Int Aquat Res (2023) 15:219–233
https://doi.org/10.22034/IAR.2023.1986215.1443

ORIGINAL RESEARCH

# Effectiveness of crude extracts from common indigo (*Indigofera tinctoria* L.) in inhibiting *Streptococcus agalactiae* in Nile tilapia

**Patcharawalai Sriyasak . Naiyana Senasri . Nattiya Chumnanka . Pongsathorn Tongkasee . Wasana Phlaetita . Pornpimol Pimolrat . Supannee Suwanpakdee**

Received: 15 May 2023 / Accepted: 03 August 2023 / Published online: 10 August 2023


**Abstract** The Nile tilapia (*Oreochromis niloticus*) culture is particularly susceptible to disease outbreaks, particularly infection by *Streptococcus agalactiae* bacteria. This study aimed to determine the efficacy of extracts of common indigo (*Indigofera tinctoria*) in inhibiting *S. agalactiae*. A completely randomized design (CRD) was carried out using pellet feed sprayed with varying concentration levels of the indigo extract: 0%, 0.1%, 0.5%, and 1%. The sample received the feed for eight weeks. The samples were analyzed to evaluate the effect of common indigo extract on growth performance, hematological parameters, biochemical parameters, and lysozyme activities. The results showed that the Nile tilapia fed pellet feed sprayed with 0.5% concentrated indigo extract exhibited the highest inhibition of *S. agalactiae*. The hematology results showed statistically higher ($P<0.05$) total red blood cells, hemoglobin counts, and hematocrit in the sample fed the pellet feed sprayed with the indigo extract than those in the control group. The sample fed pellet sprayed with 0.5% concentrated indigo extract exhibited statistically higher ($P<0.05$) white blood cells and a higher percentage of lymphocytes than those in other groups. In terms of biochemistry in blood, the sample fed pellet feed sprayed with 1% concentrated indigo extract exhibited statistically higher aspartate aminotransferase (AST) than those in other groups. The sample fed pellet feed sprayed with 0.5% and 1% concentrated indigo extracts exhibited statistically higher alkaline phosphatase (ALP) and total protein than those in other groups. Immunity test in blood showed that the sample fed pellet feed sprayed with 0.5% concentrated indigo extract exhibited statistically higher lysozyme in serum than those in other groups. The sample that was fed pellet feed sprayed with 0.1% and 0.5% exhibited statistically higher ($P<0.05$) immunoglobulin M levels than those in other groups. Overall, this study showed that the indigo extract could inhibit *S. agalactiae* and increase immunity in Nile tilapia.

**Keywords** Common indigo . *Streptococcus agalactiae* . Immunity . Nile tilapia

## Introduction

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish that is widely cultured because of its pleasant flavor,

Patcharawalai Sriyasak . Naiyana Senasri . Nattiya Chumnanka . Supannee Suwanpakdee (✉)
Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan Sakon Nakhon Campus, Sakon Nakhon 47160, Thailand
e-mail: supannee.sw@rmuti.ac.th

Pongsathorn Tongkasee
Department of Thai traditional medicine, Faculty of Natural Resources, Rajamangala University of Technology Isan Sakon Nakhon Campus, Sakon Nakhon 47160, Thailand

Wasana Phlaetita
Department of Plant science, Faculty of Natural Resources, Rajamangala University of Technology Isan Sakon Nakhon Campus, Sakon Nakhon 47160, Thailand

Pornpimol Pimolrat
Department of Coastal Aquaculture, Maejo University at Chumphon, Lamae, Chumphon 86170, Thailand

ease to raise, rapid growth, and ability to adapt well to any surrounding environment. (FAO 2018). Nowadays, the Nile tilapia culture has become highly populated and overfed. This has led to the accumulation of waste and the outbreak of bacterial diseases (Ding et al. 2021). *Streptococcus agalactiae* is a major pathogen, leading to the outbreak of diseases in Nile tilapia (Belton et al. 2009) and significant economic loss (Zhang et al. 2017). After *S. agalactiae* bacteria have colonized and multiplied in fish, they can infect different parts of the fish either externally (skin, fins, gills) or in the gastrointestinal tissues. The bacteria can also invade internal tissues and the circulatory system, resulting in a septicemic state induced by bacterial toxins. *S. agalactiae* produces numerous virulence factors that evade the host immune system's detection (Doan et al. 2022). Typically, fish farmers have prevented and treated diseases using antibiotics, causing the emergence of antibiotic residues in the environment (Rico et al. 2014). This fact has prompted researchers to investigate alternatives to the use of antibiotics. Many studies have applied local herbs as supplements for aquaculture, which has increased growth rate, immunity, and resistance to infectious diseases (Gabriel 2019; Soltani et al. 2019). Utilizing natural herbs or herbal extracts to treat bacterial infections in aquatic animals is widely popular because they are inexpensive and environmentally friendly, without chemical residues. Besides, they can be adapted for other purposes as well (Jadhav et al. 2006; Ding et al. 2021). Some studies found that many herbs can be used to treat diseases in shrimp and fish (Doan et al. 2020; Ding et al. 2021).

*Indigofera tinctoria* L. (Fabaceae Family), a tropical shrub, is one of the natural sources of indigo dye. In northeastern Thailand, local people use *I. tinctoria* to make natural indigo dye. The traditional method is used to extract indigo dye from *I. tinctoria* leaves and trunks to produce pure indigo dye (Pattanaik et al. 2019). Due to storage and waste management restrictions, most producers discard a significant amount of the indigo waste that is generated during the process. *I. tinctoria* has been well-documented in both ancient Chinese and Indian pharmacopeia. It contains major chemical components such as total phenolics, flavonoids, and terpenoids (Srinivasan et al. 2015; Rahman et al. 2018). Indigo extract possesses antioxidant (Anusuya and Manian 2013) and anti-inflammatory properties (Campos et al. 2018). The extract can interfere with parasite development (Singh et al. 2015) and inhibit bacterial infections (Vijayan et al. 2018). It can also enhance innate as well as adaptive immune responses (Boothapandi and Ramamibai 2016).

Utilizing extracts from indigo residues is one of the alternatives to antibiotics or chemical products for inhibiting bacterial infections in Nile tilapia and other aquatic animals. This research aims to study the effectiveness of crude extracts from common indigo (*I. tinctoria*) in inhibiting *S. agalactiae* in Nile tilapia as an alternative to antibiotics and chemical products. The results are expected to show that extracts from common indigo are effective in inhibiting *S. agalactiae* in Nile tilapia, and they are widely applied, which will not only reduce environmental impacts from and consumer exposure to antibiotics and chemical product residues but also increase the economic value of indigo residues as well.

## Materials and methods

### Ethical approval for animal use

This study was carried out in strict compliance with the recommendations for the use of animals regulated by the Institute of Animals for Scientific Purposes Development (IAD) of Thailand. In this research, fish handling and experimental protocols were approved by the ethics committee at Rajamangala University of Technology Isan, Thailand (Approval number: 21/2022).

### Preparation of crude extracts from common indigo

Fresh indigo waste (from the dye extraction process) was collected from the indigo dye enterprise group in Phangkhon, Sakon Nakon, Thailand. Indigo waste was air dried and put in a hot air oven at 60ºC for 72 h. The dry indigo waste was finely ground into powder and mixed with three solvents, including aqueous, 50% ethanol, and 95% ethanol at an indigo-to-solvent ratio of 1:3, with three replications for each solvent. The samples were then soaked for seven days. The samples were subsequently filtered with filter paper and concentrated with a rotary evaporator. The crude extracts were kept at 4ºC.

Calculation of the percentage yield of extracts

The following equation determined the extracts' percentage yield:

$$Yield\ (\%dry\ basis\ or\ \%db) = \frac{W1 \times 100}{W2}$$

Where W1 is the weight of the dried extracts and W2 is the weight of initial sample before extraction.

Chemical analysis of *I. tinctoria* extracts

Chemical composition analysis of *I. tinctoria* extracts was carried out using a Gas Chromatography Mass Spectrometry (GC-MS) technique, following the method described by Jemmali et al. (2016). To assess the antioxidant potential of *I. tinctoria,* different methods, including DPPH and ABTS assays, were employed. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of *I. tinctoria* was measured by the method described by Singh et al. (2018), while ascorbic acid was used as a standard antioxidant compound. Antioxidant capacity was calculated using an equation described by Mensor et al. (2001) and measured in terms of half-maximal inhibitory concentration ($IC_{50}$). The 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity of *I. tinctoria* was measured by the method described by Wang et al. (2017) and calculated using the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \frac{(1 - A_{sample})}{A_{ABTS}} \times 100$$

where $A_{sample}$ is the absorbance of the sample, and $A_{ABTS}$ is the absorbance of the ABTS solution.
Total Phenolic Content (TPC) was measured by Folin-Ciocalteu reagent following the method described by Ashraf et al. (2015). Phenolic content was expressed in terms of mg of gallic acid equivalent (GAE) per gram of extract (mg of GA/g of extract). Total Flavonoid Content (TFC) was measured and expressed in terms of quercetin equivalent per gram of dry weight (mg QE/g dry weight) following the method described by Chen et al. (2021).

Preparation of bacteria

*S. agalactiae* serotype Ia used in this experiment was obtained from the School of Agricultural Technology and Food Industry, Walailak University, Thailand. The bacteria were grown in Tryptic Soy Broth (TSB) at 37ºC for 24 h. The bacteria were then washed three times with 0.85% saline using a rotation rate of 2000 rpm for 15 min. The bacterial concentration was adjusted to $10^6$-$10^8$ CFU. $mL^{-1}$. The optical density (OD) of the sample was then measured at a wavelength of 540 nm using a spectrophotometer, the reading was equal to 0.1.

Evaluation of the antimicrobial activity

The bacteria inoculum was uniformly spread on a petri dish of Trypticase Soy Agar (TSA) using a sterile cotton swab. A volume of 30 µL of each indigo extract was dropped on 6 mm filter paper discs. The discs were placed on the surface of TSA and incubated at 37ºC for 24 h. The agar plates were evaluated by the outer diameter of the inhibition zone (recorded in mm) using a vernier caliper. The tests were repeated three times. For the control group, 100 µg/mL of ampicillin were dropped on a paper disk, and a similar process was repeated.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were determined using the broth dilution method. The extract with the highest inhibition zone was selected, and a two-fold serial dilution was performed to produce different concentrations of indigo extract (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, 0.97, and 0.49 mg/mL). Next, 0.01 mL of *S. agalactiae* solution was pipetted into 1.5 mL of TSB in a test tube. After that, the mixture was incubated at 37°C for 24 h. In the control group, no indigo extract was added. The changes in terms

of turbidity of the solution in the test tubes were observed and compared to the control group. MIC was first observed in the first test tube that gave a clear solution. The MBC for all other test tubes that gave a clear solution was calculated. Next, the bacteria were enumerated using a spread plate technique on TSA and incubated at 37ºC. After 24 hours, any concentrations that allowed the bacteria to grow on TSA were the concentrations that could not kill the bacteria but only inhibit it. The MBC was regarded as the lowest concentration of the extracts that inhibited bacterial growth on TSA.

Lethal concentration of indigo extracts required to kill 50% of Nile tilapia (LC50)

The Nile tilapia fries in size 5 cm were stocked in a glass tank with an aerator, and the fish were not fed 24 h before the experiment. The lethal concentration of the indigo extracts needed to kill 50% of Nile tilapia fries in 96 h (96-h LC50) was evaluated. The treatment was divided into five groups and a control group with three replications, each group with ten fries. The mortality rate was recorded every 24 h until 96 h. Before the experiment began and at each 24-hour interval until 96 hours, the dead fish were removed and the water's temperature, pH, dissolved oxygen, electric conductivity, and ammonia level were recorded. The 96-h LC50 was calculated using these data with a computer program.

Fish and experimental design

The Nile tilapia fries, with weighting 18.95±0.48 g, were stocked in a cement tank at the Division of Fisheries, Rajamangala University of Technology Isan, Sakon Nakhon Campus. The water was continuously aerated. Floating pelleted feed was provided twice a day, in the morning and evening, for seven days. The feed was coated with fish oil, sprayed with 500 mg/L indigo extract, and allowed to dry indoors before feeding. The indigo extract was extracted using a 95% ethanol solvent.

In the experiment, 60 fries were put in a 300 L fiber tank filled with water that had been processed with chlorine. A Completely Randomized Design (CRD) was conducted by dividing the experiment into four treatments with three replications each. The details can be described as follows: the first experimental set was the control group, which used typical pellet feed. The second experimental set used pellet feed that was sprayed with 0.1% concentrated indigo extract. The third experimental set used pellet feed that was sprayed with 0.5% concentrated indigo extract. The last experimental set used pellet feed that was sprayed with 1.0% concentrated indigo extract. Fish were fed until satiation was reached twice a day, in the morning and evening (at 8.00 AM and 17.00 PM), for eight weeks. During the experiment, fish weights and survival rates were recorded every two weeks. For each experiment, fresh pellet feed was prepared for every meal.

Bacterial challenge study

After eight weeks, antimicrobial activity testing against *S. agalactiae* was conducted on the sample in all experimental groups (three replications). In each experimental set, ten fish were injected at their belly with 0.1 mL of $10^8$ CFU.mL$^{-1}$ concentrated *S. agalactiae*. The sample in the control group was divided into two subgroups. The sample in the first subgroup was injected with 0.85% normal saline (negative control) (Schaperclaus et al. 1992). The sample in the other subgroup was injected with $10^8$ CFU.mL$^{-1}$ concentrated *S. agalactiae* (positive control). The mortality rate of the sample was recorded every day for 14 days. The relative percent survival (RPS) in each experimental group was calculated according to the method described by Amend (1981) using the following formula:

$$\text{RPS}\ (\%) = 100 \times \left[1 - \frac{\%\text{mortality of treatment group}}{\%\text{moortality of control group}}\right]$$

Blood sample and serum biochemical analysis

After eight weeks, the blood samples were collected from three fish per tank (nine fish per experimental group). Anesthesia was provided to the sample using 150 mg/L of concentrated MS-222 (Tricaine meth-

anesulfonate). For each sample, 1 mL of blood was taken from the caudal vein using a 25G needle. The blood samples were divided into two parts. The first part was collected in heparinized syringes for the evaluation of hematological parameters. The second part involved a serum sample using syringes without anticoagulant, which was allowed to clot at 4ºC for 6 h and subsequently centrifuged at 5000 rpm for 15 min at 4ºC. The serum samples were stored at -20ºC (Wangahat et al. 2022a) for serum biochemical analysis and lysozyme activities.

Total red blood cell (RBC) and total white blood cell (WBC) counts were performed using a standard Neubauer hemocytometer chamber (Stoskopf 2015). Hemoglobin contents (Hb) (g/dL) were measured by blending blood samples with Drabkin's reagent, and the absorbance of mixtures was measured at 540 nm wavelength on a spectrophotometer (Blaxhall and Daisley 1973). The hematocrit (Ht) was determined by filling hematocrit capillary tubes. Ht values were recorded using a centrifuge combo-reader (Wangahat et al. 2022a).

Total protein in the blood (g/dL) was assessed using the Biuret-blank method (Zheng et al. 2017). An albumin blood test was performed using the Bromocresol green method (Moreira et al. 2018). Globulin (g/dL) was calculated as the difference between total protein and albumin. Colorimetric determination of alanine aminotransferase (ALT, U/L) and aspartate aminotransferase (AST, U/L) was applied (Bergmeyer et al. 1978). The alkaline phosphatase (ALP, U/L) test was done following the method of McComb et al. (1981). Serum lysozyme activity (U/mL) was measured as the ability to hydrolyze Gram-positive *Micrococcus lysodeikticus* bacteria, which was determined by the decrease in turbidity at 450 nm of *Micrococcus lysodeikticus* cells using a microplate reader (Zahran et al. 2018). Immunoglobulin M (IgM, g/dL) was analyzed following the method described by Siwicki and Anderson (1993).

## Growth performance and water quality analysis

The total number, individual body length, and weight of fish from each tank were measured and used to calculate the survival rate and growth performance. Here is the list of calculations required (Bagenal 1978):

$$\text{weight gain (WG)} = \frac{\text{final body weight}}{\text{initial body weight}}$$

$$\text{average daily gain (ADG)} = \frac{(\text{average final weight} - \text{average initial weight})}{\text{feeding period (days)}}$$

$$\text{feed conversion ratio (FCR)} = \frac{\text{feed intake}}{\text{weight gain}}$$

$$\text{survival rate} = 100 \times \frac{\text{final fish number}}{\text{initial fish number}}$$

$$\text{fish output} = \text{average final weight} \times \text{number of fish at the end of the experiment}$$

The water quality, including water temperature, pH, and dissolved oxygen (DO), were recorded using the Multi Probe System (YSI 556, USA) every 15 days. The phenate method was used to determine ammonia ($NH_4$-N); diazotizing colorimetric to determine nitrite ($NO_2$-N), and cadmium reduction to determine nitrate ($NO_3$-N) based on the method described by Boyd and Tucker (1992).

## Statistical analysis

A one-way analysis of variance (ANOVA) was conducted, and the differences between the experimental groups were compared using Duncan's New Multiple Range Test (DMRT) at 95% confidence level using IBM SPSS statistics software version 22.

## Results

### Effect of extraction solvents on the yield of crude extracts

Different solvents used in the extraction process resulted in different physical appearances and percentages of yield extraction. Aqueous solvent generated blue-green a powder-like extract with a yield of 10.05±1.31%, while 50% ethanol solvent created a blue-green viscous extract with a yield of 10.78±4.56%, and 95% ethanol solvent produced a bright blue-green and viscous extract with a yield of 4.31±0.78%. The study found that 95% ethanol produced less extract compared to other solvents. The results were statistically significant ($P<0.05$).

### Chemical analysis of *I. tinctoria* extracts

### GC-MS analysis of *I. tinctoria* extracts

The GC-MS analysis was used to detect phytochemical compounds in the extracts. The analysis revealed that the aqueous extract contained cyclotrisiloxane, hexamethyl (17%); silanediol, dimethyl (12.61%); cyclotetrasiloxane, octamethyl (10.99%); and dodecanoic acid, 1,2,3-propanetriyl ester (10.15%) (Table 1). The 50% ethanol extract contained cyclotrisiloxane, hexamethyl (32.01%); cyclotetrasiloxane, octamethyl (24.87%); silanediol, dimethyl (22.05%); and cyclopentasiloxane, decamethyl (8.70%) (Table 2). The 95% ethanol extract contained dodecanoic acid, 1,2,3-propanetriyl ester (Table 3). These vital compounds found in the indigo extracts exhibited antimicrobial, antibacterial, antioxidant, antifungal, and antiviral properties, which play a critical role in the scavenging of free radicals.

### Antioxidant activity of *I. tinctoria* extracts

The antioxidant activity test using the DPPH assay method revealed that the 95% ethanol extract yielded the best antioxidant activity. The differences were statistically significant when compared to the 50% ethanol and aqueous extracts ($P<0.05$) (Table 4). All the indigo extracts exhibited higher antioxidant activity than the $IC_{50}$ of ascorbic acid (94.89±0.02 µg/mL) which was a standard antioxidant. Likewise, the antioxidant activity test using the ABTS assay method revealed that the 95% ethanol extract yielded the best antioxidant activity (Table 4).

**Table 1** GC-MS analysis of aqueous extract of common indigo (*I. tinctoria)*

| Peak# | R. Time | Area% | Name of the compound | Biological activity | References |
|---|---|---|---|---|---|
| 1 | 3.153 | 12.61 | Silanediol, dimethyl | Treatment of dermatological disorder, acne immunological disorder and viral infection | Femi-Adepoju et al. (2018) |
| 2 | 3.651 | 17.69 | Cyclotrisiloxane, hexamethyl | Antimicrobial, antibacterial, antioxidant activity, play a critical role in scavenging of free radicals | Keskin et al. (2012) Musini et al. (2013) (Prakash and Vuppu 2014) |
| 3 | 5.370 | 1.81 | Oxime-, methoxy-phenyl | Antimicrobial, antioxidant | Patil et al. (2012), Ozen and Tas (2009) |
| 4 | 6.443 | 10.99 | Cyclotetrasiloxane, octamethyl | Antifungal, antibacterial, antioxidant | Rizwana et al. (2016) |
| 5 | 7.987 | 3.67 | Cyclopentasiloxane, decamethyl | Antimicrobial | Keskin et al. (2012) |
| 12 | 26.186 | 10.15 | Dodecanoic acid, 1,2,3-propanetriyl ester | Antioxidant, antimicrobial, antiviral, candidicide, antiarthritic, hepatoprotective | Sujatha et al. (2020), Achi and Ohaeri (2015) |
| 13 | 26.302 | 7.06 | Dodecanoic acid, 1,2,3-propanetriyl ester | | |

## Total phenolic content and total flavonoid content

The total phenolic content and total flavonoid content of common indigo extract are illustrated in Table 5.

**Table 2** GC-MS analysis of 50% ethanol extract of common indigo (*I. tinctoria)*

| Peak# | R. Time | Area% | Name of the compound | Biological activity | References |
|---|---|---|---|---|---|
| 1 | 3.213 | 22.05 | Silanediol, dimethyl | Treatment of dermatological disorder, acne immunological disorder and viral infection | Femi-Adepoju et al. (2018) |
| 2 | 3.671 | 32.01 | Cyclotrisiloxane, hexamethyl | Antimicrobial, antibacterial, antioxidant activity, play a critical role in scavenging of free radicals | Keskin et al. (2012) Musini et al. (2013) (Prakash and Vuppu 2014) |
| 3 | 5.414 | 4.03 | Oxime-, methoxy-phenyl | Antimicrobial, antioxidant | Patil et al. (2012), Ozen and Tas (2009) |
| 4 | 6.442 | 24.87 | Cyclotetrasiloxane, octamethyl | Antifungal, antibacterial, antioxidant | Rizwana et al. (2016) |
| 5 | 7.454 | 0.89 | Cyclotrisiloxane, hexamethyl | As mentioned, | |
| 6 | 7.985 | 8.70 | Cyclopentasiloxane, decamethyl | Antimicrobial, antioxidant, antibacterial | Ismail et al. (2020) |
| 7 | 8.581 | 0.65 | Cyclotetrasiloxane, octamethyl | As mentioned, | |
| 8 | 9.084 | 2.46 | Cyclohexasiloxane, dodecamethyl | Antimicrobial, antioxidant, antibacterial | Ismail et al. (2020) |
| 9 | 9.408 | 0.93 | Cyclopentasiloxane, decamethyl | As mentioned, | |
| 10 | 9.920 | 0.87 | Cycloheptasiloxane, tetradecamethyl | Antifungal | Moustafa et al. (2013) |
| 11 | 10.150 | 0.82 | Cyclohexasiloxane, dodecamethyl | As mentioned, | |
| 12 | 10.619 | 0.40 | Cyclooctasiloxane, hexadecamethyl | Antimicrobial | Keskin et al. (2012) |

**Table 3** GC-MS analysis of 95% ethanol extract of common indigo (*I. tinctoria)*

| Peak# | R. Time | Area% | Name of the compound | | |
|---|---|---|---|---|---|
| 1 | 10.562 | 0.29 | Hexadecane | Antimicrobial and antioxidant activity | Yogeswari et al. (2012) |
| 2 | 12.020 | 0.65 | Hexadecanoic acid, ethyl ester | Antioxidant | Tyagi and Agarwal (2016) |
| 3 | 12.644 | 0.61 | Z,Z-8,10-Hexadecadien-1-ol | No activity reported | Banakar and Jayaraj (2018) |
| 4 | 38.540 | 1.20 | Dodecanoic acid, 1,2,3-propanetriyl ester | Antioxidant, antimicrobial, antiviral, candidicide, antiarthritic, hepatoprotective | Sujatha et al. (2020), Achi and Ohaeri (2015) |
| 5 | 38.660 | 2.98 | Dodecanoic acid, 1,2,3-propanetriyl ester | | |
| 7 | 38.923 | 3.47 | Dodecanoic acid, 1,2,3-propanetriyl ester | | |
| 8 | 39.020 | 8.63 | Dodecanoic acid, 1,2,3-propanetriyl ester | | |
| 9 | 39.230 | 7.51 | Dodecanoic acid, 1,2,3-propanetriyl ester | | |
| 10 | 39.381 | 15.79 | Dodecanoic acid, 1,2,3-propanetriyl ester | | |

**Table 4** Antioxidant activity of common indigo (*I. tinctoria)* extract

| Extraction solvent | Antioxidant activity | |
|---|---|---|
| | DPPH assay $IC_{50}$ (µg/mL) | ABTS assay $IC_{50}$ (µg/mL) |
| Aqueous | $91.09\pm0.39^{c}$ | $4.10\pm0.15^{b}$ |
| 50% Ethanol | $87.96\pm0.98^{b}$ | $4.02\pm0.01^{b}$ |
| 95% Ethanol | $78.78\pm0.03^{a}$ | $2.58\pm0.01^{a}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different *(*$P<0.05$)

**Table 5** Total phenolic content and total flavonoid content of common indigo (*I. tinctoria*) extract

| Extraction solvent | Total phenolic content (mg GAE/g E) | Total flavonoid content (mg QE/g DW) |
|---|---|---|
| Aqueous | $4.60\pm0.00^{a}$ | $0.122\pm0.001^{a}$ |
| 50% Ethanol | $6.47\pm0.00^{b}$ | $0.128\pm0.002^{a}$ |
| 95% Ethanol | $7.47\pm0.67^{c}$ | $0.443\pm0.011^{b}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different ($P<0.05$)

The 95% ethanol extract contained the highest phenolic and total flavonoid contents. The results were statistically significant (P<0.05) when compared to extracts that used the other solvents.

## Antimicrobial activity testing of common indigo (*I. tinctoria*) against *S. agalactiae*

### Evaluation of the antimicrobial activity

The antimicrobial activity testing of the common indigo against *S. agalactiae* using the agar disk diffusion method revealed that the 50% and 95% ethanol extracts exhibited a statistical difference in their ability to inhibit bacterial growth (P<0.05). The 95% ethanol extract possessed the best ability to inhibit *S. agalactiae* growth (Table 6).

Based on the MIC value derived from this experiment, the 95% ethanol extract possessed the best ability to inhibit *S. agalactiae* growth with an MIC value equal to 250 mg/mL, followed by the 50% ethanol extract with an MIC value equal to 500 mg/mL, and the aqueous extract with an MIC value equal to 1,000 mg/mL. The MBC was derived from the bacteria from the test tube that demonstrated no growth of *S. agalactiae* by a spread plate technique on TSA. The study showed that *S. agalactiae* could be killed by the 95% ethanol extract with concentration levels of 1000 mg/mL and 500 mg/mL and the 50% ethanol extract with concentration levels of 500 mg/mL. Based on these preliminary trials, the 95% ethanol extract with concentration levels of 500 mg/mL was selected to test the effectiveness of the indigo extract in inhibiting *S. agalactiae* growth in Nile tilapia fries because it possessed the best performance in killing *S. agalactiae.*

### Lethal concentration of indigo extracts required to kill 50% of Nile tilapia (LC50) in 96 h

The result showed that the mortality rates of Nile tilapia in the experiments that used 0, 2000, 4000, 6000, 8000, and 10000 mg/L concentrated indigo extracts were statistically different (P<0.05). The highest concentration level required to kill 100% of the fish in 96 h was equal to 10000 mg/L (Table 7). The concentration level required to kill 50% of the fish in 96 h was 4902 mg/L. The cumulative death rate is represented in the following equation:

$y = 0.0102x$ ($R^2 = 0.9766$)

The safe concentration level of the indigo extracts was calculated based on the application factor of 0.1 according to Chen et al. (1990), which was equal to 490.2 mg/L.

**Table 6** Inhibition zone against *S. agalactiae* growth

| Extraction solvent | Inhibition zone (mm) |
|---|---|
| Ampicillin (100 µg/mL) | 22.83±1.81$^{a}$ |
| Aqueous | 0.49±0.36$^{b}$ |
| 50% Ethanol | 3.88±1.41$^{c}$ |
| 95% Ethanol | 8.20±1.27$^{d}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different (p<0.05)

**Table 7** Cumulative death rate of Nile tilapia exposed to different levels of concentrations of indigo extract for 96 h

| Concentration of common indigo extract (mg/L) | Lethal concentration (%) | | | | |
|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 96 h |
| 0 | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| 2,000 | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 10.00±0.00$^{b}$ | 16.67±5.77$^{b}$ |
| 4,000 | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 16.67±5.77$^{b}$ | 30.00±10.00$^{c}$ |
| 6,000 | 0.00±0.00$^{a}$ | 43.33±5.77$^{b}$ | 53.33±5.77$^{b}$ | 60.00±10.00$^{c}$ | 66.67±5.77$^{d}$ |
| 8,000 | 0.00±0.00$^{a}$ | 53.33±5.77$^{c}$ | 76.67±5.77$^{c}$ | 83.33±11.55$^{d}$ | 86.67±5.77$^{e}$ |
| 10,000 | 0.00±0.00$^{a}$ | 100.00±0.00$^{d}$ | 100.00±0.00$^{d}$ | 100.00±0.00$^{e}$ | 100.00±0.00$^{f}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different (P<0.05)

## Bacterial *(S. agalactiae*) challenge study in Nile tilapia

### Mortality rate and relative percent survival of Nile tilapia

This bacterial challenge study in Nile tilapia was divided into 4 experimental groups and carried out by injecting 0.1 mL of $10^8$ CFU.mL$^{-1}$ concentrated *S. agalactiae* into the fish belly. The sample in the control group was divided into two subgroups. The sample in the first subgroup was injected with 0.85% normal saline (a negative control). The sample in the other subgroup was only injected with $10^8$ CFU.mL$^{-1}$ concentrated *S. agalactiae* (a positive control). The results revealed that the fish that consumed pellet feed sprayed with indigo extracts exhibited higher survival rate than the control groups that did not receive any indigo extracts. The fish that consumed pellet feed sprayed with 0.5% concentrated pellet feed exhibited a higher antimicrobial activity against *S. agalactiae*, with a 70% mortality rate and a relative percent survival of 36.67%. The results were statistically significant when compared to other experimental groups ($P<0.05$) (Table 8).

### Hematology, biochemistry, and immunology in fish blood

Total red blood cells, hemoglobin counts, and hematocrit of fish that received pellet feed sprayed with the indigo extract were statistically higher than those of the control group ($P<0.05$). The results showed no differences among the experimental groups that received pellet feed sprayed with different concentrations of the indigo extract. White blood cells and the percentage of lymphocytes in fish that received pellet feed sprayed with the 0.5% concentrated indigo extract were statistically higher than those of the control group ($P<0.05$). However, the results showed no statistical differences in neutrophils or eosinophils among all experimental groups (Table 9).

The biochemical parameters of Nile tilapia blood fed with different types of feed are displayed in Table 10. Fish that received pellet feed sprayed with 1% concentrated indigo extract exhibited statis-

**Table 8** Mortality rate and relative percent survival of Nile tilapia exposed to *S. agalactiae* in different experimental groups for the bacterial challenge study.

| Experimental group | Mortality rate (%) | Relative percent survival (%) |
|---|---|---|
| Control group, injected with 0.85% NaCl (negative control) | 0.00±0.00$^{d}$ | 100±0.00$^{a}$ |
| Control group, injected with $10^8$ CFU.mL$^{-1}$ concentrated bacteria (positive control) | 100±0.00$^{a}$ | 0.00±0.00$^{d}$ |
| Pellet feed sprayed with 0.1 % concentrated indigo extract | 76.67±5.77$^{b}$ | 23.33±5.77$^{c}$ |
| Pellet feed sprayed with 0.5 % concentrated indigo extract | 70.00±10.00$^{c}$ | 36.67±10.00$^{b}$ |
| Pellet feed sprayed with 1.0 % concentrated indigo extract | 73.33±5.77$^{b}$ | 26.67±5.77$^{c}$ |

**Table 9** Hematological parameters of Nile tilapia fed with different feed for 8 weeks

| Parameter | Control group (0%) | Pellet feed sprayed with indigo extract | | |
|---|---|---|---|---|
| | | 0.1% Concentration | 0.5% Concentration | 1% Concentration |
| Hematocrit (%) | 17.67±1.52$^{a}$ | 19.67±2.08$^{ab}$ | 22.67±2.51$^{ab}$ | 24.00±4.00$^{b}$ |
| Haemoglobin (g/dL) | 5.80±0.26$^{a}$ | 5.97±0.42$^{ab}$ | 7.27±0.68$^{ab}$ | 7.60±1.45$^{b}$ |
| RBC (x10$^6$ cells/mm$^3$) | 2.37±0.06$^{a}$ | 2.53±0.58$^{ab}$ | 2.73±1.52$^{b}$ | 2.83±0.32$^{b}$ |
| WBC (x10$^3$ cells/mm$^3$) | 1.39±1.00$^{a}$ | 1.52±1.25$^{a}$ | 1.90±0.92$^{b}$ | 1.36±1.02$^{a}$ |
| Lymphocytes (%) | 5.67±1.15$^{a}$ | 7.67±2.08$^{a}$ | 16.33±1.52$^{c}$ | 11.67±2.08$^{b}$ |
| Neutrophils (%) | 88.67±3.51$^{a}$ | 91.67±2.51$^{a}$ | 92.67±3.21$^{a}$ | 87.33±5.13$^{a}$ |
| Eosinophils (%) | 0.33±0.57$^{a}$ | 0.33±0.57$^{a}$ | 0.33±0.57$^{a}$ | 0.33±0.57$^{a}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different ($P<0.05$)

**Table 10** Biochemical parameters in Nile tilapia blood fed with different feed for 8 weeks

| Parameter | Control group (0%) | Pellet feed sprayed with indigo extract | | |
|---|---|---|---|---|
| | | 0.1% Concentration | 0.5% Concentration | 1% Concentration |
| ALT (U/L) | 19.33±1.53$^{a}$ | 21.33±3.21$^{a}$ | 25.33±7.02$^{a}$ | 26.33±3.79$^{a}$ |
| AST (U/L) | 34.00±7.81$^{a}$ | 35.67±6.51$^{a}$ | 41.00±5.57$^{ab}$ | 48.33±3.51$^{b}$ |
| ALP (U/L) | 36.00±8.54$^{a}$ | 36.33±6.66$^{a}$ | 51.00±7.21$^{b}$ | 51.33±2.08$^{b}$ |
| Total protein (g/dL) | 2.67±0.27$^{a}$ | 3.06±0.33$^{ab}$ | 3.35±3.23$^{b}$ | 3.23±0.22$^{b}$ |
| Albumin (g/dL) | 1.41±0.29$^{a}$ | 1.42±0.14$^{a}$ | 1.43±0.07$^{a}$ | 1.51±0.26$^{a}$ |
| Globulin (g/dL) | 0.36±0.11$^{a}$ | 0.41±0.05$^{a}$ | 0.41±0.67$^{a}$ | 0.38±0.09$^{a}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different ($P<0.05$)

tically higher AST than those in other experimental groups (P<0.05). Fish that received pellet feed sprayed with 0.5% and 1% concentrated indigo extracts exhibited statistically higher ALP and total protein than those in other experimental groups (P<0.05). However, the results showed no statistical differences in ALT, albumin, or globulin among all experimental groups (P<0.05).

This research analyzed immunity in the blood of fish that received different types of feed for eight weeks. The results revealed that fish that received feed sprayed with 0.5% concentrated indigo extract exhibited statistically higher levels of lysozyme in their blood than those in other experimental groups (P<0.05) (Fig. 1). Fish that received feed sprayed with 0.1% and 0.5% concentrated indigo extracts exhibited statistically higher IgM than those in other groups (P<0.05) (Fig. 2).

## Nile tilapia growth and water quality

After eight weeks, the results showed that fish fed with feed sprayed with 0.5% concentrated indigo extract exhibited a higher final weight, increased weight, average daily gain (ADG), and feed conversion ratio (FCR) than those in the control group (P<0.05). However, these were not statistically different from those in the experimental groups that used feed sprayed with 0.1% and 1% concentrated indigo extract. In addition, the results showed no statistical differences in survival rate or fish output among all experimental groups (P<0.05) (Table 11).

In terms of water quality, the results showed no statistical differences in pH, dissolved oxygen,

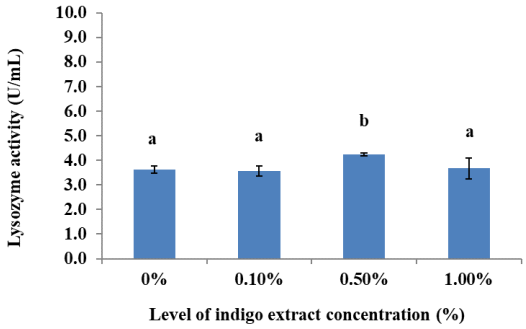


**Fig. 1** Serum lysozyme activity of Nile tilapia fed with different feed for 8 weeks. The values displayed in the graph as Mean±SD with difference letters are significantly different (P<0.05).

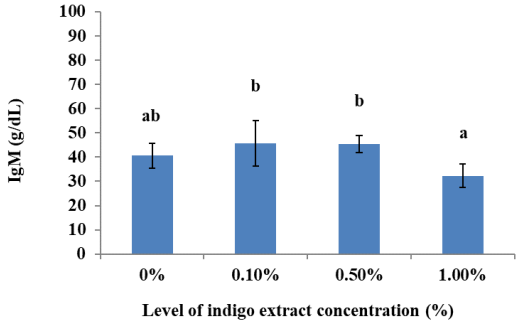


**Fig. 2** Immunoglobulin M (IgM) in Nile tilapia blood fed with different feed in 8 weeks. The values displayed in the graph as Mean±SD with difference letters are significantly different (P<0.05).

**Table 11** Initial weight, final weight, increased weight, average daily gain, feed conversion ratio, survival rate, and fish output.

| Parameter | Control group (0%) | Pellet feed sprayed with indigo extract | | |
|---|---|---|---|---|
| | | 0.1% Concentration | 0.5% Concentration | 1% Concentration |
| Initial weight (g) | 18.75±0.57$^{a}$ | 19.00±0.35$^{a}$ | 18.85±0.85$^{a}$ | 19.20±0.49$^{a}$ |
| Final weight (g) | 34.33±0.11$^{a}$ | 35.98±0.53$^{ab}$ | 38.25±1.70$^{b}$ | 36.95±2.19$^{ab}$ |
| Weight gain (g) | 15.58±0.67$^{a}$ | 16.98±0.18$^{ab}$ | 19.05±1.20$^{b}$ | 18.10±1.34$^{ab}$ |
| ADG (g/fish/day) | 0.260±0.011$^{a}$ | 0.283±0.003$^{ab}$ | 0.318±0.020$^{b}$ | 0.302±0.022$^{ab}$ |
| FCR | 2.10±0.09$^{a}$ | 1.94±0.02$^{ab}$ | 1.78±0.11$^{b}$ | 1.95±0.14$^{ab}$ |
| Survival rate (%) | 81.82±5.14$^{a}$ | 84.55±1.29$^{a}$ | 86.36±1.29$^{a}$ | 85.45±5.14$^{a}$ |
| Fish output (g/tank) | 1,545±102$^{a}$ | 1,673±50$^{a}$ | 1,800±188$^{a}$ | 1,754±78$^{a}$ |

The values shown as Mean±SD with different superscript letters in a column are significantly different (P<0.05)

temperature, ammonia, nitrite, or nitrate among all experimental groups ($P<0.05$). In this entire experiment, pH ranged between 7.43 and 7.69, dissolved oxygen ranged between 6.32 and 8.29 mg/L, the temperature ranged between 26.00 and 28.79 °C, ammonia ranged between 0.095 and 0.279 mg-N/L, nitrite concentration ranged between 0.031 and 0.553 mg-N/L, and nitrate concentration ranged between 0.020 and 0.167 mg-N/L.

## Discussion

This research aimed to study the utilization of indigo extracts in inhibiting bacterial infection in Nile tilapia. The results showed that the 95% ethanol indigo extract exhibited the highest ability to kill *S. agalactiae*. The results from the bacterial challenge study revealed that Nile Tilapia that consumed feed sprayed with 0.5% concentrated indigo extract could best inhibit *S. agalactiae*. Indigo extracts could inhibit bacteria because they contain phytochemical components including alkaloids, flavonoids, tannins, phenols, saponins, glycosides, and terpenoids that possess antioxidant potential (Chandra et al. 2014; Srinivasan et al. 2016; Vijayan et al. 2018). The results from the GC-MS analysis, total phenolic content, total flavonoid content, and antioxidant activity testing revealed various vital compounds in the indigo extracts that exhibited antimicrobial, antibacterial, and antioxidant activity properties. The results showed that 95% ethanol extract exhibited higher total phenolic content and total flavonoid content and consequently, the highest antioxidant activity. Previous research has shown that various plants contain secondary metabolites and other components that can inhibit bacteria (Cluz et al. 2019; Zofia et al. 2020).

Hematology results showed that the Nile tilapia in the group that ate pellet food sprayed with indigo extract had more red blood cells, more hemoglobin, and a higher hematocrit than those in the control group. However, there were no statistical differences among the experimental groups that received pellet feed sprayed with different concentrations of the indigo extract. White blood cells and the percentage of lymphocytes in fish that received pellet feed sprayed with the 0.5% concentrated indigo extract were statistically higher than those of other experimental groups. Hematological parameters, including total red blood cells, hemoglobin counts, hematocrit, total white blood cells, and types of white blood cells, are widely used for assessing the health status and antioxidative capacity of fish when adapting to the environment (Kim et al. 2021). Various studies found that mixing herbs with fish feed could help improve the hematological parameters of many types of fish (Adel et al. 2015; Dadras et al. 2016; Ebrahimi et al. 2020).

This study revealed that indigo extracts could help improve the hematological parameters of Nile tilapia. The study showed that the total white blood cells and the percentage of lymphocytes in fish that received pellet feed sprayed with the 0.5% concentrated indigo extract for eight weeks were statistically higher than those of other experimental groups. Leukocytes are part of the immune system, and white blood cells can be an indication of aquatic animal health. This research aligns with Boothapandi and Ramanibai (2016), who found that *I. tinctoria* extract could increase pinocytic activity, which could further enhance the phagocytic activity of macrophages. Macrophages play a dominant role in phagocytosis, which carries out the intracellular killing of antigen (Delcenserie et al. 2008). Furthermore, some previous studies also used plant extracts such as turmeric (Abdelrazek et al. 2017), bael fruit (*Aegle marmelos*) (Wangkahat et al. 2022b), jujube leaves (*Ziziphus mauritiana*) (Asely et al. 2020), and miswak (*Salvadora persica*) (El-latif et al. 2021) to increase immunity in Nile tilapia. Sadeghi et al. (2021) also found that herbs could boost immunity and increase white blood cells in common carp. Overall, this research found that using pellet feed sprayed with indigo extract could help boost innate immunity in Nile tilapia.

Blood biochemistry is important for assessing pathology and indicating fish health (Casanovas et al. 2021). The amount of ALT, AST, and ALP is an indication of illness and reflects the level of liver injury in fish (Zhang et al. 2020). The fish provided with pellet feed sprayed with 1% concentrated indigo extract exhibited statistically higher AST than those in other groups. The fish provided with 0.5% and 1% concentrated indigo extracts exhibited statistically higher ALP than those in other groups. This study found higher ALT, AST, and ALP than the normal values found in Nile tilapia blood in Wangkahat et al. (2022a) and Monir et al. (2020) reports. This can be interpreted as meaning that using higher indigo extract concentrations could affect liver cells.

Total protein, albumin, and globulin are used in assessing nutritional status or diagnosing liver and kidney abnormalities and other diseases. Globulins are a group of proteins in the blood that play an important role in liver function, blood clotting, and immune responses (Wangkahat et al. 2022a). Higher total protein levels reflect improved responses to the innate immune system (Hassaan et al. 2021). In this study, fish that received 0.5% and 1% concentrated indigo extracts exhibited statistically higher total protein than those in the control group. This illustrates the ability of indigo extract to boost immunity.

The innate immune response in fish is typically assessed by the release of bactericidal or hydrolyzing enzymes called lysozymes in fish serum. Lysozyme is an important enzyme for the innate immune system. It is produced by a monocyte, macrophages, and neutrophils (Saurabh and Sahoo 2008; Carbone and Faggio 2016). This enzyme breaks down bacterial cell walls through cell lysis. Lysozyme activity can boost leucocytes and macrophages, which causes phagocytosis (Magnadottir 2006). It possesses the ability to kill bacteria and plays an important role in inhibiting the spread of bacteria, especially gram-positive (Tahmasebi-Kohyani et al. 2011). Based on the results of this study, fish that received feed sprayed with 0.5% concentrated indigo extract exhibited statistically higher levels of lysozyme in their blood than those in other experimental groups. The results showed that fish that received feed sprayed with 0.1% and 0.5% concentrated indigo extract exhibited statistically higher IgM than those in other experimental groups. This shows that fish that received feed mixed with indigo extracts had higher innate immunity than those in the groups that received feed without indigo extracts. This aligns with Boothapandi and Ramanibai (2016), who found that *I. tinctoria* extracts enhanced innate as well as adaptive immune response.

In this study, Nile tilapia were provided pellet feed sprayed with different concentration levels (0%, 0.1%, 0.5%, and 1%) of the indigo extract for eight weeks. The results showed that the final weight, weight gain, ADG, and FCR were statistically higher in the sample that received pellet feed sprayed with 0.5% concentrated indigo extract than those in the control group. This could be because indigo extract contains tannins and saponins, which typically lead to less feed consumption. Tannins can interfere with the digestive system (Halawany 2012). Receiving high levels of tannins can suppress enzyme activity, including amylase, lipase, and protease, in the digestive system, causing slow growth in fish (Omnes et al. 2017). In addition, regularly consuming herbs that contain bioactive compounds such as saponins could cause too much of an immune response that could obstruct the metabolism of fish (Talpur and Ikhwanuddin et al. 2013).

## Conclusion

This study found that the 95% ethanol indigo extract could best inhibit *S. agalactiae* growth. The best hematological parameters, immunity, and growth of Nile tilapia were obtained when pellet feed was sprayed with 0.5% concentrated indigo extract. The results revealed that indigo extracts could inhibit *S. agalactiae* and increase immunity in Nile tilapia. However, common indigo extract may be used in essential oil or powder form, but experimental studies of the effectiveness of indigo extracts in inhibiting bacterial infection in fish are still limited. To ensure safe and effective use of the common indigo extract, further studies are needed on how it enters the body when used in fish feed as a powder, essential oil, or capsule. These studies will also help to better understand how common indigo extract works to improve fish health.

**Competing interest** The authors declare no competing interests.

**Authors' contributions** SS and PS, designing, conceptualizing, data analysis, writing and editing; NS, NC, and WP, formal analysis, review and writing; PT, chemical analysis of the extracts and reviewing; PP, interpretation of data and writing. All authors read and approved the final version of the manuscript.

**Acknowledgements** This research project is supported by Thailand Science Research and Innovation (TSRI) (Contract No. FRB650059/SNK/08-3). The authors are thankful the Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus for research facilities support.

**References**


Abdelrazek HMA, Tag HM, Kilany OE, Reddy PG, Hassan AM (2017) Immuomodulatory effect of dietary turmeric supplementation on Nile tilapia (*Oreochromis niloticus*). Aquac Nutr 23:1048-1054. https://doi.org/10.1111/anu.12472

Achi NK, Ohaeri OC (2015) GC-MS determination of bioactive constituents of the methanolic fractions of *Cnidoscolus aconitifolius*. J Pharm Res Int 5(3):163-172

Adel M, Amiri AA, Zorriehzahra J, Nematolahi A, Esteban MA (2015) Effects of dietary peppermint (*Mentha piperita*) on growth performance, chemical body composition and hematological and immune parameters of fry Caspian white fish (*Rutilus frisii kutum*). Fish Shellfish Immunol 45:841-847. https://doi.org/10.1016/j.fsi.2015.06.010

Amend DF (1981) Potency testing of fish vaccines. Fish biologics: Serodiagnostics and vaccines. Dev Biol Stand 49:447-454

Anusuya N, Manian S (2013) Antioxidant and free radical scavenging potential of different solvent extracts of *Indigofera tinctoria* L. leaves. Int J Pharm Pharm Sci 5(1):142-147

Asely AE, Amin A, El-Naby SA, Samir F, El-Ashram A, Dawood M (2020) *Ziziphus mauritiana* supplementation of Nile tilapia (*Oreochromis niloticus*) diet for improvement of immune response to *Aeromonas hydrophila* infection. Fish Physiol Biochem 46:1561-1575. https://doi.org/10.1007/s10695-020-00812-w

Ashraf A, Sarfraz RA, Anwar F, Shahid SA, Alkharfy KM (2015) Chemical composition and biological activities of leaves of *Ziziphus mauritiana* L. native to Pakistan. Pak J Bot 47(1):367-376

Bagenal T (1978) Methods for the assessment of fish production in fresh waters. Blackwell scientific publications, London

Banakar P, Jayaraj M (2018) GC-MS analysis of bioactive compounds from ethanol leaf extract of *Waltheria indica* Linn. and their pharmacological activities. Int J Pharm Sci Res 9(5):2005-2010

Belton B, Turongruang D, Bhujel R, Little DC (2009) The history, status, and future prospects of monosex tilapia culture in Thailand. Aquac Asia Mag 14(2):16-19

Bergmeyer HU, Scheibe P, Wahlefeld AW (1978) Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem 24(1):58-73. https://doi.org/10.1093/clinchem/24.1.58

Blaxhall PC, Daisley KW (1973) Routine haematological methods for use with fish blood. J Fish Biol 5(6):771-781. https://doi.org/10.1111/j.1095-8649.1973.tb04510.x

Boothapandi M, Ramamibai R (2016) Immunomodulatory activity of *Indigofera tinctoria* leaf extract on invitro macrophage responses and lymphocyte proliferation. Int J Pharm Pharm Sci 8(7):58-63

Boyd CE, Tucker CS (1992) Water quality and pond soil analyses for aquaculture. Agricultural Experiment Station, Alabama

Campos JKL, Araujo TF da S, Brito TG da S, da Silva APS, da Cunha RX, Martin MB, da Silva NH, Santos BS, da Silva CA, Lima VL de M (2018) *Indigofera suffruticosa* Mill. (Anil): Plant profile, phytochemistry and pharmacology review. Adv Pharmacol Sci 2018:1-6. https://doi.org/10.1155/2018/8168526

Carbone D, Faggio C (2016) Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrachus labrax*. Fish Shellfish Immunol 54:172-178. https://doi.org/10.1016/j.fsi.2016.04.011

Casanovas P, Walker SP, Johnston H, Johnston C, Symonds JE (2021) Comparative assessment of blood biochemistry and haematology normal ranges between Chinook salmon (*Oncorhynchus tshawytscha*) from seawater and freshwater farms. Aquaculture 537:1-11. https://doi.org/10.1016/j.aquaculture.2021.736464

Chandra S, Saklani S, Mishra AP, Rana G (2014) Nutritional, anti-nutritional profile and phytochemical screening of flowers of *Indigofera tinctoria* from Garhwal Himalaya. Int J Herb Med 1(5):23-27

Chen JC, Liu PC, Lei SC (1990) Toxicities of ammonia and nitrite to *Penaeus monodon* adolescents. Aquaculture 89:127-137

Chen XQ, Li ZH, Lui LL, Wang H, Yang SH, Zhang JS, Zhang Y (2021) Green extraction using deep eutectic solvents and antioxidant activities of flavonoids from two fruits of *Rubia* species. LWT-Food Sci 148:1-9. https://doi.org/10.1016/j.lwt.2021.111708

Cruz JER, Guerra JFC, Gomes MS, Freitas GRO, Morais ER (2019) Phytochemical analysis and evaluation of antimicrobial activity of *Peumus boldus*, *Psidium guajava*, *Vernonia polysphaera*, *Persea Americana*, *Eucalyptus citriodora* leaf extracts and *Jatropha multifida* raw sap. Curr Pharm Biotechnol 20(5):433-444. https://doi.org/10.2174/1389201020666190409104910

Dadras H, Hayatbakhsh MR, Shelton WL, Golpour A (2016) Effects of dietary administration of rose hip and safflower on growth performance, haematological, biochemical parameters and innate immune response of beluga, *Huso huso* Linnaeus, 1758. Fish Shellfish Immunol 59:109-114. https://doi.org/10.1016/j.fsi.2016.10.033

Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, Roy D (2008) Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol 10:37-54. https://doi.org/10.21775/cimb.010.037

Ding ZH, Hong JM, Guo WL, Li GH, Zhao ZC, Zhou Y, Wang SF, Sun Y, Li JL, Zhang DD, Zhou YC (2021) The screen herbal immunopotentiator and research on its effect on the innate immune system and disease resistance of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae*. Aquaculture 541:736-778. https://doi.org/10.1016/j.aquaculture.2021.736778

Doan HV, Hoseinifar SH, Jaturasitha S, Dawood MAO, Harikrishnan R (2020) The effects of berberine powder supplementation on growth performance, skin mucus immune response, serum immunity, and disease resistance of Nile tilapia (*Oreochromis niloticus*) fingerlings. Aquaculture 520:1-8. https://doi.org/10.1016/j.aquaculture.2020.734927

Doan, HV, Soltani M, Leitao A, Shafiei S, Asadi S, Lymbery AJ, Ringo E (2022) Streptococcosis a re-emerging disease in aquaculture: significance and phytotherapy. Animals 12: 2443. https://doi.org/10.3390/ani12182443

Ebrahimi E, Haghjou M, Nematollahi A, Goudarzian F (2020) Effects of rosemary essential oil on growth performance and hematological parameters of young great sturgeon (*Huso huso*). Aquaculture 521:1-6. https://doi.org/10.1016/j.aquaculture.2019.734909

El-latif AMA, Eman AE, Eman IS, Ramy MS, Zahran E (2021) Dietary supplementation with miswak (*Salvadora persica*) improves the health status of Nile tilapia and protects against *Aeromonas hydrophila* infection. Aquac Rep 19:1-9. https://doi.org/10.1016/j.aqrep.2021.100594

FAO (2018) The state of world fisheries and aquaculture 2018-meeting the sustainable development goals. Food and Agriculture Organization of the United Nations, Rome

Femi-Adepoju AG, Adepoju AO, Durodola FA, Akanbi-Gada MA (2018) Phytochemical, antimicrobial and bio-active component analysis of *Platycerium Superbum* (L.) methanolic extract. Int J Sci: Basic Appl Res 40(1):98-107

Gabriel NN (2019) Review on the progress in the role of herbal extracts in tilapia culture. Cogent Food Agric 5:1-21. https://doi.org/

10.1080/23311932.2019.1619651
Halawany HS (2012) A review on miswak (*Saladora persica*) and its effect on various aspects of oral health. Saudi Dent J 24(2):63-69. https://doi.org/10.1016/j.sdentj.2011.12.004
Hassaan MS, Mohammady EY, Soaudy MR, Sabae SA, Mahmoud AM, El-Haroun ER (2021) Comparative study on the effect of dietary β-carotene and phycocyanin extracted from *Spirulina platensis* on immune-oxidative stress biomarkers, genes expression and intestinal enzymes, serum biochemical in Nile tilapia, *Oreochromis niloticus*. Fish Shellfish Immunol 108:63-72. https://doi.org/10.1016/j.fsi.2020.11.012
Ismail GA, Gheda SF, Abo-shady AM, Abdel-karim OH (2020) In vitro potential activity of some seaweeds as antioxidants and inhibitors of diabetic enzymes. Food Sci Technol 40(3):681-691
Jadhav VS, Khan SI, Girkar MM, Gitte MJ (2006) The role of immunostimulants in fish and shrimp aquaculture. Aquac Asia Mag 11(3):24-27
Jemmali Z, Chartier A, Elkafir C (2016) Development of a gas chromatography-mass spectrometry method to monitor in a single run, mono-to triterpenoid compounds distribution in resinous plant materials. J Chromatogr A 1443:241-253. https://doi.org/10.1016/j.chroma.2016.03.031
Keskin D, Ceyhan N, Ugur A, Dbeys A (2012) Antimicrobial activity and chemical constitutions of West Anatolian olive (*Olea europaea* L.) leaves. J Food Agric Environ 10(2):99-102. https://doi.org/10.1234/4.2012.2896
Kim JH, Sohn S, Kim SK, Kim SR, Kim SK, Kim SM, Kim NY, Hur YB (2021) Effects on the survival rates, hematological parameters, and neurotransmitters in olive flounders, *Paralichthys olivaceus*, reared in bio-floc and seawater by *Streptococcus iniae* challenge. Fish Shellfish Immunol 113:79-85. https://doi.org/10.1016/j.fsi.2021.03.013
Magnadottir B (2006) Innate immunity of fish (overview). Fish Shellfish Immunol 20:137-151. https://doi.org/10.1016/j.fsi.2004.09.006
McComb RB, Bowers GN, Posen S (eds) (1981) Alkaline phosphatase. Plenum press, New York
Mensor LL, Menezes FS, Leitao GG, Reis AS, Santos TC, Coube CS, Leitao SG (2001) Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res 15(2):127-130. https://doi.org/10.1002/ptr.687
Monir W, Abdel-Rahmana M, Hassana S, Mansourb ES, Awadc S (2020) Pomegranate peel and moringa-based diets enhanced biochemical and immune parameters of Nile tilapia against bacterial infection by *Aeromonas hydrophila*. Microb Pathog 145:104202. https://doi.org/10.1016/j.micpath.2020.104202
Moreira VG, Vaktangova NB, Martinez Gago MD, Gonzalez BL, Alonso SG, Rodriguez EF (2018) Overestimation of albumin measured by bromocresol green vs bromocresol purple method: influence of acute-phase globulins. Lab Med 49(4):355-361. https://doi.org/10.1093/labmed/lmy020
Moustafa MFM, Alamri SA, Taha TH, Alrumman SA (2013) In vitro antifungal activity of *Argemone ochroleuca* sweet latex against some pathogenic fungi. Afr J Biotechnol 12(10):1132-1137
Musini A, Prakash RMJ, Giri A (2013) Phytochemical investigations and antibacterial activity of *Salacia oblonga* Wall ethanolic extract. Ann Phytomed 2:102-107
Omnes MH, Le Goasduff J, Le Delliou H, Le Bayon N, Quazuguel P, Robin JH (2017) Effects of dietary tannin on growth, feed utilization and digestibility and carcass composition in juvenile European seabass (*Dicentrarchus labrax* L.). Aquac Rep 6:21-27. https://doi.org/10.1016/j.aqrep.2017.01.004
Ozen T, Tas M (2009) Screening and evaluation of antioxidant activity of some amido-carbonyl oxime derivatives and their radical scavenging activities. J Enzyme Inhib Med Chem 24(5):1141-1147. http://doi.org/10.1080/14756360802669981. PMid:19772487
Pattanaik L, Naik SN, Hariprasad P (2019) Valorization of waste *Indigofera inctoria* L. biomass generated from indigo dye extraction process-potential towards biofuels and compost. Biomass Convers Biorefin 9:445-457. http://doi.org/ /10.1007/s13399-018-0354-2
Patil SS, Jadhav SD, Deshmukh MB (2012) Synthesis and antimicrobial activities of new oxime carbamates of 3-aryl-2-thioquinazolin-4(3H)-one. J Chem Sci 124(5):1043-1048. http://doi.org/10.1007/s12039-012-0302-9
Prakash A, Vuppu S (2014) *Punica granatum* (pomegranate) rind extract as a potent substitute for L-ascorbic acid with respect to the antioxidant activity. Res J Pharm Biol Chem Sci 5:597-603
Rahman UT, Zeb AM, Liaqat W, Sajid M, Hussain S, Choudhary MI (2018) Phytochemistry and pharmacology of genus *Indigofera*: A review. Rec Nat Prod 12(1):1-13
Rico A, Oliveira R, McDonough S, Matser A, Khatikarn J, Satapornvanit K, Nogueira JA, Soares A, Omingues I, Van den Brink P (2014) Use fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand. Environ Pollut 191:8-16. https://doi.org/10.1016/j.envpol.2014.04.002
Rizwana H, Alwhibi MS, Soliman DA (2016) Antimicrobial activity and chemical composition of flowers of *Matricaria aurea* a native herb of Saudi Arabia. Int J Pharmacol 12(6):576-586. http://doi.org/10.3923/ijp.2016.576.586
Sadeghi F, Ahmadifar E, Shahriari M, Ghiyasi M, Dawood MA, Yilmaz S (2021) Lemon, *Citrus aurantifolia*, peel and *Bacillus licheniformis* protected common carp, *Cyprinus carpio*, from *Aeromonas hydrophila* infection by improving the humoral and skin mucosal immunity, and antioxidative responses. J World Aquac Soc 52:124-137. https://doi.org/10.1111/jwas.12750
Saurabh S, Sahoo S (2008) Lysozyme: an important defence molecule of fish innate immune system. Aquac Res 39(3):223-239. https://doi.org/10.1111/j.1365-2109.2007.01883.x
Schaperclaus W, Kulow H, Schreckenbach K (eds) (1992) Fish Disease. A.A. Balkema, Rotterdam
Singh R, Sharma S, Sharma V (2015) Comparative and quantitative analysis of antioxidant and scavenging potential of *Indigofera tinctoria* Linn. extracts. J Integr Med 13(4):269-278. https://doi.org/10.1016/S2095-4964(15)60183-2
Singh DP, Verma S, Prabha R (2018) Investigations on antioxidant potential of phenolic acids and flavonoids: The common phytochemical ingredients in plants. J Plant Biochem Physiol 6(3):1-5. https://doi.org/10.4172/2329-9029.1000219
Siwicki A, Anderson D (1993) An easy spectrophotometric assay for determining total protein and immunoglobulin levels in fish sera: correlation to fish health. Tech Fish Immunol 3:23-30
Soltani M, Lymbery A, Song SK, Hosseini P, Shekarabi HP (2019) Adjuvant effects of medicinal herbs and probiotics for fish vaccines. Rev Aquac 11:1325-1341. https://doi.org/10.1111/raq.12295
Srinivasan S, Wankhar W, Rathinasamy S, Rajan R (2015) Larvicidal potential of *Indigofera tinctoria* (Fabaceae) on dengue vector

(*Aedes aegypti*) and its antimicrobial activity against clinical isolates. Asian J Pharm Clin Res 8(30):316-319

Srinivasan S, Wankhar W, Rathinasamy S, Rajan R (2016) Free radical scavenging potential and HPTLC analysis of *Indigofera tinctoria* linn (Fabaceae). J Pharm Anal 6(2):125-131. https://doi.org/10.1016/j.jpha.2015.04.003

Stoskopf MK (2015) Biology and management of laboratory fishes. In Fox J (ed) Laboratory animal medicine, 3rd edn. Academic press, London

Sujatha S, Sara SC, Gayathiri M, Roselin IR, Ruby RGD (2020) Analysis of bioactive compounds present in methanolic extract of *Phymatosorus scolopendria* (BURM. F.) Pic. Serm. through gas chromatography and mass spectroscopy. Int J Pharm Sci Res 11(7):3294-3299

Tahmasebi-Kohyani A, Keyvanshokooh S, Nematollahi A, Mahmoudi N, Pasha-Zanoosi H (2011) Dietary administration of nucleotides to enhance growth, humoral immune responses, and disease resistance of the rainbow trout (*Oncorhynchus mykiss*) fingerlings. Fish Shellfish Immunol 30:189-193. https://doi.org/10.1016/j.fsi.2010.10.005

Talpur AD, Ikhwanuddin M (2013). *Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*. Fish Shellfish Immunol 34(1):254-264. https://doi.org/10.1016/j.fsi.2012.11.003

Tyagi T, Agarwal M (2016) Phytochemical screening and GC-MS analysis of bioactive constituents in the ethanolic extract of *Pistia stratiotes* L. and *Eichhornia crassipes* (Mart.) solms. J Pharmacogn Phytochem 6(1):195-206

Vijayan R, Joseph S, Mathew B (2018) Anticancer, antimicrobial, antioxidant, and catalytic activities of green-synthesized silver and gold nanoparticles using *Bauhinia purpurea* leaf extract. Bioprocess Biosyst Eng 42(2):305-319. https://doi.org/10.1007/s00449-018-2035-8

Wang X, Wang M, Cao J, Wu Y, Xiao J, Wang Q (2017) Analysis of flavonoids and antioxidants in extracts of ferns from Tianmu Mountain in Zhejiang Province (China). Ind Crops Prod 97:137-145. https://doi.org/10.1016/j.indcrop.2016.12.013

Wangkahart E, Bruneel B, Wisetsri T, Nontasan S, Martin SA, Chantiratikul A (2022a) Interactive effects of dietary lipid and nutritional emulsifier supplementation on growth, chemical composition, immune response and lipid metabolism of juvenile Nile tilapia (*Oreochromis niloticus*). Aquaculture 546:1-12. https://doi.org/10.1016/j.aquaculture.2021.737341

Wangkahart E, Wachiraamonloed S, Lee PT, Subramani PA, Qi Z, Wang B (2022b) Impacts of *Aegle marmelos* fruit extract as a medicinal herb on growth performance, antioxidant and immune responses, digestive enzymes, and disease resistance against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol 120:402-410. https://doi.org/10.1016/j.fsi.2021.11.015

Yogeswari S, Ramalakshmi S, Neelavathy R, Muthumary J (2012) Identification and comparative studies of different volatile fractions from *Monochaetia kansensis* by GCMS. Glob J Pharmacol 6(2):65-71

Zahran E, El-Gawad EAA, Risha E (2018) Dietary *Withania sominefera* root confers protective and immunotherapeutic effects against *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol 80:641-650. https://doi.org/10.1016/j.fsi.2018.06.009

Zhang DF, Yuan W, Ke XL, Liu ZG, Cao JM, Lu MX, Wang M, Yi MM (2017) Molecular characteristics and transmission of *Streptococcus agalactiae* in a major tilapia culturing area of China. J Fish Sci China 24(03):606–614

Zhang X, Sun Z, Cai J, Wang J, Wang G, Zhu Z, Cao F (2020) Effects of dietary fish meal replacement by fermented moringa (*Moringa oleifera* Lam.) leaves on growth performance, nonspecific immunity and disease resistance against *Aeromonas hydrophila* in juvenile gibel carp (*Carassius auratus gibelio* var. CAS III). Fish Shellfish Immunol 102:430-439. https://doi.org/10.1016/j.fsi.2020.04.051

Zheng K, Wu L, He Z, Yang B, Yang Y (2017) Measurement of the total protein in serum by biuret method with uncertainty evaluation. Measurement 112:16-21. https://doi.org/10.1016/j.measurement.2017.08.013

Zofia NL, Martyna ZD, Aleksandra Z, Tomasz B (2020) Comparison of the antiaging and protective properties of plants from the *Apiaceae* family. Oxid Med Cell Longev 9:1-16. https://doi.org/10.1155/2020/5307614

**Publisher's Note**