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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
This study investigated the effects of essential oils from *Houttuynia cordata* Thunb as diet supplements on growth performance and immune responses of Hybrid red tilapia. *H. cordata* Thunb essential oils (HEO) were extracted using hydrodistillation and analyzed by gas chromatography-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 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 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. 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). 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.

Keywords: *Houttuynia cordata* Thunb, Hybrid red tilapia, growth performance, immune response

1. Introduction
Tilapia is one of the most important freshwater fish in aquaculture [1]. Hybrid red tilapia is now becoming very popular for freshwater aquaculture in Thailand, 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 [1]. Hybrid red tilapia is cultured in ponds and floating cages in rivers or reservoirs where infectious diseases are common. 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. 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 [2]. Moreover, thyme oil (0.25%) was also reported to have an antibacterial effect against pathogens of Nile tilapia [3].

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, fatty acids, sterols, flavonoid and alkaloids [4]. Moreover, it possesses a variety of pharmacological functions including anti-platelet aggregation, anti-bacterial, anti-tumor, anti-microbial, anti-inflammatory, anti-leukemic and immunomodulatory effects [5-8]. Decanoyl acetaldehyde (Houttuynin) 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 [9]. Essential oils from this plant have antibacterial activity against *Staphylococcus aureus* and *Sarcina ureae* [10]. 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.

2. Materials and Methods

2.1 Essential oil extraction

Houttuynia cordata Thunb was collected from Pasao district of Lamphun province, Thailand (Latitude 18.639539, Longitude 99.032919) (Fig. 1). The whole fresh plants were cut into small segments and subjected to hydrodistillation in distilled water for 3 hours. The extracted oil product was stored at 4 ºC until further analysis.

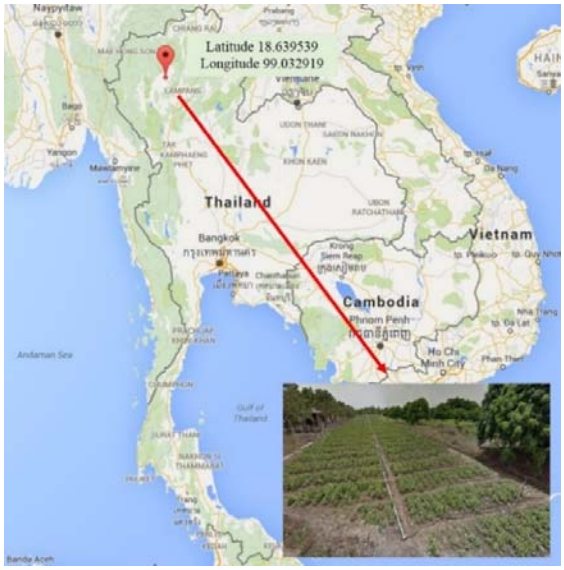

Fig 1: Map of study area in Pasao district of Lamphun province, Thailand. Arrow indicates *H. cordata* Thunb culture locations

2.2 Gas Chromatography-Mass Spectrometer Analysis (GC-MS)

GC-MS analysis was performed using Agilent 6890N gas chromatography instrument coupled to an Agilent 5873 mass spectrometer and equipped with a HP INNOWAX capillary column (30 m x 0.25 mm I.D., film thickness 0.25 mm). The column was initially maintained at 35 ºC for 3 min and programmed to 250 ºC at flow rate at 5 ºC/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.

2.3 *In vitro* study

2.3.1 Bacterial species

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.

2.3.2 Antibacterial testing (Minimum inhibitory concentration: MIC)

The integrated assay (growth and non-growth of bacteria) was assessed by visual inspection [7]. Minimum inhibitory concentration (MIC) is the observed lowest concentration of an essential oil to effectively prevent the growth of bacteria.

Aeromonas hydrophila, *Streptococcus* sp. and *Flavobacterium columnare* were grown for 24 hours at 37 ºC and total counts were adjusted to approximately 10^7 CFU/mL. A 4,000 ppm stock solution of *Houttuynia cordata* Thunb essential oils (HEO) was prepared by 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 concentrations were prepared by serial broth dilution method [7]. 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 37 ºC for 20 hours.

2.4 Fish study (*In vivo* study)

2.4.1 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 *Houttuynia 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* [11]. To test the effect of HEO on Hybrid red tilapia, 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 [12]: 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 1). The diets were mixed well by hand and dried up at room temperature. All diets contained 30% protein. The fishes were fed three times a day with an amount that is 5% of their body weight.

Table 1: 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

2.4.2 Fish and experimental design

The trials were carried out in Mae Taeng reservoir. The dimension of floating cages was 1x1x1.5 m (Fig. 2). A total of 270 Hybrid red tilapia fishes (average 24 g) were randomly chosen and distributed into 9 cages. There were 3 replicate

cages with 30 fishes per cage. Weight gain was monitored every 15 days until 60 days feeding experiment. Growth performance was determined and feed utilization was calculated as follows:
Weight gain (WG) = Final weight - Initial weight (g/fish)
Food 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 x 100/ Number of fishes initial stocked (%)

Fig 2: Floating cages in Mae Taeng reservoir

2.4.3 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. 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 4 ºC until blood clotted then centrifuged 10,000 revolutions per 10 min. After non-hemolysed serum, samples were stored at -20 ºC until further analysis.

2.4.3.1 Lysozyme assay

Plasma lysozyme activity of serum was measured using the turbidometric assay [13]. 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 minutes 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).

2.4.3.2 Hematocrits (Packed cell volume: PVC)

PVC was determined after the blood has been transferred to microcapillary tubes and centrifuged at 4,000 revolutions per 5 min. It was expressed using the formula [14]:
Percent hematocrit = (Packed cell volume/ Total blood volume) x 100

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

Total red blood cells (RBC) were counted using an improved Neubauer haemocytometer method [15]. Blood has been diluted with 5% EDTA with the ratio of 1:250. Erythrocytes were measured via haemocytometer chamber then calculated to x10^{6} mm^{-3} [16]. For the total white blood cells (WBC), blood was diluted with Dacie' s fluid with a ratio of 1: 100 and measured using haemocytometer then calculated to x10^{3} mm^{-3} [17]. Both measurements were operated under 100× microscope (Olympus).

2.4.3.4 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 cells count [18].

2.5 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. Result

3.1 GC-MS Analysis

Hydrodistillation of *Houttuynia cordata* Thunb produced a clear colorless to pale yellow oil with a yield of 0.057%. GC-MS analysis of essential oils from *H. cordata* Thunb identified 28 phytochemicals. Results were further investigated and the major components were found to be beta-myrcene (44.220%) and 2-undecanone (21.985%) (Fig. 3) (Table 2).

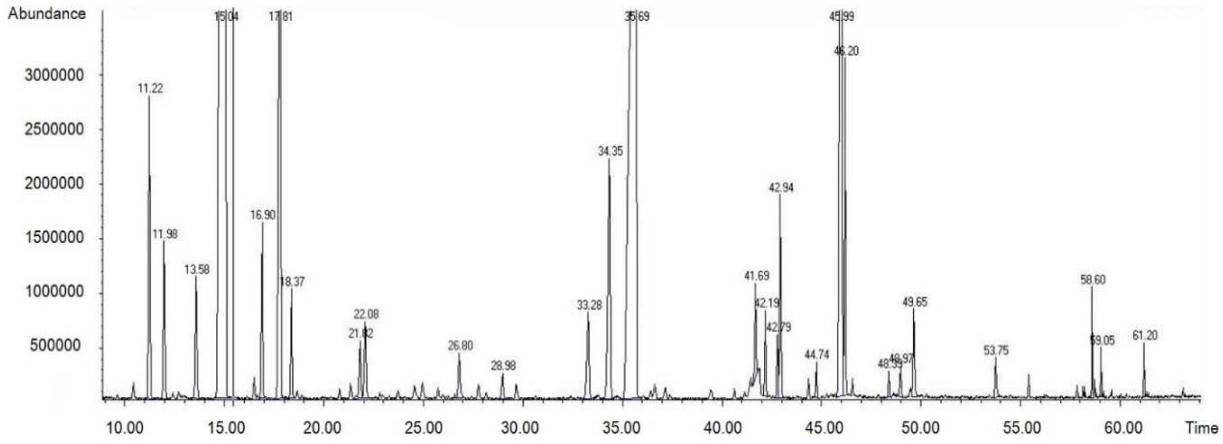

Fig 3: Chromatograms of essential oil components of *Houttuynia cordata* Thunb extract

Table 2: Chemical composition of essential oils from *Houttuynia cordata* Thunb extract

RT (min.)	Percent (%)	Compound name
11.223	2.124±0.048	1R-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-, (E)
18.367	0.687±0.005	1, 3, 6-Octatriene, 3, 7-dimethyl-, (Z)
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, (E)
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-, (Z)
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

3.2 *In vitro* study

In this study, the *Houttuynia 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. It should be noted that HEO was effective at the same concentration as Ampicillin sodium against *Aeromonas hydrophila, Streptococcus* sp. and *Flavobacterium columnare* (Table 3).

Table 3: Antibacterial activity against fish pathogens

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

HEO = The *Houttuynia cordata* Thunb essential oils
MIC = Minimum Inhibitory Concentration

3.3 Fish study

3.3.1 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 Fig. 4 and data on growth performance of the fishes during the experiment were summarized in Table 4. Based on the results obtained, the survival rate was not affected by the differences among fish diets (p>0.05) because no mortality in all treatments was observed. 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.

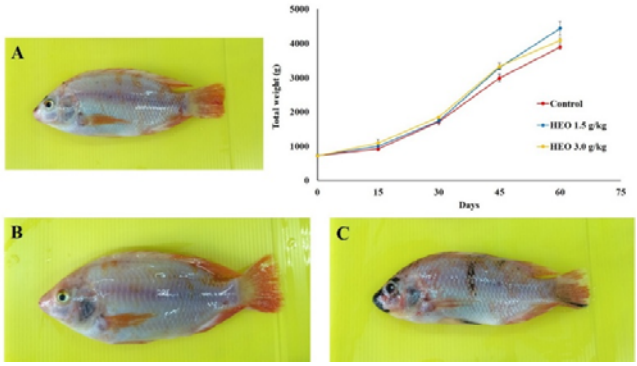

Fig 4: Hybrid red tilapia after 60 days experimental and total weight; Control (A), HEO 1.5 g/kg (B) and HEO 3.0 g/kg (C)

Table 4: 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 = The *Houttuynia cordata* Thunb essential oils
ADG = Average Daily Growth
FCR = Feed Conversion Rate

3.3.2 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 5). 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 6) (Fig. 5).

Table 5: 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 (%)	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 = The *Houttuynia cordata* Thunb essential oils
RBC = Total count of red blood cells
WBC = White blood cells

Table 6: 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 = The *Houttuynia cordata* Thunb essential oils

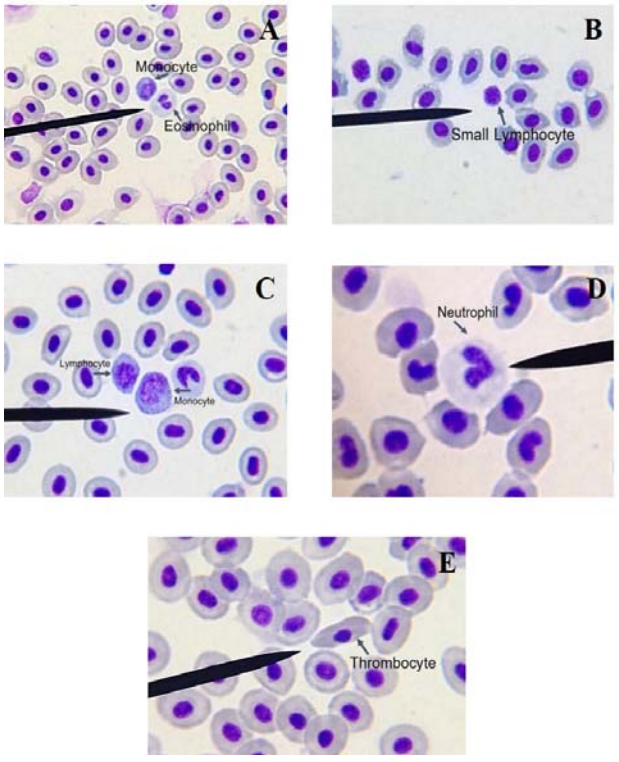

Fig 5: The hematological profile of Hybrid red tilapia after 60 days experimental period; Monocyte and Eosinophil (A), Small Lymphocyte (B), Monocyte and Lymphocyte (C), Neutrophil (D) and Thrombocyte (E)

4. Discussion

4.1 GC-MS Analysis

The GC-MS analysis of essential oils from *Houttuynia 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 essential oil from the belowground parts (root) was found to be dominated by methyl nonyl ketone (2-undecanone) (23.96%) and β-myrcene (14.29%) [7] and leaves were found to contain β-myrcene (30.8%) and 2-undecanone (19.7%) as major components [19]. While, [7] reported that the essential oil from aboveground parts (stem and leave) was characterized by high methyl nonyl ketone (2-undecanone) (36.07%) and β-myrcene (12.57%). However, the use of different instrument and analysis techniques may have an effect on the chemical components of this plant [20]. Reported that various sampling techniques were compared for the gas chromatography-mass spectrometry 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). According to [21] 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.

4.2 *In vitro* study

In this study, the *Houttuynia 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 *Aeromonas hydrophila*, *Streptococcus* sp. and *Flavobacterium columnare* disease treatments instead of using chemotherapeutic methods which can pose harm to humans.

The antibacterial activity of essential oils 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 essential oil. 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 essential oil 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 [22-24]. 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 [20]. This component has activities that can cause a moderate inhibition of *Escherichia coli* [25], *Salmonella* sp. [26], *Staphylococcus aureus* [7-8, 10] and *Sarcina ureae* growth [7]. Some minor components in essential oils of *H. cordata* Thunb such as α-pinene, β-pinene and limonene also have a strong antibacterial activity [27-30].

The mechanism of action of this antibacterial effect concerns the cell wall structure as well as denaturing and coagulating proteins. In gram-negative bacteria, the cell wall is constituted by a thin peptidoglycan layer adjacent to cytoplasmic membrane and an outer membrane composed by phospholipids and lipopolysaccharides [31]. On the other hand, gram-positive bacteria, lack the outer membrane but the cell wall is 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 [32]. 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 [33].

4.3 Fish study (*In vivo* study)

4.3.1 Growth performance

Houttuynia cordata Thunb essential oils (HEO) showed antimicrobial activity against fish pathogens in laboratory condition (*in vitro*). Based on Navarrete *et al.* [11] 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 [34-36]. 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 [37]. 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 and antibacterial effect [38-40], increased digestibility and absorption of nutrients [41] and improvement of immune response [42]. Thyme oil showed strong antibacterial activity (*Pseudomonas aeruginosa*) and increased growth performance in Nile tilapia fingerling [3]. Diets supplemented with essential oil extracted from *Aloysia triphylla* increases silver catfish growth [43]. The herbal product act as growth promoters to help induce the transcription rate which lead to increased RNA, total amino acid and protein production in fish cell [44].

4.3.2 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 [45]. 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 [46-47].

Regarding the hematological parameters (RBC and PCV) in Hybrid red tilapia fed with HEO 1.5 g/kg were increased 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 they are involved in the defense disease mechanism and control of immune response in fish [48]. 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) [49].

5. Conclusion

Based on the results obtained from Hybrid red tilapia fed with *Houttuynia cordata* Thunb essential oils (HEO) 1.5 g/kg, it can be concluded that essential oils from *H. cordata* Thunb can be a potential feed supplement for aquaculture as it can enhance the growth performance and immune response of fish. However, further tests are needed to determine if commercial diet containing HEO 1.5 g/kg will have the same effect on other aquaculture species before developing HEO as an alternative to antibiotics.

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