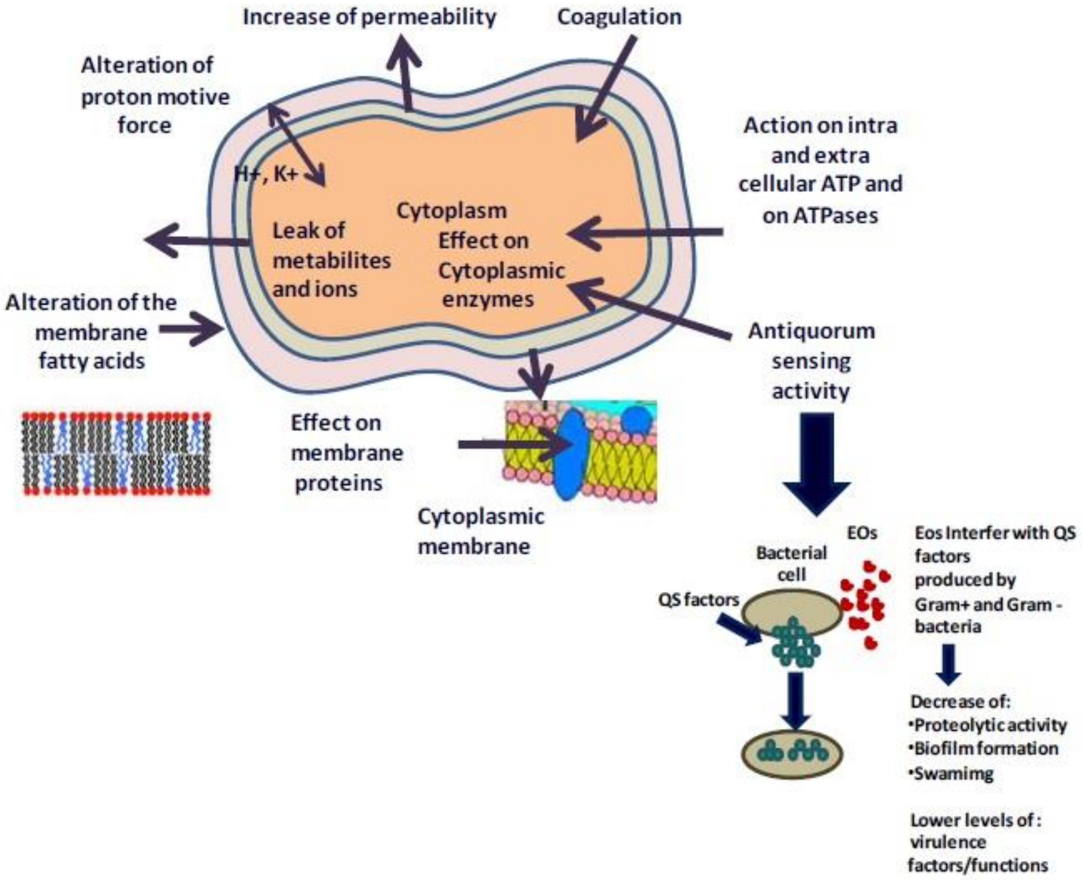


Figure 10 Mechanism of action and target sites of EOs on microbial cells; Nazzaro et al., 2013

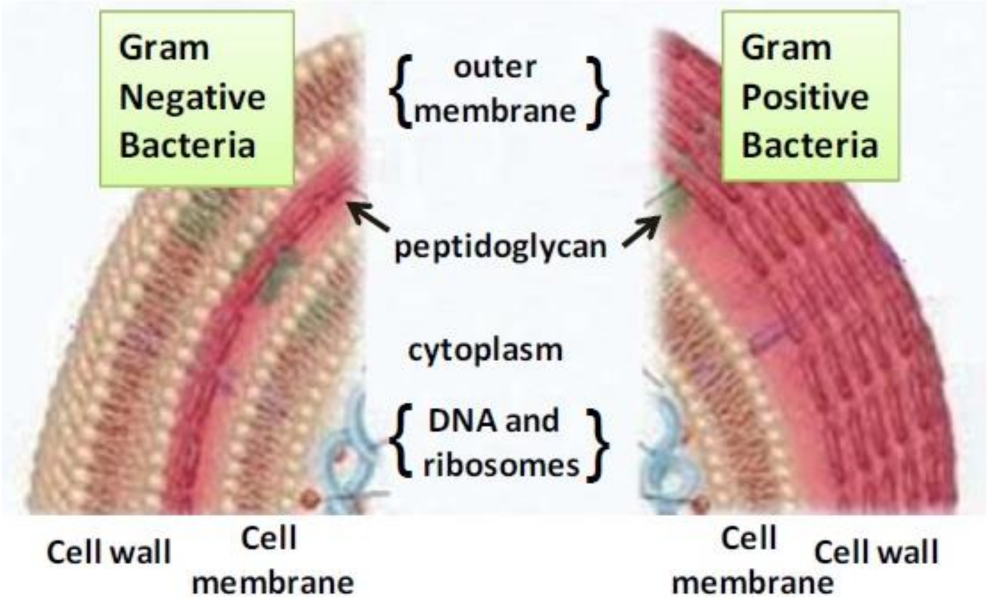


Figure 11 The difference between cell membranes of Gram-positive (on the right) and Gram-negative bacteria (on the left); Nazzaro et al., 2013

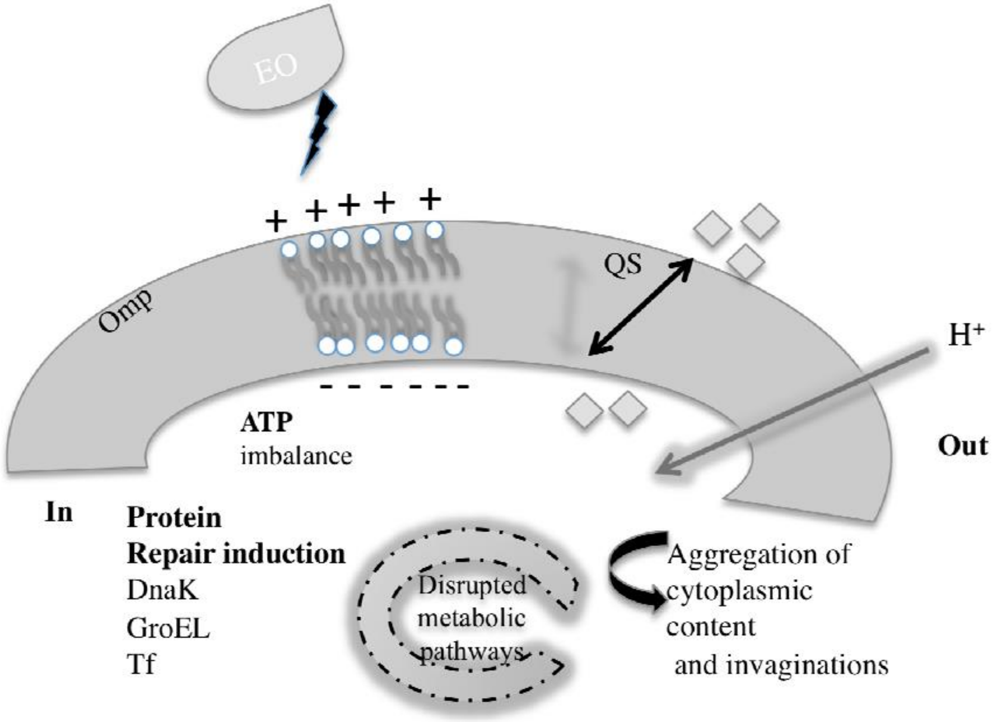


Figure 12 Identified bacterial cell structures and cellular processes disrupted by the action of EOs or their components. EOs treated cells are more permeable to protons, experience an ATP imbalance and induced synthesis of chaperones. OMP (Outer membrane protein); QS (quorum sensing). Metabolic pathways can be disrupted; Faleiro, 2011

CHAPTER 2

Gas Chromatography-Mass Spectrometry (GC-MS) for Analysis of Chemical Composition of Essential Oils (EOs) from *Houttuynia cordata* Thunb (dokudami)

2.1 Introduction

Houttuynia cordata Thunb is a flowering plant native to Japan, Southern China and Southeast Asia and is commonly known as Chinese lizard tail. It contains volatile oil, fatty acids, sterols, flavonoid and alkaloids (Bauer et al., 1996). Decanoyl acetaldehyde (houttuynin) is one of the main components of *H. cordata* Thunb EOs. It was reported that houttuynin has various medicinal effect, but it is unstable and easily oxidized between the process of distillation and storage. The complex and time-consuming process of sample preparation is sometimes very complicated which may lead to various results due to many factors involved. Some studies reported about the chemical composition of *H. cordata* Thunb EOs extracted by steam distillation and analyzed using GC-MS (Do et al., 2015; Liang et al., 2005; Yang et al., 2010; Xu et al., 2011). However, the properties of the metabolites found in EOs of *H. cordata* Thunb growing in North Thailand have rarely been investigated. This study aimed to identify the chemical composition of *H. cordata* Thunb essential oils by gas chromatography-mass spectrometry (GC-MS).

2.2 Methods

2.2.1 Plant material and EOs extraction

H. cordata Thunb was collected from Pasao district of Lamphun province, Thailand (Latitude 18.639539, Longitude 99.032919). Total area was 0.48 hectare **(Fig. 13)**. Amount of 300 g of whole fresh plants were cut into small pieces (10 cm) introduced into the distillation flask (2 L), which was connected to the steam generator via a glass tube and to a condenser to retrieve the oil. EOs were volatilized with boiling water at temperature 100°C for 3 hours. After the steam distillation process, the product was collected and separated using separatory funnel. EOs settled at the bottom layer of the separatory funnel and were separated several times until no oil was left in the separatory funnel. EOs product was stored at 4ºC until further analysis **(Fig. 14)**.

2.2.2 GC-MS analysis

GC-MS analysis was performed using Agilent 6890N gas chromatography instrument coupled to an Agilent 5873 mass spectrometer and equipped with an HP INNOWAX capillary column (30 m x 0.25 mm I.D., film thickness 0.25 mm). The column was initially maintained at 35ºC for 3 min and programmed to 250ºC at flow rate at 5ºC/min, then held for 5 min. The temperature of the injection port and interface was set at 250°C. Helium was used as the carrier gas with a flow rate of 1 mL/min. One µL of samples was used during injection. Mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70 eV with the scan rate of 2.69 scan/s from 50 to 600 atomic mass units. The ionization source temperature was 230 °C. The chemical composition of essential oils was identified using the NIST Mass Spectral Database. The relative responses of the individual components were expressed as percent peak area relative to total peak area (Lu et al., 2006a).

Figure 13 Map of study area in Pasao district of Lamphun province, Thailand. Arrow indicates *Houttuynia cordata* Thunb culture locations

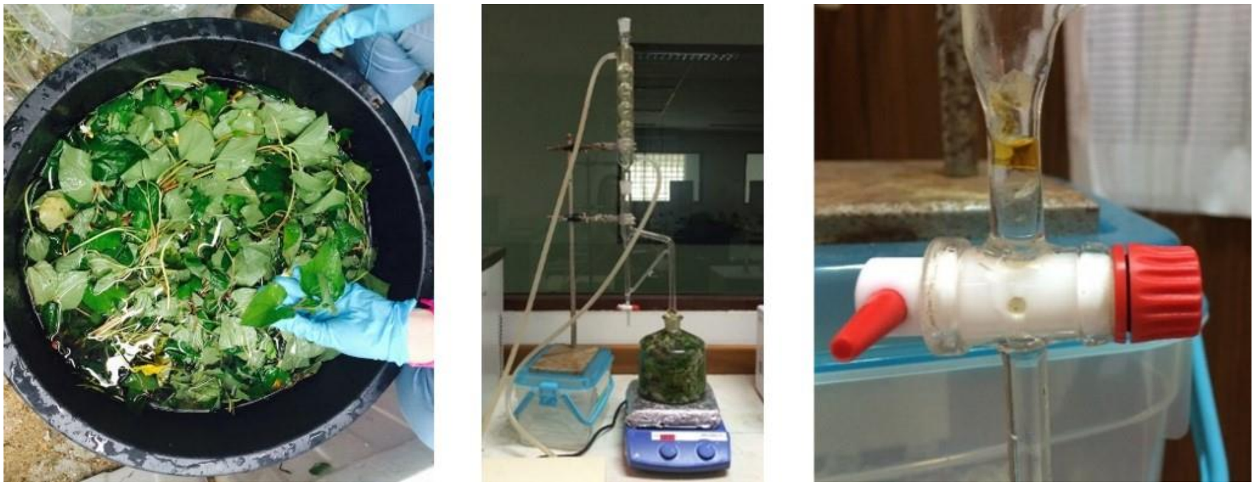


Figure 14 Preparation of sample for hydrodistillation

2.3 Results

Hydrodistillation of *Houttuynia cordata* Thunb produced a clear colorless to pale yellow oil with a yield of 0.057%v/w **(Fig. 15)**. GC-MS analysis of essential oils from *H. cordata* Thunb identified 28 phytochemicals **(Fig. 16) (Table 5)**. Results were further investigated and the major components found were beta-myrcene (44.220%) and 2-undecanone (21.985%) **(Fig. 17)**.

2.4 Discussion

EOs yield is a product of biomass yield and concentration present in plants. The EOs yield of *Houttuynia cordata* Thunb was 0.057%v/w which implies that 1,000 kg fresh *H. cordata* Thunb can produce 57 kg of EOs. The average harvest per year of fresh *H. cordata* in a farm this study is 15,000 kg/ha/year yielding to 855 kg/ha/year essential oil. This amount is sufficient for aquaculture application of *H. cordata* EOs as feed supplement for Hybrid red tilapia. The GC-MS analysis of EOs from *H. cordata* Thunb identified 28 phytochemicals. The major components were found to be beta-myrcene (44.220%) and 2-undecanone (21.985%). This result differed from previous reports, which can be attributed to the difference of plant parts used, extraction method, condition method and environmental condition.

Whole plant of *H. cordata* Thunb was used to extract essential oil and the main component found was indeed 2-undecanone (21.985%). This quantity of 2-undecanone is quite similar when belowground parts and leaves were used in the extraction as reported by previous studies. The EOs from the belowground parts was found to be dominated by methyl nonyl ketone (2-undecanone) (23.96%) and beta-myrcene (14.29%) (Lu et al. 2006a) and leaves were found to contain beta-myrcene (30.8%) and 2-undecanone (19.7%) as major components (Do et al., 2015). While, Lu et al. (2006a) reported that the EOs from aboveground parts was characterized by high methyl nonyl ketone (2-undecanone) (36.07%) and beta-myrcene (12.57%). However, the use of different instrument and analysis techniques may have an effect on the chemical components of this plant. Agreed with Liang et al. (2005) reported that various sampling techniques were compared for the GC-MS of volatile oil in *H. cordata* Thunb (HCT). 2-undecanone (22.21%) and houttuynum (7.23%) were the predominant components of HCT samples obtained by headspace solid-phase micro-extraction technique, whereas the levels were 3.95% and 3.60% from the same samples in flash evaporation technique and 25.93% and 6.60%, respectively, in steam distillation.

Differences in the growing conditions of *H. cordata* Thunb influence the production of metabolites. In this study, the samples were collected from Lamphun province, Thailand (Latitude 18.639539, Longitude 99.032919). Yang et al. (2010) studied volatile compounds in *H. cordata* Thunb populations from different altitudes in Guizhou province, China by GC-MS and HPLC (high performance liquid chromatography). The relative percentage of 2-undecanone increased from 13.75% to 79.42% when the altitude increased from 200 - 300 m at Tongren to 700 - 800 m at Yinjiang.

2.5 Conclusion

Houttuynia cordata Thunb from Northern Thailand produced a clear colorless to pale yellow oil with a yield of 0.057% v/w and GC-MS analysis of EOs identified 28 phytochemicals. 2-Undecanone, one of the major components, is known to have pharmacological effect.

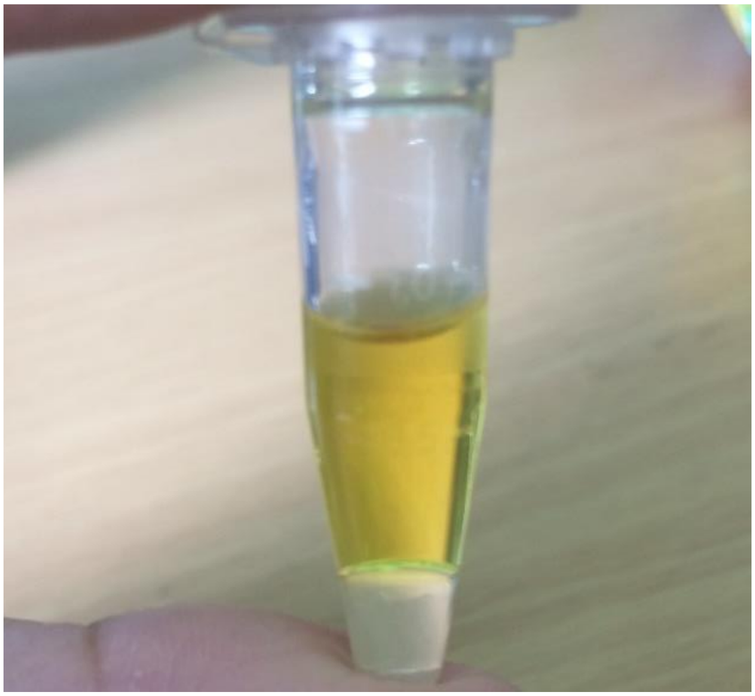


Figure 15 EOs of *Houttuynia cordata* Thunb extract

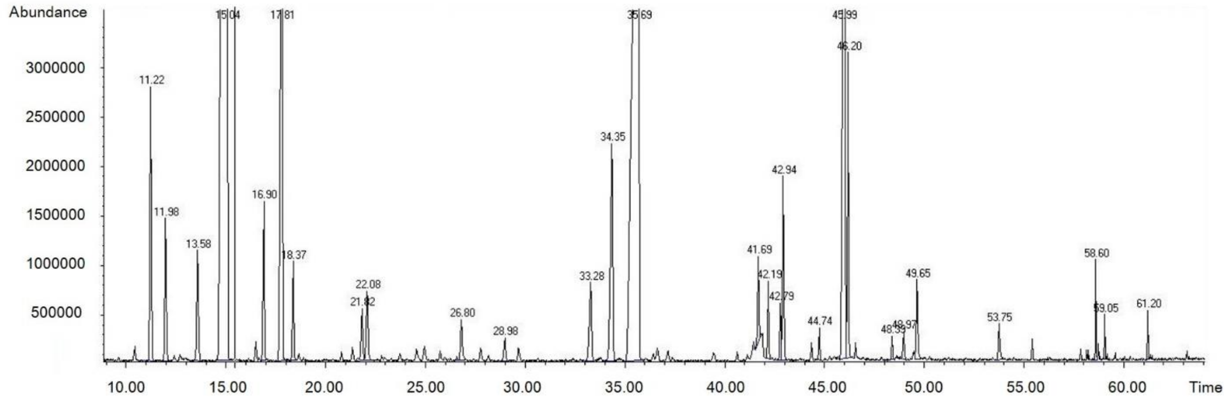


Figure 16 Chromatogram of EOs components of *Houttuynia cordata* Thunb

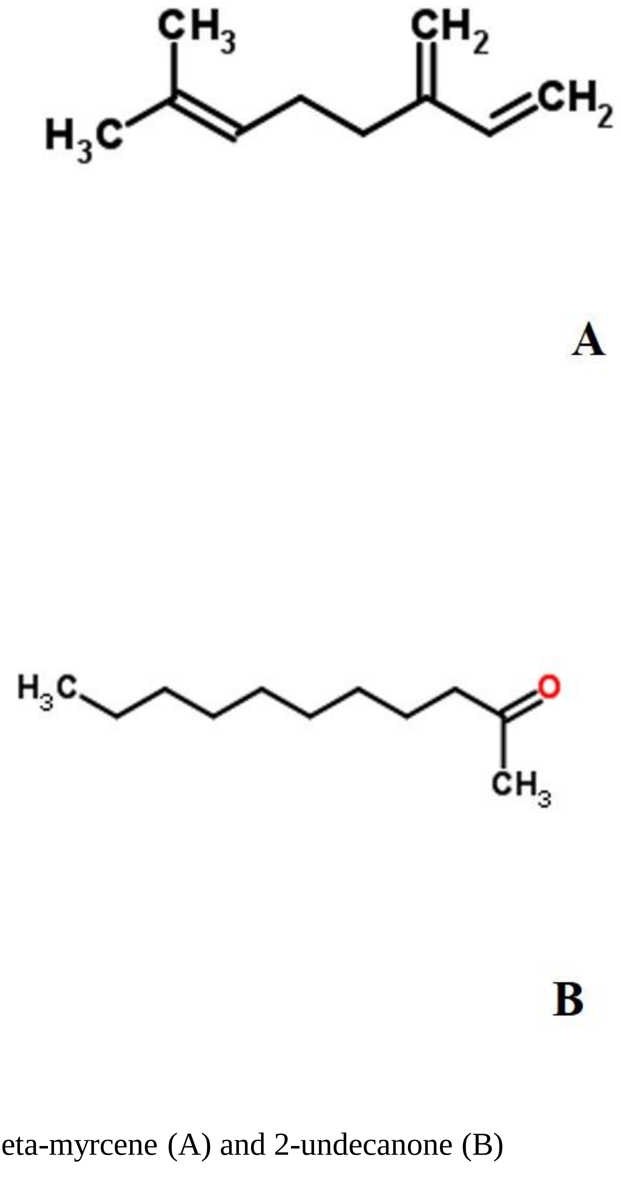


Figure 17 Structure of beta-myrcene (A) and 2-undecanone (B)

Table 5 Chemical composition of EOs from *Houttuynia cordata* Thunb extract

RT (min.)	Percent (%)	Compound name
11.223	2.124±0.048	Alpha-pinene
11.974	1.125±0.023	Camphene
13.582	1.011±0.020	Beta-pinene
15.045	**44.220±0.240**	**Beta-myrcene**
16.897	1.125±0.008	D-limonene
17.810	7.424±0.014	1,3,6-Octatriene, 3,7-dimethyl-
18.367	0.687±0.005	1,3,6-Octatriene, 3,7-dimethyl-
21.820	0.502±0.002	1,6-Octhadien-3-ol, 3,7dimethyl-
22.076	0.713±0.002	2-Hexenoic acid, 6-(2-methylenecyclopropyl)-, methyl ester
26.799	0.424±0.008	1-Nonanol
28.982	0.215±0.011	Decanal
33.280	0.956±0.021	Dimethyl (1-methylvinyl) chloroilane
34.349	2.685±0.014	Bicyclo[2.2.1]heptan-2-ol,1,7,7-trimethyl, acetate (1S-endo)
35.700	**21.985±0.146**	**2-Undecanone**
41.687	0.561±0.025	2,9-Octadien-1-ol, 3,7-dimethyl-, acetate
42.187	0.501±0.016	2-Dodecanone
42.794	0.363±0.011	Tridecanal
42.938	1.266±0.042	Caryophyllene
44.739	0.193±0.023	1,6,10-Dodecatriene, 7,7-dimethyl-3-methlene-
45.984	8.510±0.217	1-Propene, 1-methoxy-2-methyl
46.197	1.830±0.055	2-Tridecanone
48.392	0.141±0.002	1,6,10-Dodecatrien-3-ol, 3,7-trimethyl
48.968	0.197±0.002	Caryophyllene oxide
49.644	0.540±0.042	Heptane, 3-ethyl-5-methylene
53.747	0.318±0.011	L-leucine, N-acetyl-, methyl ester
58.601	0.255±0.009	2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene
59.052	0.129±0.005	Cyclohexene,1-methyl-4-(5-methyl-1-methyl)
61.203	0.131±0.002	Hexadecanoic acid,1,1-dimethylethyl ester
	100.00	

RT = Retention time

CHAPTER 3

Antibacterial Activities of Essential Oils (EOs) from *Houttuynia cordata* Thunb (dokudami) Against Fish Pathogens

3.1 Introduction

In Thailand, as aquaculture production becomes more intensive, the cases of infectious diseases have also increased. Fish diseases caused by *Aeromonas hydrophila, Streptococcus* sp. and *Flavobacterium columnare* are the common ones. Among these, *Aeromonas hydrophila*, Gram-negative bacteria, cause widespread infection and highest fish mortality (Starliper et al., 2015). In addition, *Streptococcus* sp., a Gram-positive bacteria, cause various clinical signs, such as haemorrhages at the gill plate, eyes and base of the fins and opercula, loss of appetite, spine displacement and corneal opacity (Amal and Zamri-Saad, 2011). *Flavobacterium columnare*, a Gram-negative rod-shaped bacteria, affects the skin and gills of fish with elevation of water temperature which quickly initiates the columnaris infection (Rattanachaokunsopon and Phumkhachorn, 2009).

The use of antibiotics for controlling diseases has been criticized for their negative impacts (environmental contamination). Heavy antibiotics used in aquaculture need to be reduced and replaced with alternative processes in treating fish diseases. Treatment of bacterial diseases with EOs from herbs has been known to be safe for use in aquaculture. Previously published studies reported the effect of EOs from herbs such as cinnamon (*Cinnamomum cassia)*, oregano (*Origanum vulgare)*, lemongrass (*Cymbopogon citratus)*, thyme (*Thymus vulgaris)*, *Hesperozygis ringen*, *Ocimum gratissimum* and *Ocimum americanum* on disease resistance against *Aeromonas* spp. (Starliper et al., 2015; Sutili et al., 2015; Pongsak and Parichat, 2010). Moreover, it was found out that Chinese chive (*Allium tuberosum*) had a bacteriocidal effect on *F. columnare* (Rattanachaokunsopon and Phumkhachorn, 2009).

H. cordata Thunb is a flowering plant native to Japan, Southern China and Southeast Asia and is commonly known as Chinese lizard tail. It contains volatile oil, fatty acids, sterols, flavonoid and alkaloids (Bauer et al., 1996). Decanoyl acetaldehyde (houttuynin) is one of the main components of *H. cordata* Thunb EOs. It was reported that houttuynin has various medicinal effect and well known for its antibacterial effects (Lu et al., 2013). Furthermore, the EOs from this plant have antibacterial activity against *Staphylococcus aureus* and *Sarcina ureae* (Zhang et al., 2008). The aim of this study was to determine the antibacterial activity of *H. cordata* Thunb EOs against fish pathogen.

3.2 Methods

3.2.1 EOs extraction

H. cordata Thunb was collected from Pasao district of Lamphun province, Thailand (Latitude 18.639539, Longitude 99.032919). Total area was 0.48 hectare **(Fig. 13)**. Amount of 300 g of whole fresh plants were introduced into the distillation flask (2 L), which was connected to the steam generator via a glass tube and to a condenser to retrieve the oil. EOs were volatilized with boiling water at temperature 100°C for 3 hours. After the steam distillation process, the product was collected and separated using separatory funnel. EOs settled at the bottom layer of the separatory funnel and were separated several times until no oil was left in the separatory funnel. EOs product was stored at 4ºC until further analysis **(Fig. 14)**.

3.2.2 Determination of the antibacterial activity (*in vitro* study)

3.2.2.1 Preparation of the bacteria

The pathogenic bacteria that were used, which were commonly found in Tilapia include *Aeromonas hydrophila*, *Streptococcus* sp. and *Flavobacterium columnare*. They were isolated from infected tilapia and cultured at Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Thailand. For preparation of the inocula, *Aeromonas hydrophila* and *Streptococcus* sp. were taken from the stock culture and grown separately in 50 mL nutrient agar and incubated at 37ºC for 24 hours. On the other hand, *Flavobacterium columnare* was grown in 50 mL CPA 1 broth and incubated at 37ºC for 24 hours. For the bacterial suspension used in the experiment, phosphate buffer solution (PBS) was used as the suspending liquid. An appropriate volume of bacterial suspension was transferred to a cuvette and absorbance was read at 610 OD using a spectrophotometer. *Aeromonas hydrophila* and *Streptococcus* sp. had an absorbance reading of 0.08-0.1 while *Flavobacterium columnare* was at 0.04-0.1, which produce a viable count $1 x 10^7$ CFU/mL.

3.2.2.2 Minimum inhibitory concentration (MIC)

The integrated assay (growth and non-growth of bacteria) was assessed by visual inspection (Lu et al., 2006a). Minimum inhibitory concentration (MIC) is the observed lowest concentration of an essential oil to effectively prevent the growth of bacteria **(Fig. 18)**.

Aeromonas hydrophila, *Streptococcus* sp. and *Flavobacterium columnare* were grown for 24 hours at 37ºC and total counts were adjusted to approximately 10^7 CFU/mL. A 4,000 ppm stock solution of *H. cordata* Thunb essential oils (HEO) was prepared by

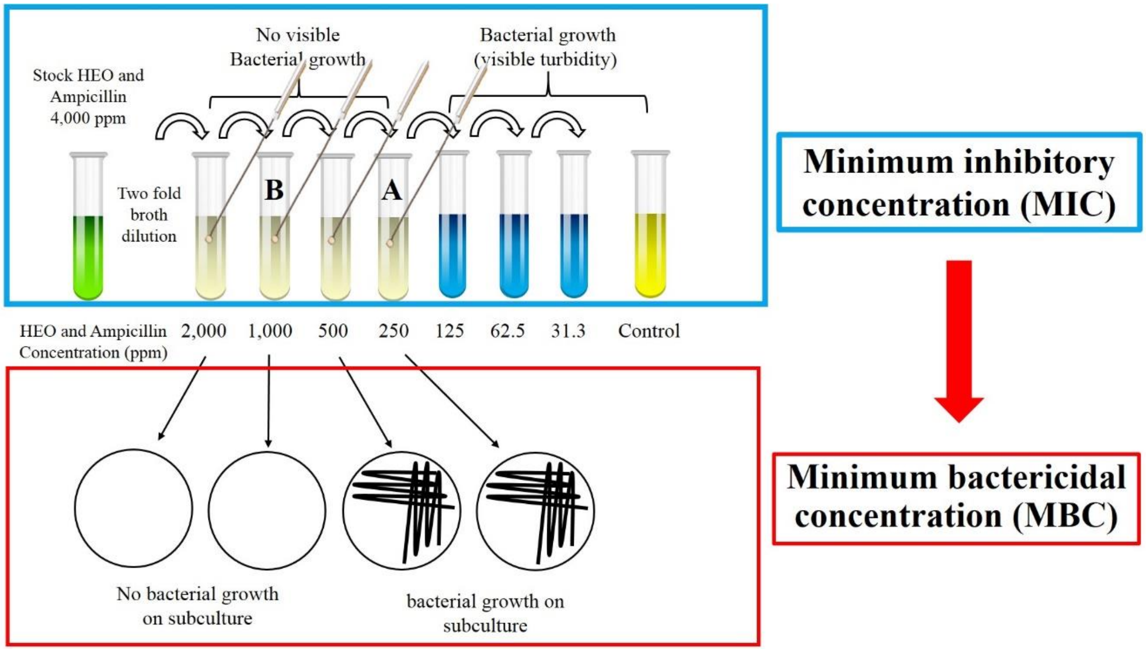


Figure 18 The minimum inhibitory concentration and minimum bactericidal concentration testing. In this example, MIC of the *Houttuynia cordata* Thunb essential oils (HEO) is 250 ppm (tube A) and MBC is 1,000 ppm (tube B)

diluting 0.2 µL essential oil to 5 mL sterile distilled water: Tween 80 (Sigma 0.5%, v/v). From the stock solution 2,000, 1,000, 500, 250, 125, 62.5 and 31.3 ppm (Lu et al., 2006a) concentrations were prepared by serial broth dilution method. Ampicillin sodium served as the positive control with same amounts as HEO used. After the serial two-fold broth dilution method, 0.1 mL of bacterial suspension was added to 0.5 mL of each serial two-fold dilution tube mixed and incubated at 37ºC for 20 hours.

3.2.2.3 Minimum bactericidal concentration (MBC)

Referring to the results of MIC assay, all tubes showing complete absence of growth were compared with control. One loopful from each tube was transferred to a nutrient agar plate and incubated at 37ºC for 20 hours. The lowest concentration of an EOs to totally kill the bacteria was considered as the MBC **(Fig. 18)**.

3.2.3 Statistical analysis

Data obtained from the experiment were expressed as mean ± standard error (S.E.) of triplicates. One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (p<0.05) were used to analyze the significant statistical differences between the treatments.

3.3 Results

In this study, the *H. cordata* Thunb essential oils (HEO) also expressed antibacterial activities against *Aeromonas hydrophila, Streptococcus* sp. and *Flavobacterium columnare* with minimum inhibitory concentration (MIC) of 250, 250 and 500 ppm, respectively. On the other hand, minimum bactericidal concentration (MBC) was not determined for all concentration. It should be noted that HEO was effective at the same concentration as ampicillin sodium against *Aeromonas hydrophila, Streptococcus* sp. and *Flavobacterium columnare* **(Table 6) (Fig. 19 and 20)**.

Table 6 Antibacterial activity against fish pathogens

Species	HEO		Ampicillin sodium	
	MIC (ppm)	MBC (ppm)	MIC (ppm)	MBC (ppm)
Aeromonas hydrophila	250	ND	250	ND
Streptococcus sp.	250	ND	250	ND
Flavobacterium columnare	500	ND	500	ND

HEO = *Houttuynia cordata* Thunb essential oils
MIC = Minimum Inhibitory Concentration
MBC = Minimum Bactericidal Concentration
ND = Not detected

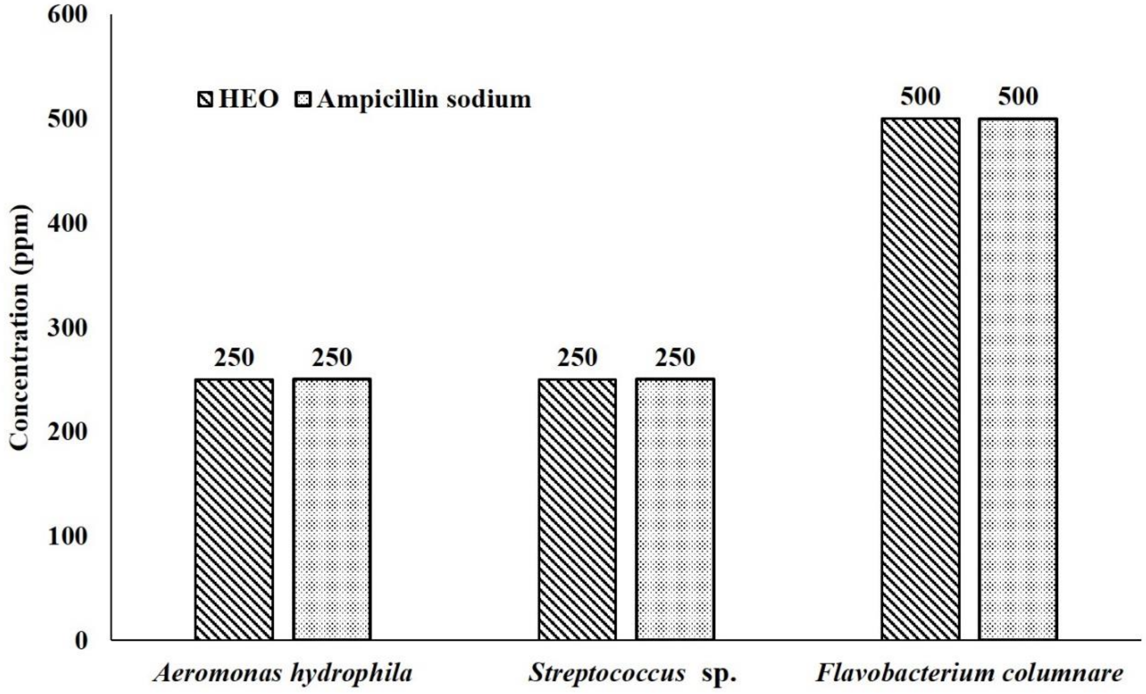


Figure 19 Minimum inhibitory concentration (MIC) of HEO compared with ampicillin sodium

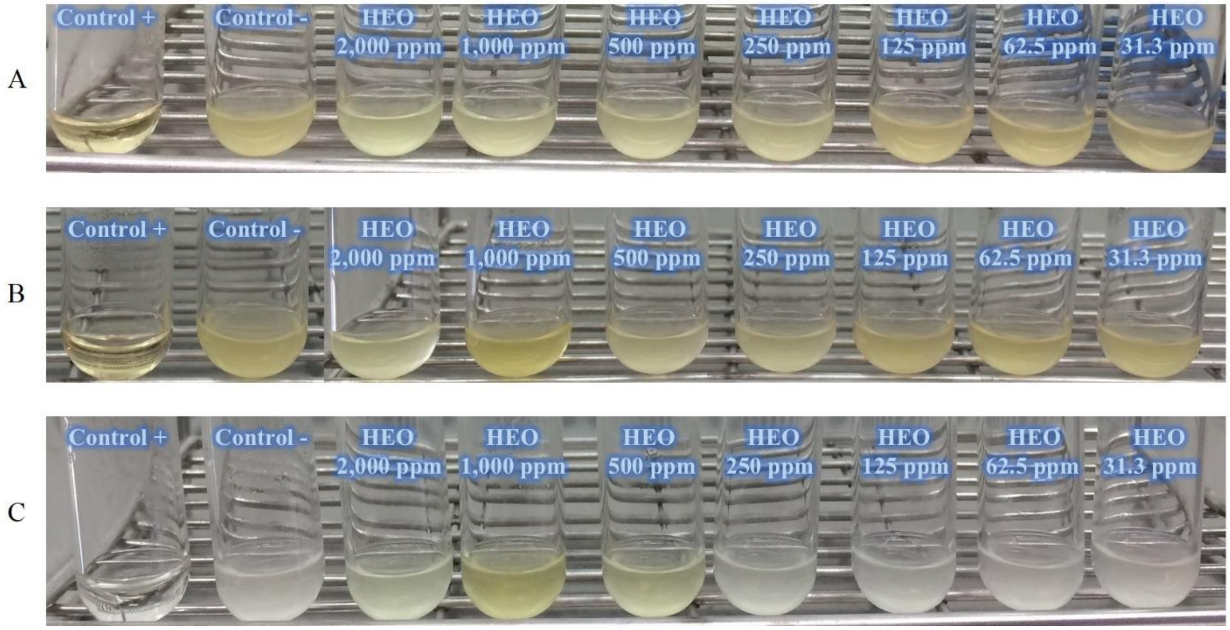


Figure 20 Complete absence of *Aeromonas hydrophila* (A) *Streptococcus* sp. (B) and *Flavobacterium columnare* (C) were compared with control

3.4 Discussion

In this study, the *H. cordata* Thunb essential oils (HEO) also expressed a wide range of MIC against *Aeromonas hydrophila* (250-2,000 ppm), *Streptococcus* sp. (250-2,000 ppm) and *Flavobacterium columnare* (500-2,000 ppm). It was noted that the HEO was effective at the same concentration as ampicillin sodium. Certain HEO can be a promising alternative as antibacterial agents against fish pathogens such as *A. hydrophila*, *Streptococcus* sp. and *F. columnare* disease treatments instead of using chemotherapeutic methods which can pose harm to humans.

The antibacterial activity of EOs against bacteria varies depending on the concentration of the components and strain of bacteria. These activities may be attributed to the presence of chemical compounds in essential oils of *H. cordata* Thunb. One of the major components, beta-myrcene did not show observable antibacterial activity on its own. However, it provided enhanced activities when mixed with other components in EOs. Interestingly the antibacterial activities were not as effective when the constituent beta-myrcene was taken out. This showed that the chemical components which make up an EOs were complementary to each other and actually perform better as a whole. Hence, beta-myrcene shows good antibacterial activity due to the synergistic effect combine with other main components (Tajadod et al., 2012; Ghorab et al., 2013; Chibani et al., 2013). The second main component, 2-undecanone (methyl nonyl ketone) is oxidized from decanoyl acetaldehyde. It is known to have a pharmacological effect, but unstable and easily oxidized (Liang et al., 2005). This component has activities that can cause a moderate inhibition of *Escherichia coli* (Egyud, 1967), *Salmonella* sp. (Kim et al., 2008), *Staphylococcus aureus* (Lu et al., 2013; Lu et al., 2006a) and *Sarcina ureae* growth (Lu et al., 2006a). Some minor components in essential oils of *H. cordata* Thunb such as α-pinene, β-pinene and limonene also have a strong antibacterial activity (Magiatis et al., 1999; Martin et al., 2000; Tzakou et al., 2001; Sökmen et al., 2003).

The mechanism of action of this antibacterial effect involves cell wall structure as well as the denaturing and coagulating proteins. In Gram-negative bacteria, the cell wall is composed of a thin peptidoglycan layer adjacent to cytoplasmic membrane and an outer membrane made up of phospholipids and lipopolysaccharides (Nazzaro et al., 2013). On the other hand, Gram-positive bacteria, lack the outer membrane but has a cell wall composed of a thicker peptidoglycan layer. Generally, Gram-positive bacteria were more sensitive to herbal essential oils than gram-negative bacteria, due to their outer membrane barriers (Burt, 2004). However, in this study, essential oils of *H. cordata* Thunb showed activity against both Gram-positive and Gram-negative bacteria which imply that the presence of an outer membrane is irrelevant for antimicrobial resistance. Moreover, the cell shape may also be involved in the susceptibility of bacteria. Normally, rod-shaped bacterial cells are more sensitive to essential oils than coccoid cells (Hajlaoui et al., 2009).

3.5 Conclusion

HEO showed proven antimicrobial activity against fish pathogens. Thus, the use of EOs from herbs could be an efficient tool to achieve an economically efficient and environment-friendly approach in solving problems related to aquaculture production.

CHAPTER 4

Effect of Essential Oils (EOs) from *Houttuynia cordata* Thunb (dokudami) Supplemented Diets on Growth Performance and Immune Response of Hybrid Red Tilapia (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.)

4.1 Introduction

Tilapia is one of the most important freshwater fish in aquaculture. In Thailand, the annual report stated that, more than 188,000 tons of tilapia were produced in 2014 (Food and Agriculture Organization of the United Nation, 2016). Hybrid red tilapia is cultured in ponds and floating cages in river and reservoir where infectious diseases are common. This is the major cause of huge economic loss in commercial aquaculture. The aquaculture industry began to focus on the prevention of diseases rather than treating them with chemotherapeutics and antibiotics (the use of which has been criticized for their negative side effects). In avoiding chemotherapeutics and antibiotics, natural products are perceived as the best alternative to control infectious diseases.

Currently, Thai herbs have been widely used in commercial aquaculture as growth-promoting substances, antibiotics, antimicrobial agents, nutrient source and many more. There are a lot of herbal medicines found to be effective growth promoters and agents against fish pathogens. Ramudu and Dash (2013) and Citarasu (2010) reviewed the ability of herb extracts and spices in feed to improve animal performance (by stimulating action on gut secretions or by having a direct bactericidal effect on gut microflora) and stimulate salivation (amylase production). Good results in fish have been attained with garlic (Harada, 1990; Lee and Gao, 2012; Zeng et al., 1996), green tea (Hwang et al., 2013) and ginseng (Goda, 2008), which showed an enhanced growth and increase in immune factors which indirectly raises fish resistance to various stresses (Chakraborty and Hancz, 2011; Syahidah et al., 2015).

H. cordata Thunb is a flowering plant native to Japan, Southern China and Southeast Asia and commonly known as Chinese lizard tail. It contains volatile oil, fatty acids, sterols, flavonoid and alkaloids (Bauer et al., 1996). There are only a few studies about the use of this plant in aquaculture. Lee et al. (2015) used 1% powder of *H. cordata* Thunb that was mixed to the diet of juvenile olive flounder but it showed no effect on weight gain and serum chemistry. On the other hand, the addition of essential oils to the diet was found to cause an improvement to animals. Essential oil of *Aloysia triphylla* added to the diet of silver catfish at 2 mL/kg increases growth (Zeppenfeld et al., 2015) while *Zataria multiflora* essential oil had an effect on antibody, total white blood cells and serum bactericidal activity of common carp (Soltani et al., 2010). However, no information is available regarding the potential of essential oils from *H. cordata* Thunb as growth promoter and immunity enhancer in fish. Therefore, the objective of this study was to evaluate *H. cordata* Thunb essential oils in terms of stimulation of immune function of Hybrid red tilapia as well as its effect on the growth performance.

4.2 Methods

4.2.1 Essential oil (EOs) extraction

H. cordata Thunb was collected from Pasao district of Lamphun province, Thailand (Latitude 18.639539, Longitude 99.032919). Total area was 0.48 hectare **(Fig. 13)**. Amount of 300 of whole fresh plants were introduced into the distillation flask (2 L), which was connected to the steam generator via a glass tube and to a condenser to retrieve the oil. EOs were volatilized with boiling water at temperature 100°C for 3 hours. After the steam distillation process, the product was collected and separated using separatory funnel. EOs settled at the bottom layer of the separatory funnel and were separated several times until no oil was left in the separatory funnel. EOs product was stored at 4ºC until further analysis **(Fig. 14)**.

4.2.2 Fish study (*in vivo* study)

4.2.2.1 Preparation of fish diets

This study was performed in both laboratory condition (*in vitro*) and field fish study (*in vivo*), thus, this difference could have important biological effects which explains the sensitivity of *H. cordata* Thunb essential oils (HEO) in environmental conditions. Moreover, it is for this reason that the concentrations of HEO used for the *in vivo* study were higher than the *in vitro* (Navarrete et al., 2010). To test the effect of HEO on Hybrid red tilapia, MIC of fish pathogen (500 ppm) obtained *in vitro* by previous studies was used. In this study, three times of this concentration was used due to the fact that in fish field study, it is very difficult to control the environmental condition. Three diets 0, 1.5 and 3.0 g/kg, containing HEO feed were prepared using a base of commercial pellets. Three diets containing HEO 0, 1.5 and 3.0 g/kg feed were prepared using a base of commercial pellets. The diets were sprayed with different concentrations (Harikrishnan et al., 2011): control diet (soybean oils 10 g/kg), treatment 2 (soybean oils 8.5 g/kg + HEO 1.5 g/kg) and treatment 3 (soybean oils 7.0 g/kg + HEO 3.0 g/kg). Afterwards, all feed groups were coated with soybean oils 10 g/kg **(Table 7)**. The diets were mixed well by hand and dried up at room temperature **(Fig.21)**. All diets contained 30% protein. The fishes were fed three times a day with an amount that is 5% of their body weight.

Table 7 Composition of soybean oils and HEO on diets

Supplementation diet (g/kg)	Control	Treatment 2	Treatment 3
Soybean oils	20.0	18.5	17.0
Houttuynia cordata Thunb essential oils (HEO)	0.0	1.5	3.0

Figure 21 Preparation of fish diets

4.2.2.2 Fish and experimental design

The trials were carried out in Mae Taeng reservoir. The dimension of floating cages was 1x1x1.5 m **(Fig. 22)**. A total of 270 Hybrid red tilapia fishes (average 24 g) were randomly chosen and distributed into 9 cages **(Fig. 23)**. There were 3 replicate cages with 30 fishes per cage. Weight gain was monitored every 15 days until 60 days feeding experiment **(Fig. 24)**. Growth performance was determined and feed utilization was calculated as follows:

Weight gain (WG) = Final weight - Initial weight (g/fish)

Feed conversion ratio (FCR) = Total feed / Total weight gain

Average daily gain (ADG) = Final weight - Initial weight / Experimental period (g/fish)

Survival rate = (Number of fishes at the end / Number of fishes initial stocked) x 100 (%)

Figure 22 Floating cages in Mae Taeng reservoir, Thailand

Figure 23 Hybrid red tilapia were randomly chosen into cages

Figure 24 The body weight was monitored every 15 days

4.2.2.3 Proximate analysis of fish

After 60 days, 3 fishes were taken from each treatment. The fish fillets (dorsal muscle) were minced in the blender. The proximate composition of fillets (dorsal muscle) were analyzed according to the procedures described in AOAC (1990). Analysis included determination:

- Moisture content was determined by oven drying at 110ºC overnight.
- Ash content was determined by loss on ignition in a muffle furnace at 550ºC overnight.
- Crude protein was determined by kjeldahl using a Tecator Kjeldahl Auto 1030 using standard procedures as described by AOAC (1990).
- Crude lipid was determined by the Soxhlet method using petroleum ether as a solvent.

4.2.2.4 Immunological and hematological parameters

By the end of the feeding experiment (60 days), blood samples were collected from the caudal vein of the fish and were divided into two parts **(Fig. 25)**. One part was collected with 5% EDTA and used for blood cell counting (red blood cells and white blood cells). The other part was collected for serum analysis. The samples were left overnight at 4ºC until blood clotted then centrifuged 10,000 revolutions per for 10 min. After non-hemolysed serum, samples were stored at -20ºC until further analysis.

Lysozyme assay

Plasma lysozyme activity of serum was measured using the turbidometric assay (Parry et al., 1965). Briefly, a standard suspension of 0.75 mg/mL *Micrococcus lysodeikticus* lyophilized cells (Sigma, St. Louis, MO) was prepared in a 0.1 M phosphate buffer (pH 6.2). Hybrid red tilapia serum (10 µL) was placed in triplicate into a 96 well microtiter plate together with 250 µL of bacterial suspension at 25ºC. The reduction in absorbance at 450 nm was recorded at 1 and 5 min using microplate reader (Metertech M965$^+$). A lysozyme activity unit was defined as the amount of enzyme produced which decreases absorbance at 0.001/min (initial abs – final abs) **(Fig. 26)**.

Hematocrits (packed cell volume: PVC)

PVC was determined after the blood has been transferred to microcapillary tubes and centrifuged at 4,000 revolutions per for 5 min (Heraeus, Biofuge haemo). It was expressed using the formula (Snieszko, 1960) **(Fig. 26):**

Percent hematocrit = (packed cell volume/ Total blood volume) x 100

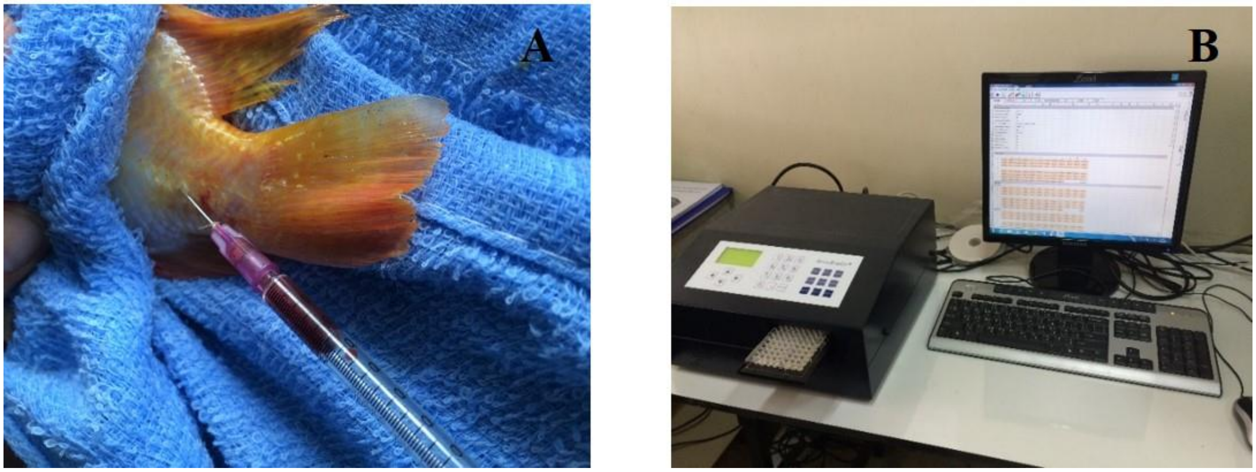


Figure 25 The blood samples were collected from the caudal vein of the fish (A) and microplate reader (Metertech M965^{+}) (B)

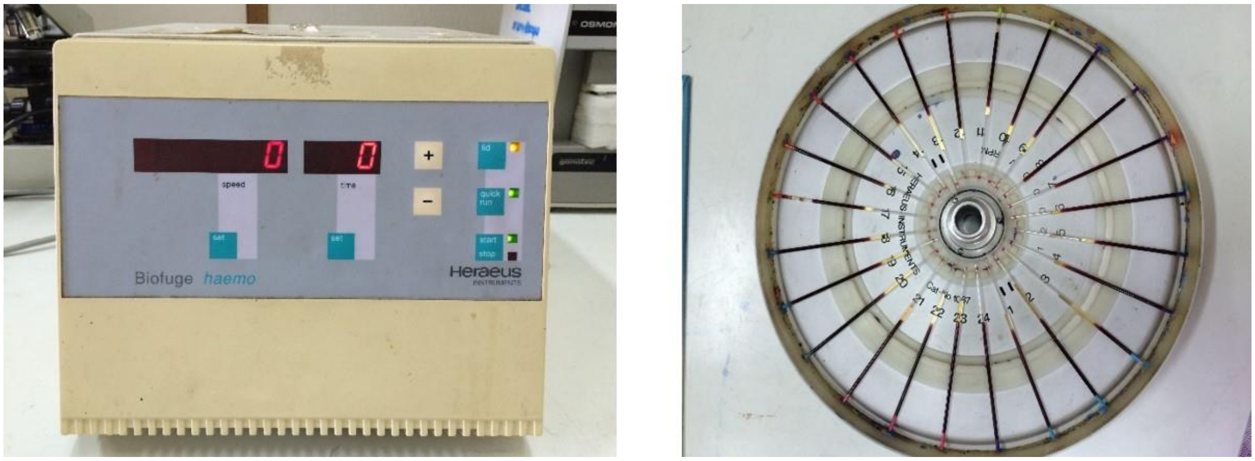


Figure 26 The Biofuge haemo and hematocrit dish for read hematocrit values

Total count of red blood cells (RBC) and white blood cells (WBC)

Total red blood cells (RBC) were counted using an improved Neubauer hemocytometer method (Shah and Altindağ, 2004). Blood was diluted with 5% EDTA with the ratio of 1:250. Erythrocytes were measured via hemocytometer chamber then calculated to $\times10^6$ mm^{-3} (Wintrobe, 1976). For the total white blood cells (WBC), blood was diluted with Dacie's fluid with a ratio of 1:100 and measured using hemocytometer then calculated to $\times10^3$ mm^{-3} (Mgbenka et al., 2003). Both measurements were operated under 100x microscope (Olympus).

Morphology of blood cells (WBC differential)

Blood samples were used to prepare the blood smears for morphological observation. The samples were stained using the Drip Quick Technique by Wright instant stain set (Bio-Medical Laboratory, Bangkok). The blood smears were used for the total white blood cell count (Tavares-Dias and Moraes, 2007).

4.2.3 Statistical analysis

Data obtained from the experiment were expressed as mean ± standard error (S.E.) of triplicates. One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test ($p<0.05$) were used to analyze the significant statistical differences between the treatments.

4.3 Results

4.3.1 Growth performance

Actual photographs and total weight of Hybrid red tilapia fed with the different diets after the 60 days experiment period were shown in **Figs. 27 and 28**. The data on growth performance of the fish during the experiment are shown in **Table 8**. Based on the results obtained, the survival rate was not affected by the differences among fish diets ($p>0.05$). In contrast, weight gain and average daily growth of fishes fed with HEO 1.5 g/kg were the highest among the other setups ($p<0.05$). Moreover, feed conversion ratio of HEO 1.5 g/kg was significantly ($p<0.05$) lower than the control and HEO 3.0 g/kg. The proximate composition of Hybrid red tilapia fillet showed that the protein content of fish fed with HEO 1.5 g/kg was significantly ($p<0.05$) higher than HEO 3.0 g/kg and control. On the other hand, fish fed with HEO 3.0 g/kg yielded to a lipid content that is significantly ($p<0.05$) higher than HEO 1.5 g/kg and control **(Table 9)**.

4.3.2 Immunological and hematological

The immunological and hematological parameters of Hybrid red tilapia showed that lysozyme activity after the 60 days experiment period in HEO 1.5 g/kg are significantly higher ($p<0.05$) compared to the control and HEO 3.0 g/kg. Hybrid red tilapia fed with HEO 1.5 g/kg had higher red blood cell and hematocrits counts than control and HEO 3.0 g/kg but this finding is not statistically significant ($p>0.05$) as the values are not hugely different. Whereas, the white blood cells showed that HEO 3.0 g/kg are significantly higher ($p<0.05$) compared to control and HEO 1.5 g/kg **(Table 10)**. The types of white blood cells present were found to be lymphocyte, monocyte, neutrophil, eosinophil and thrombocyte. However, the counts of each type showed no significant differences ($p>0.05$) among all treatments **(Table 11) (Fig. 29)**.

Table 8 Effect of fish diets on growth performance of Hybrid red tilapia

	Fish Diet		
	Control	**HEO 1.5 g/kg**	**HEO 3.0 g/kg**
Initial weight (g/fish)	24.10±0.10 ^{a}	24.80±0.80 ^{a}	23.65±0.55 ^{a}
Final weight (g/fish)	129.70±0.07 ^{c}	147.81±2.75 ^{a}	136.13±0.07 ^{b}
Total weight (g/cage)	3,891.00±68.30 ^{c}	4,434.30±199.86 ^{a}	4,083.90±78.31 ^{b}
Weight Gain (g/fish)	105.60±0.24 ^{c}	123.01±1.95 ^{a}	112.48±0.62 ^{b}
ADG (g/fish/day)	1.76±0.00 ^{b}	2.05±0.03 ^{a}	1.87±0.15 ^{b}
FCR	1.57±0.01 ^{c}	1.34±0.02 ^{a}	1.47±0.01 ^{b}
Survival rate (%)	100±0.00 ^{a}	100±0.00 ^{a}	100±0.00 ^{a}

Note: Different letter (a, b and c) in the same row show significant statistical differences ($p<0.05$)
HEO = *Houttuynia cordata* Thunb essential oils
ADG = Average Daily Growth
FCR = Feed Conversion Rate

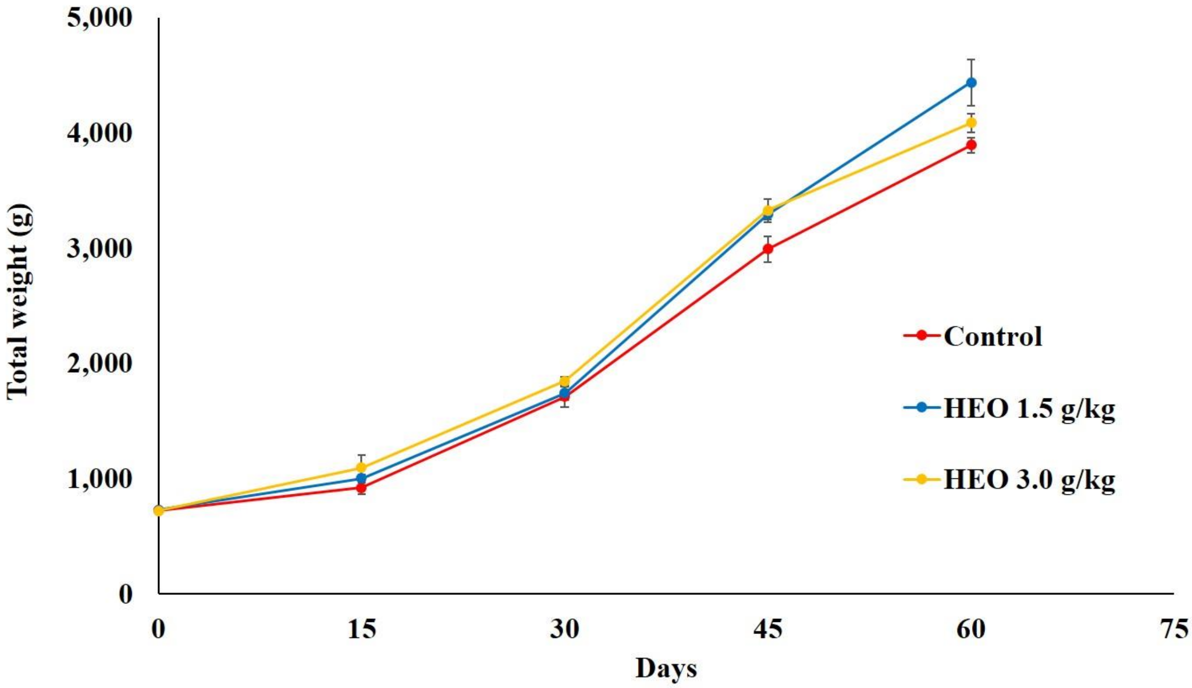


Figure 27 Total weight (g/cage) of Hybrid red tilapia monitored every 15 days

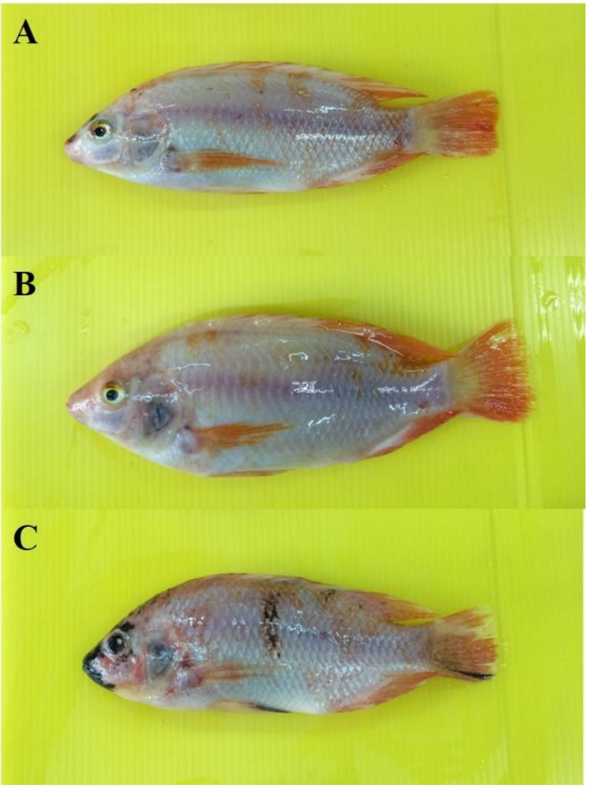


Figure 28 Hybrid red tilapia after 60 days experimental; Control (A), HEO 1.5 g/kg (B) and HEO 3.0 g/kg (C)

Table 9 Proximate composition of fillet of Hybrid red tilapia (%; on fresh weight basis)

	Hybrid red tilapia fillet		
	Control	**HEO 1.5 g/kg**	**HEO 3.0 g/kg**
Moisture (%)	68.04±1.51 ^{a}	66.55±1.43 ^{a}	68.31±1.12 ^{a}
Ash (%)	0.25±0.08 ^{b}	0.46±0.10 ^{a}	0.41±0.05 ^{a}
Crude Lipid (%)	0.22±0.02 ^{c}	0.33±0.08 ^{b}	0.46±0.09 ^{a}
Crude Protein (%)	15.91±0.81 ^{c}	27.74±1.29 ^{a}	23.55±0.62 ^{b}

Note: Different letter (a, b and c) in the same row show significant statistical differences ($p<0.05$)
HEO = *Houttuynia cordata* Thunb essential oils

Table 10 Immunological of Hybrid red tilapia after 60 days feeding period

	Fish Diet		
	Control	**HEO 1.5 g/kg**	**HEO 3.0 g/kg**
Lysozyme (Unit/mL)	1.65±0.24 ^{b}	2.26±0.16 ^{a}	1.41±0.10 ^{b}
Hematocrits (PVC) (%)	37.61±1.99 ^{a}	40.97±0.22 ^{a}	39.04±2.15 ^{a}
RBC (x 10^6 mm^{-3})	2.29±0.28 ^{a}	2.46±0.04 ^{a}	2.09±0.07 ^{a}
WBC (x 10^3 mm^{-3})	36.63±3.82 ^{b}	43.75±1.56 ^{b}	79.69±1.56 ^{a}

Note: Different letter (a, b, c) in the same row show significant statistical differences ($p<0.05$)
HEO = *Houttuynia cordata* Thunb essential oils
PVC = Packed cell volume
RBC = Total count of red blood cells
WBC = White blood cells

Table 11 Differential morphology counting of white blood cells (%)

	Fish Diet		
	Control	**HEO 1.5 g/kg**	**HEO 3.0 g/kg**
Lymphocyte	82.89±3.93 ^{a}	82.89±1.49 ^{a}	81.33±1.92 ^{a}
Monocyte	12.78±3.56 ^{a}	11.22±1.89 ^{a}	13.22±2.76 ^{a}
Neutrophil	0.33±0.33 ^{a}	0.11±0.11 ^{a}	0.78±0.39 ^{a}
Eosinophil	0.33±0.19 ^{a}	0.11±0.11 ^{a}	0.33±0.33 ^{a}
Thrombocyte	3.67±0.96 ^{a}	5.67±0.51 ^{a}	4.33±1.02 ^{a}

Note: Different letter (a, b, c) in the same row show significant statistical differences ($p<0.05$)
HEO = *Houttuynia cordata* Thunb essential oils

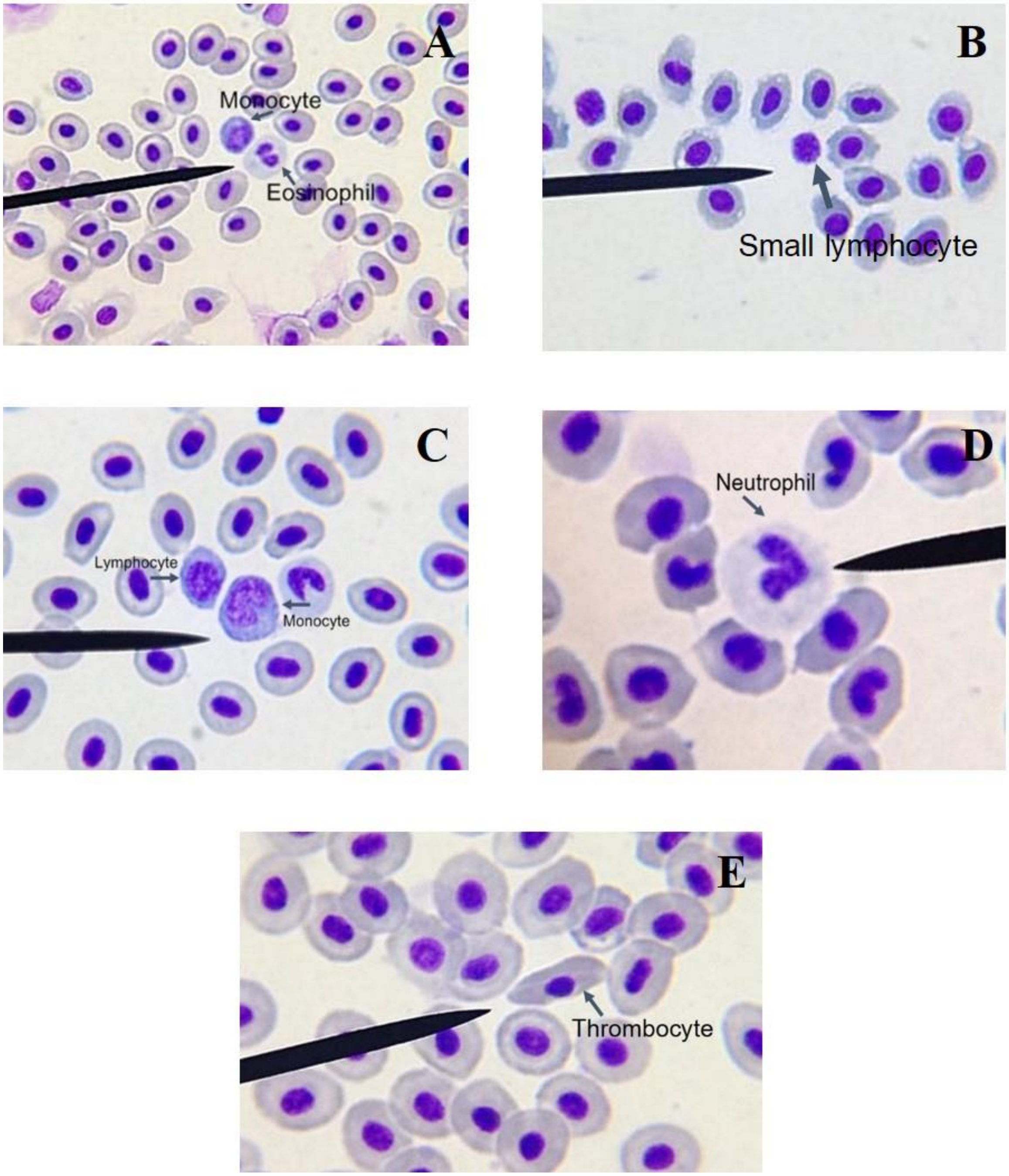


Figure 29 The hematological profile of Hybrid red tilapia during 60 days experimental period; monocyte and eosinophil (A), small lymphocyte (B), monocyte and lymphocyte (C), neutrophil (D) and thrombocyte (E)

4.4 Discussion

4.4.1 Growth performance

The previous studies of *Houttuynia cordata* Thunb essential oils (HEO) showed antimicrobial activity against fish pathogens in laboratory condition (*in vitro*). Based on Navarrete et al. (2010) the *in vitro* antibacterial activity of *Thymus vulgaris* essential oil (TVEO) was assessed using a range of normal intestinal isolates and fish pathogens and it was found out that the inhibitory concentrations for all the tested bacteria were higher than the TVEO levels used in trout (microbiota isolates from faeces). This may imply that HEO is more effective *in vivo* than *in vitro*. A possible reason for this is that the conditions (such as normal flora, pH and temperature) inside the body of fish can have a negative effect on the activity of essential oil by suppressing its antimicrobial activity. Therefore, further studies are needed to evaluate the activity of HEO *in vivo* by using higher concentrations of HEO than *in vitro*.

This study used soybean oils for the control and mixed with HEO on the other two diets because soybean oils do not compromise fish growth and non-specific immune function (Mourentea et al., 2007; Stephan, 2011; Mayra et al., 2016). Data on growth performance of the fishes during the experiment showed that the survival rate was not affected by any of diet. While, weight gain and average daily growth of HEO 1.5 g/kg fed Hybrid red tilapia were the highest among the other setups. Feed conversion rate of HEO 1.5 g/kg fed Hybrid red tilapia were lower than control and HEO 3.0 g/kg. Some publications reported about the use *H. cordata* Thunb in aquaculture. As stated, the use of 1% powdered *H. cordata* Thunb feed additives on olive flounder diets did not affect the feed efficiency ratio and protein efficiency ratio (Lee et al., 2015).

The result of the proximate composition analysis of fish fillets showed that fish fed with HEO 1.5 g/kg had more protein content compared to the other treatments which proves that there was an improved efficiency in nutrient transfer from the feed to the fish. Furthermore, fish fed with HEO 3.0 g/kg showed higher lipid content than HEO 1.5 g/kg fed and control ($p<0.05$). Similarly, fishes fed with a diet of mixed 75% fish oil and 25% vegetable oils had the highest body protein content and lowest body fat content (Sagne et al., 2013). 1% of powdered thyme was added to the feed of sea bass (*Dicentrarchus labrax*) and showed an improve fillet protein levels ($p<0.05$) (Yilmaz et al., 2012). Zakęŝ et al. (2008) observed that supplementing diets with *Astragalus radix* and *Lonicera japonica* had a significant effect on body proximate composition of pikeperch.

Many reported that other herbs have the ability to make fishes more active, consume more food and have increase in growth. The positive effect of essential oils on weight gain, average daily growth and feed conversion rate may be due to its antioxidant activity, antibacterial effect (Botsoglou et al., 2002, 2004; Milos et al., 2000), increased digestibility and absorption of nutrients (Namkung et al., 2004) and improvement of immune response (Oetting et al., 2006). Thyme oil showed strong antibacterial activity (*Pseudomonas aeruginosa*) and increased growth performance in Nile tilapia fingerling (Shehata et al., 2013). Diets supplemented with essential oil extracted from *Aloysia triphylla* increases the growth of silver catfish (Zeppenfeld et al., 2015). The herbal product act as growth promoter to help induce the transcription rate which lead to increased RNA, total amino acid and protein production in fish cell (Citarasu, 2010).

4.4.2 Immunological and hematological parameters

Fish lysozymes possess a high potential for non-specific defense against pathogens and due to this, it is desirable for cultured fish to have a high lysozyme activity so it can fight infection (Saurabh and Sahoo, 2008). In this study, it was detected that the immunological parameter (measured by the lysozyme activity of serum assay), there was an increase in lysozyme activity in Hybrid red tilapia that was fed with HEO 1.5 g/kg. A similar finding was documented in tilapia fed with garlic (*Allium sativam*), *Ganoderma lucidium* and *Lonicera japonica* where there was an enhanced lysozyme activity (Ndong and Fall, 2011; Yin et al., 2008).

When it comes to the hematological parameters, Hybrid red tilapia fed with HEO 1.5 g/kg showed the highest packed cell volume and total red blood cells count compared to the other setups. After studying the morphology of blood cells, it was found out that WBCs consist of different type of cells, each with its own role in protecting the body. The lymphocytes were detected easily because are involved in the defense disease mechanism and control of immune response in fish (Giuseppe, 2013). Previous reports showed that Nile tilapia fed with diets supplemented with thyme, rosemary and fenugreek showed increased in WBC, RBC, PCV, neutrophil and monocyte (p<0.05) (Gültepe et al., 2014).

4.5 Conclusion

In conclusion, the results of the present study demonstrated that supplementing diet with essential oil from *Houttuynia cordata* Thunb at the concentration of 1.5 g/kg has the potential of enhancing immune system and promoting growth of Hybrid red tilapia. Thus, it’s use as a feed additive is recommended in aquaculture.

CHAPTER 5

Summary

Houttuynia cordata Thunb could be used as a medicinal plant in aquaculture. This study investigated the effects of essential oils from *H. cordata* Thunb as diet supplements on growth performance and immune responses of Hybrid red tilapia (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.). Essential oils from *H. cordata* Thunb were extracted using hydrodistillation and analyzed by GC-MS. Beta-myrcene (44.220%) and 2-undecadone (21.985%) were the major components out of the 28 components present. The *in vitro* antibacterial activity of HEO showed minimal inhibitory concentration (MIC) against *Aeromonas hydrophila*, *Streptococcus* sp. and *Flavobacterium columnare* at 250, 250 and 500 ppm, respectively (Ampicillin sodium exhibited similar MIC values against the bacteria used). The *in vivo* analysis on growth performance of fish, Hybrid red tilapia (24 g) fed with three diet containing HEO 0, 1.5 and 3.0 g/kg showed no mortality after the 60 days experiment period. On the other hand, weight gain and average daily growth of Hybrid red tilapia fed with HEO 1.5 g/kg were the highest among the other setups ($p<0.05$). Feed conversion ratio of HEO 1.5 g/kg was significantly ($p<0.05$) lower than the control and HEO 3.0 g/kg. The proximate composition of Hybrid red tilapia fillet showed that the protein content of fish fed with HEO 1.5 g/kg was significantly ($p<0.05$) higher than HEO 3.0 g/kg and control. In contrast, fish fed with HEO 3.0 g/kg yielded to a lipid content that is significantly ($p<0.05$) higher than HEO 1.5 g/kg and control. It was also found out that the immunological parameter (lysozyme activity) of Hybrid red tilapia fed with commercial diet containing HEO 1.5 g/kg is significantly ($p<0.05$) higher than the control and 3.0 g/kg. However, no significant difference was observed in hematocrits, red blood cells and white blood cell values among all treatments (p>0.05). These results indicate that essential oils from *H. cordata* Thunb has a good potential as growth enhancer and can possibly replace antibiotics in enhancing fish immune response.

CHAPTER 6

Conclusion and Future prospects

Houttuynia cordata Thunb collected from Northern Thailand produced a clear colorless to pale yellow oil with a yield of 0.057% v/w. Among the substances obtained, beta-myrcene and 2-undecadone were the major components. 2-undecanone is oxidized from decanoyl acetaldehyde. It is also well known for its antibacterial effects. The *in vitro* antibacterial activity of HEO showed minimal inhibitory concentration (MIC) against *Aeromonas hydrophila*, *Streptococcus* sp. and *Flavobacterium columnare* at 250, 250 and 500 ppm, respectively. Based on the results obtained from Hybrid red tilapia fed with *H. cordata* Thunb essential oils (HEO), the concentration of 1.5 g/kg showed the highest weight gain, average daily growth, protein content and lysozyme activity. Thus, it can be concluded that this concentration can be a potential feed supplement in aquaculture as it enhances the growth performance and immune response of fish. However, before developing HEO as an alternative to antibiotics, further tests should be performed to determine if commercial diet containing HEO 1.5 g/kg will have the same effect on other aquaculture species. In order to further prove and support the results of this study, other researchers are encouraged to optimize the condition for HEO extraction, test the resistance of bacteria against HEO by prolonging incubation time, challenge test fish pathogen on Hybrid red tilapia and determine possible side effects of HEO to fish flesh.

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ADDENDUM

The following is the publication and conferences of the candidate which are related the theme of this thesis.

Publication

Supranee Wigraiboon, Nakao P Nomura and Niwooti Whangchai (2016) Effect of essential oils from *Houttuynia cordata* Thunb supplemented diets on growth performance and immune response of hybrid red tilapia (*Oreochromis mossambicus* Linn. × *Oreochromis niloticus* Linn.). International Journal of Fisheries and Aquatic Studies (IJFAS) 4(3): 677-684

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