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Efficacy of *Excoecaria agallocha* on hematological parameters in hybrid tilapia (*Oreochromis niloticus*) after experimental challenge with *Streptococcus agalactiae*

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Abstract

The potential of milky mangrove *Excoecaria agallocha* leaf extracts has been utilized in traditional medicine in various parts of the world. Blood is an important indicator of health and is a pathological mirror of the entire body. Therefore, the effects of *E. agallocha* leaf extract on hematology indices of hybrid tilapia (*Oreochromis niloticus*) were investigated. Experimental fish were randomly divided into seven groups. Groups 1 to 5 were fed medicated feed at five different concentrations (10, 20, 30, 40 and 50 mg kg^{-1}) of *E. agallocha* leaf extract. Group 6 was given Flumequine (25 mg kg^{-1}) and group 7 was fed with untreated feed (control) for 28 days before they were intraperitoneally exposed to $4x10^5$cfu CFU ml^{-1} *S. agalactiae*. The results revealed that the group fed medicated feed at 50 mg kg^{-1} was the most effective concentration and showed no significant difference (p>0.05) compared to control and antibiotic groups on blood parameters (red blood cell, globulin, total serum protein, mean cell hemoglobin, and mean cell hemoglobin concentration) with the highest survival rate 99.67% within experimental groups. Overall, our results indicated the potential of *E. agallocha* could prove to be a hematological profile enhancer which can help fish combat bacterial infections. It was concluded that increase in the white blood cell count observed in fish administered with *E. agallocha* leaf extract at 50 mg kg^{-1} suggests that *E. agallocha* extract contains agents that could stimulate the production of leucocytes and serve as an immune booster and prevent the risk of anemia.

Keywords: *Streptococcus agalactia,* Hematology, Hybrid tilapia, *Excoecaria agallocha,* Survival**.**

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Introduction

Major goals of the aquaculture industry are to maintain fish health as well as to improve fish performance for increasing of production. The use of plant extracts in practical diets for fish is a very important concept in the aquaculture industry. Therefore, medicinal plants are being increasingly reported to favour various activities for their antimicrobial properties. They create specific bioactive molecules that enable them to react with other organisms in the environment, resulting in inhibition of bacterial growth (Rai *et al*., 2010).

Milky mangroves *Excoecaria agallocha* have many bioactivities including; antioxidant, antibacterial, antiviral, and anticancer activities due to the presence of numerous phytochemical metabolites (Yin *et al.*, 2008; Vadlapudi *et al.*, 2009; Boopathy *et al.*, 2011; Batsa *et al.*, 2013; Laith *et al.*, 2014). The extracts of *E. agallocha* preparation have been suggested as being useful in the treatment of various diseases because of their possession of anti-oxidant agents and free radical scavenging efficiency (Thirunavukkarasu *et al.*, 2009; Laith *et al.*, 2016). The application of plant extracts to enhance disease resistance in animals is expanding at the present time (Takaoka *et al*., 2011). Previously, therapeutic applications of *E. agallocha* on diabetes mellitus was observed by Rahman *et al*. (2010) who used 400 mg kg^{-1} body weight of *E. agallocha* methanolic extract orally administrated to experimental mice and showed significant reduction in serum glucose level. Laith *et al*. (2010) recorded the antibacterial activity of *E. agallocha* against *Flavobacterium indicum*, *Chryseobacterium indologenes*, *Chryseobacterium gleum* and *Elizabethkingia meningoseptica*. Moreover, studies by Gultepe *et al*. (2014) revealed the effects of dietary thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*) and fenugreek (*Trigonella foenum graecum*) as a feed additive on hematology, innate immune response, and disease resistance of tilapia (*Oreochromis mossambicus*) challenged with *Streptococcus iniae* at concentration of 10^8 CFU ml^{-1}. Their results revealed that a dietary herbal extracts level of 1% provides the best survival rate for tilapia. On the other hand, Gabriel *et al.* (2015) investigated the herb extracts of *Aloe vera* at concentration 0.5% 1%, 2%, and 4% kg feed to tilapia for 8 weeks and challenged with *S. iniae*. Their results revealed significant improved hemato-biochemical parameters of tilapia.

Streptococcus agalactiae is an emerging pathogen associated with severe economic losses due to high mortality rates in fish farms worldwide (Duremdez *et al*., 2004; Mian *et al*., 2009). This pathogen has also been shown to compromise food safety and they represent a zoonotic hazard (Pereira *et al*., 2010). Sporadically, *S. agalactiae* has been associated with diseases in various other hosts including chickens, camels, dogs, horses, cats, frogs, hamsters, mice and monkeys. *Streptococcus* spp have been associated with fatal outcomes in tilapia

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making it an important fish pathogen in Malaysia (Marcel *et al*., 2013). The past several years showed numerous *S. agalactiae* infection outbreaks which is documented in several farms in Malaysia (Suanyuk *et al*., 2005; Najiah *et al.,* 2012) and having a mortality rate of 70% in Red hybrid tilapia (*Oreochromis niloticus*) in cages of Kenyir, Pedu and Pergau Lakes in Malaysia (Siti-Zahra *et al.,* 2004; Siti-Zahra *et al*., 2005; Amal *et al*., 2008).

Hematology is a tool which makes it possible to study organisms' physiological responses to pathogens. It assists in the diagnoses and prognoses of diseases among fish populations (Sebastiao *et al.,* 2011). The hematological parameters of fish may be important in relation to fish farming because of their potential to be used as indicators of physiological conditions and for monitoring diseases and the stress caused by handling (Sebastiao *et al.,* 2011). Although there are several plants whose extracts have been reported to have a potential impact on pathogenic bacteria, very few of them have been investigated for their potential on blood parameters after bacterial infection in addition to being safe and sustainable feed additives. Therefore, the present study was carried out to determine whether *E. agallocha* extracts would be influential on blood parameters of hybrid tilapia infected with *S. agalactiae*.

Materials and methods

Fish

Ten apparently naturally infected hybrid tilapia weighing 200-300 g were collected from farm ponds in Temerloh Pahang Province, Malaysia. Disease signs were observed and recorded. Ten fish with clinical signs were transferred in plastic bags with an oxygen supply to the Fish Health laboratory (AQUATROP) in University Malaysia Terengganu (UMT) for further study. The fish were anesthetized with Tricaine Methanesulfonate (MS-222), dissected following the methods of Wilson *et al*. (2009), and submitted for autopsy.

Bacterial isolates

Samples were taken for routine bacteriological examination from eye, brain, liver, spleen and kidney of hybrid tilapia. They were then inoculated onto brain heart infusion (BHI) agar (MERCK, Germany) and incubated at 30 °C for 24 h. The dominant colonies were sub cultured on the same media to check the purity of the isolate. After incubation at 30 °C for 24 h, bacterial colonies were picked and plated on blood agar (MERCK, Germany) plates until pure cultures were obtained. Pure stock isolates were stored at −20 °C in 15% glycerol (final concentration) supplied with BHI broth (Wang *et al*., 2013).

Biochemical characteristics of the isolates

Biochemical characteristics of the isolates were confirmed by microbial biochemical identification basis of standard phenotypic testing criteria, Gram stain, motility, oxidase activity, growth characteristics and hemolysis test. The phenotypic systems examined

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in this study using the VITEK 2 Systems Version: 5.04 ID card (bioMérieux, Inc., Hazelwood, MO) with reference to Berger's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Molecular approaches

The identified *Streptococcus agalactiae* from the eye was subjected to 16S rRNA gene PCR amplification by universal primers for confirmation of *S. agalactiae* (Evans *et al*., 2006a).

Bacterial culture and DNA extraction

The isolate was cultured in 3 ml of Tryptic soya broth (TSB, MERCK, Germany) overnight at 37 °C. The bacterial culture was centrifuged (14,000 ×g for 5 min at room temperature), pellet was harvested and total genomic DNA (gDNA) of the isolates were extracted using Wizard® Genomic DNA Purification Kit, A1120 (Promega, USA) according to manufacturer's protocol for gram positive bacteria. The extracted DNA was used as the template for PCR (Yang *et al*., 2013).

Polymerase Chain Reaction (PCR)

The PCR reaction mixture of 16s rRNA was done in 25 µl total reaction by using 2X MyTaq mix (Bioline, UK) with 10 µM of each primer. Negative control served as non-template mixture (Fay *et al*., 2016). The gDNA of the isolate was amplified for 16S rRNA by bacterial universal primers 8F (5'-GTTTACCTTGTTACGACTT-3') and 1492R (5'-AGAGTTTGATCCTGGATGCTCAG-3'). The PCR reaction was performed in a Biothermal cycler (Bio-Rad, USA) with an initial denaturing step at 95°C for 5 min; 26 cycles of 95°C for 30s, 55°C for 1 min and 72°C for 2 min; followed by 72°C for 10 min. Six microlitre of the amplified products were electrophoresed by 1.2% (w/v) agarose gel in 1x TBE electrophoresis buffer. Standard DNA ladder, 1kb and 100bp (Invitrogen, Germany) were used to confirm the size of the amplified PCR products at 1,500 bp. The gel was stained with ethidium bromide (Promega, USA) and documented by UV-transilluminator (Bio-Rad, USA). Sequences obtained were analysed and compared with sequences from GenBank using BLAST NCBI citation (http://blast.ncbi.nlm.nih.gov). The accession number of the *S. agalactiae* was deposited in Genbank.

Collection and extraction

The leaves of *E. agallocha* were collected from rural areas in Terengganu, Malaysia (5˚24'38.29˚ N and 103˚05'31.32˚ E). The plant was identified at the Plant Taxonomy Laboratory, University Malaysia Terengganu (UMT). The extract of *E. agallocha* was prepared by standardized procedure as described by Laith *et al* (2014).

Preparation of medicated feed

The LC_{50} value was 94.19 mg/ml, calculated by probit method as previously described by Laith (2014). The feed containing extracts was prepared before the experiment. Extracts were calculated according to

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animal weight, diluted in methanol and mixed with previously-weighed feed. After total methanol evaporation at room temperature, the feed was weighed once again. The number of pellets containing the crude extract was calculated according to the daily pellet consumption by the fish during the adaptation week. Averages of 3 to 4 pellets were administered containing the methanolic crude extract of *E. agallocha* in an adequate dosage for each fish.

Experiment lay-out

Hybrid tilapias weighing from 250 - 400 g were collected from a local cage-culture farm in Marang River Terengganu, Malaysia. Clinical and post mortem (P.M) examination were achieved through the methods described by Laith *et al*. (2016). The fish were divided into 7 groups, each containing 30 fish. Water properties (mean±SE) were measured daily as salinity 0.25±0.5 ppt, pH 7.2±0.2, temperature 26±2°C and dissolved oxygen 4.4±0.1 mg L^{-1}; photoperiod: light-dark cycle of 14:10 h. Tanks were provided with continuous aeration. Groups 1 to 5 were fed crude leaf extract of *E. agallocha* at the concentrations of 10, 20, 30, 40 and 50 mg kg^{-1} respectively. Group 6 was given antibiotics- Flumequine 25 mg kg^{-1}. Group 7 was considered as control group and fed normal feed (no additive of extract of plant). Groups 1 to 6 were injected with 0.1 ml^{-1} of bacterial culture of *S. agalactiae* at dose of $15x10^5$ CFU ml^{-1}. The fish were fed twice daily at the rate of 3% perbody weight with 3-5 mm dry pelleted diet (10 % carbohydrate, 20 % lipid, 55 % protein, 12 % ash and 3% vitamins and minerals) during acclimatisation for 14 days and experimental periods for 28 days and challenge test was done on day 29. Then fish were anesthetized with Tricaine Methanesulfonate (MS-222) and dissected (Wilson *et al*., 2009).

Determination of the median lethal dose (LD_{50})

Experimental Fish

Healthy juvenile hybrid tilapias were obtained from University Malaysia Terengganu (UMT) hatchery. Fish were randomly tested and screened to ensure that they were disease-free and pathogen-free. Juveniles weighed as 3.5±0.2 g with an average total body length of 6±2 cm. All fish were maintained in a 20 L fibreglass tank supplied with flow-through water at 0.5 L h^{-1} and held at 26 °C with aeration.

Prepare bacteria inoculum

The stock bacteria of *S. agalactiae* was first passed through healthy fish to potentiate its virulence and then grown on blood agar (Oxoid, U.K.) at 28-30 °C for 24 to 48 h. Bacterial cells were washed twice with physiological saline and then re-suspended in the same solution to obtain a bacterial suspension. The bacteria suspension was adjusted to McFarland turbidity standard No.5 equivalent to 15×10^8 CFU ml^{-1}. Ten-fold serial dilutions were done to obtain a *S. agalactiae* concentration of 15×10^4 CFU ml^{-1}. The fish were anesthetized using MS-222 and later divided into two groups: an

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infected group and a control group. One hundred micro litre of the bacterial suspension of *S. agalactiae* was injected intraperitoneally into each fish fed medicated feed and antibiotic, while, the control group injected with the same volume of physiological saline instead of the bacterial suspension.

Estimation of the dose range and percentage of mortality

The LD_{50} value was determined to obtain the lowest bacterial dose of *S. agalactiae*, which would cause 50% mortality in the hybrid tilapia population. Five doses were given by intraperitoneal injection to 5 groups of 10 hybrid tilapia per group (Table 1). The experiment was conducted in a 20 L aquarium supplied with adequate aeration. The environmental condition was maintained as optimum as possible. The temperature was kept at 26±2 °C, dissolved oxygen was 4.4±0.1 mg L, and pH was 7.2±0.2. The fish in groups 1, 2, 3, 4 and 5 were artificially infected by intraperitoneal injection with 0.1 ml of culture suspension of pathogenic *S. agalactiae* containing 15×10^8, 15×10^7, 15×10^6, 15×10^5 and 15×10^4 CFU ml^{-1}, respectively. The 6th group was the control group and injected with physiological saline. Fish mortality was recorded every 24 h for 5 days. Dead fish were removed from the aquarium daily. Probit analysis was used to determine 120 h LD_{50} using SPSS 16.0.

Table 1: Biochemical characteristics of *Streptococcus agalactiae* isolated from naturally infected hybrid tilapia (*Oreochromis* spp).

Characteristics	Result
Gram stain	Positive
Shape	Coccus
Motility	Negative
Oxidase	Negative
Catalase	Negative
Starch	Negative
Lactose	Negative
Esculin	Negative
Glucose	Positive
Blood hemolysis	β-Hemolytic

Blood collection

Blood samples were collected according to the method of Kori-Siakpere *et al.* (1997) from the caudal vein with the volume of 1 ml using sterile non-heparinised syringe. The blood samples were divided into 0.5 ml and immediately transferred into a tube containing EDTA (Ethylene Diamine Tetra-acetic Acid) as anticoagulant. Hematological parameters were then analysed including red blood cells (RBC, 10^6 mm^3), white blood cells (WBC, 10^3 mm^3), hemoglobin (Hb, g dl^{-1}), and hematocrit (PCV, %). Another 0.5 ml of blood sample was transferred into a test tube without anticoagulant. This test tube was placed on ice-chilled water for 2 h and blood was allowed to clot, and then centrifuged at 500×g at 4°C for 5 min. Serum aliquots were stored at -20°C until further utilisation

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in determining immune parameters such as total serum protein (TSP), albumin (A), and globulin (G). Blood smears were air dried, fixed in absolute methanol, and stained with Giemsa stain for 10 min, respectively. All run were carried out in triplicate for each sample.

Blood analysis

Determination of red blood cells (RBC, 10^6 mm^3) and white blood cells (WBC, 10^3 mm^3) were conducted using the Neubauer hemocytometer. Hematocrit (PCV, %) was acquired using the microhematocrit capillary tube (IRIS-USA). Hemoglobin (Hb, gdl^{-1}) concentration values were achieved according to Blaxhall *et al*. (1973) using the cyanomethaemoglobin method. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Baker (1982). Blood smears were stained with Giemsa stain to determine WBC and differential WBC counts according to Blaxhall *et al*. (1972). Total serum protein was analysed according to Lowry *et al.* (1951). Albumin content was detected according to the methods of Doumas *et al.* (1971), and globulin content was determined by subtracting the albumin values from the total protein values.

Data analysis

Data for each parameter was taken and the mean value was calculated as the total average. Data were subjected to analysis of variance (ANOVA) and the mean was compared with least significant difference (L.S.D) (p<0.05) using Gestate 12.1 program.

Results

Morphological and biochemical characteristics

The isolates were Gram-positive, cocci in chains bacteria. After incubation on brain heart infusion (BHI) agar (MERCK, Germany) at 30 ºC for 24 h, the colonies were raised and glossy, with a diameter of 1.5–2 mm. Biochemical characteristics of the isolates were consistent with *S. agalactiae* (Tables 1, 2).

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Table 2: Biochemical characteristics of suspected *Streptococcus agalactiae* isolated from naturally infected hybrid tilapia (*Oreochromis* spp) using Vitek 2.

Well	Biochemical Test	Result
2	D-AMYGDALIN (AMY)	–
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C (PIPLC)	–
5	D-XYLOSE (dXYL)	–
8	ARGININE DIHYDROLASE 1 (ADH1)	+
9	BETA-GALACTOSIDASE (BGAL)	–
11	ALPHA-GLUCOSIDASE (AGLU)	–
13	Ala-Phe-Pro ARYLAMIDASE (APPA)	–
14	CYCLODEXTRIN (CDEX)	–
15	L-Aspartate ARYLAMIDASE (AspA)	–
16	BETA GALACTOPYRANOSIDASE (BGAR)	–
17	ALHA-MANNOSIDASE (AMAN)	–
19	PHOSPHATASE (PHOS)	(+)
20	Leucine ARYLAMIDASE (LeuA)	+
23	L-Proline ARYLAMIDASE (ProA)	–
24	BETA GLUCURONIDASE (BGURr)	–
25	ALPHA-GALACTOSIDASE (AGAL)	–
26	L-Pyrrolidonyl-ARYLAMIDASE (PyrA)	–
27	BETA-GLUCURONIDASE (BGUR)	–
28	Alanine ARYLAMIDASE (AlaA)	–
29	Tyrosine ARYLAMIDASE (TyrA)	–
30	D-SORBITOL (dSOR)	–
31	UREASE (URE)	–
32	POLYMIXIN B RESISTANCE (POLYB)	+
37	D- GALACTOSE (dGAL)	+
38	D-RIBOSE (dRIB)	+
39	L-LACTATE alkalinization (ILATk)	–
42	LACTOSE (LAC)	–
44	N-ACETYL-D-GLUCOSAMINE (NAG)	+
45	D-MALTOSE (dMAL)	+
46	BACITRACIN RESISTANCE (BACI)	+
47	NOVOBIOCIN RESISTANCE (NOVO)	+
50	GROWTH IN 6.5 % NaCl (NC6.5)	+
52	D-MANNITOL (dMAN)	–
53	D-MANNOSE (dMNE)	+
54	METHYL-B-D-GLUCOPYRANOSIDE (MBdG)	+
56	PULLULAN (PUL)	–
57	D-RAFFINOSE (dRAF)	–
58	O/129 RESISTANCE (comp. vibrio) (O129R)	–
59	SALICIN (SAL)	–
60	SACCHAROSE/SUCROSE (SAC)	+
62	D-TREHALOSE (dTRE)	+
63	ARGININE DIHYDROLASE2 (ADH2s)	+
64	OPTOCHIN RESISTANCE (OPTO)	+

16S rRNA sequence analysis

The 16S rRNA sequence of the isolate was analyzed via BLAST network services. Sequence alignments with known sequences in the GenBank database showed that the brain isolate had high similarity (99.9%) to *S. agalactiae*. The DNA of the isolate was analyzed by PCR using the 16S rRNA universal primers that amplified fragments of approximately 1,500 bp in size. The sequencing result of the isolate showed the sequence length of the PCR product at 1,216 bp and was deposited in GenBank (Accession No. KT869025) (Fig. 1).

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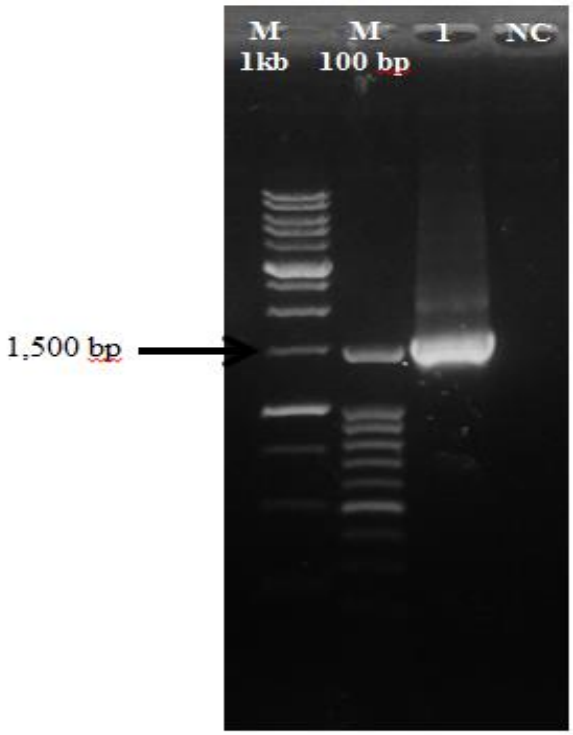


Figure 1: 16s rRNA PCR product of *Streptococcus agalactiae* (Lane 1) amplified at 1,500 bp with standard molecular weight marker (M 1 kb; M 100 bp) and negative control (NC).

Median lethal dose (LD_{50})

In the LD_{50} trial, fish in groups 1 to 5 showed clinical characteristics and mortalities such as lethargy and loss of appetite during 24h post-inoculation (hpi). Some fish died without any clinical signs. Mortality started within 24h (hpi) in groups 1 to 4 and cumulative mortality reached 30%. The fish injected with 10^8, 10^7, 10^6, 10^5, 10^4 CFU ml^{-1} bacteria showed 100, 70, 50, 20, 10% mortality, respectively. The calculated LD_{50} of the isolated bacteria was $4x10^5$ CFU ml^{-1} (Table 3).

Table 3: Mortality recorded in Juvenile red hybrid tilapia (*Oreochromis niloticus*) injected intraperitoneally with *Streptococcus agalactiae*.

		Post-infection (hours)						
Group	**CFU ml^{-1}**	**24**	**48**	**72**	**96**	**120**	**Total no. Dead**	**Mortality (%)**
1	10^8	10	0.0	0.0	0.0	0.0	10/10	100
2	10^7	3	2	1	1	0.0	7/10	70
3	10^6	1	2	1	1	0.0	5/10	50
4	10^5	1	1	0.0	0.0	0.0	2/10	20
5	10^4	0.0	1	0.0	0.0	0.0	1/10	10
6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mortality	Per day	30%	12%	4%	4%	0%		

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Blood parameters

The effect of different concentrations of *Excoecaria agallocha* methanolic crude extract on blood parameters was significantly difference. In general, significantly increased was observed in albumin and monocytes but decreased the percentage of hemoglobin, packed cell volume, erythrocytes, and globulin. In the present study, the result revealed that the application of 50 mg kg^{-1} *Excoecaria agallocha* showed no significant difference as compared to the control and antibiotic groups on the blood parameters (red blood corpuscular, globulin, total serum protein, lymphocytes, neutrophil, MCH, and MCHC). On the other hand, application of 50 mg kg^{-1} *Excoecaria agallocha* significantly increased the percentage of monocytes in comparison to control and antibiotic groups. Furthermore, it was increased by about 36.62 % and 23.88% as compared to the control and antibiotic groups, respectively (Fig. 2).

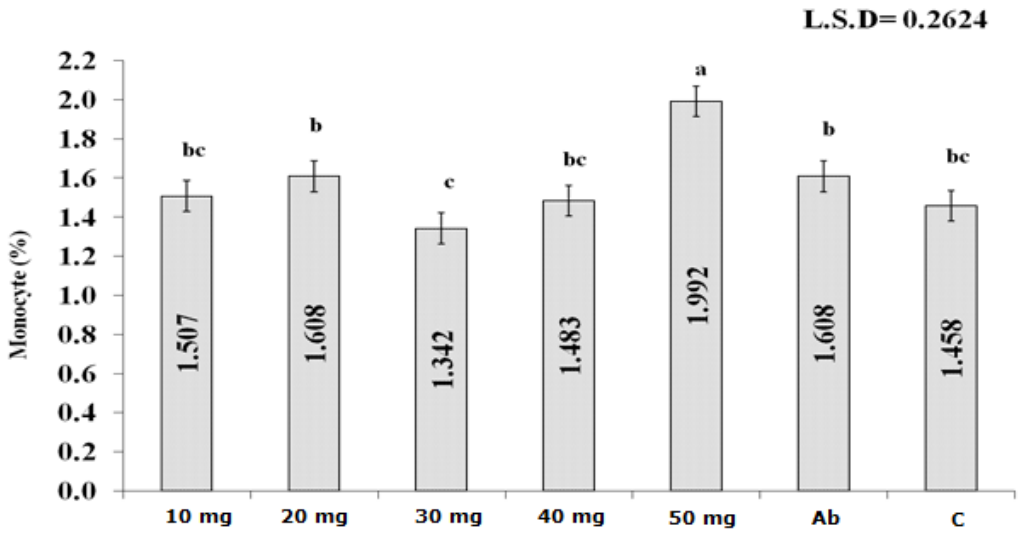


Figure 2: The effect of different levels of *Excoecaria agallocha* on the percentage of monocytes (%). Columns with the different alphabets are considered a significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

The value of WBC in the application of 50 mg kg^{-1} *Excoecaria agallocha* showed significant difference as compared to the antibiotic group. Furthermore, it was higher by about 3.90 % and 3.02% as compared to the antibiotic and control groups, respectively (Fig. 3).

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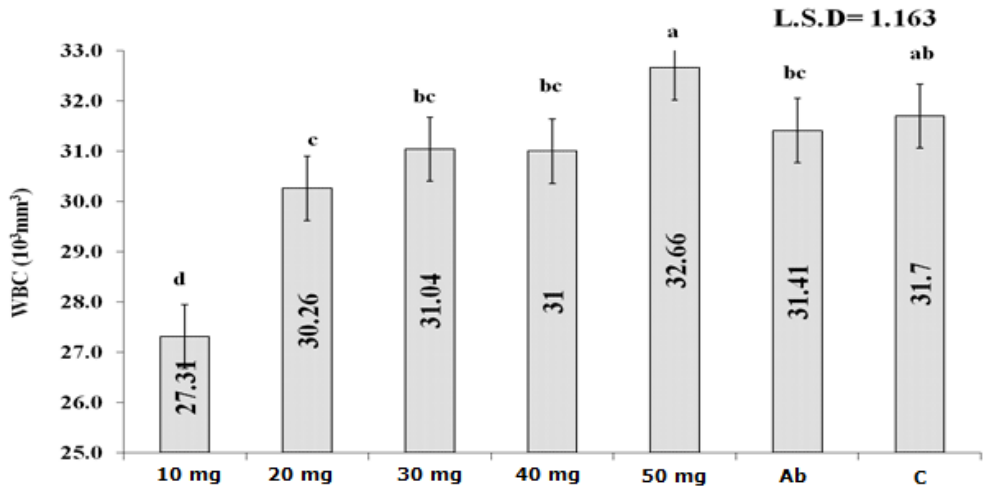


Figure 3: The effect of different concentrations of *Excoecaria agallocha* on the percentage of WBC (10^3 mm^3). Columns with the different alphabets are considered a significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

Although the application of 50 mg kg^{-1} *Excoecaria agallocha* significantly increased the percentage of albumin by about 18.87% and 9.82% as compared to the control and antibiotic groups, respectively (Fig. 4).

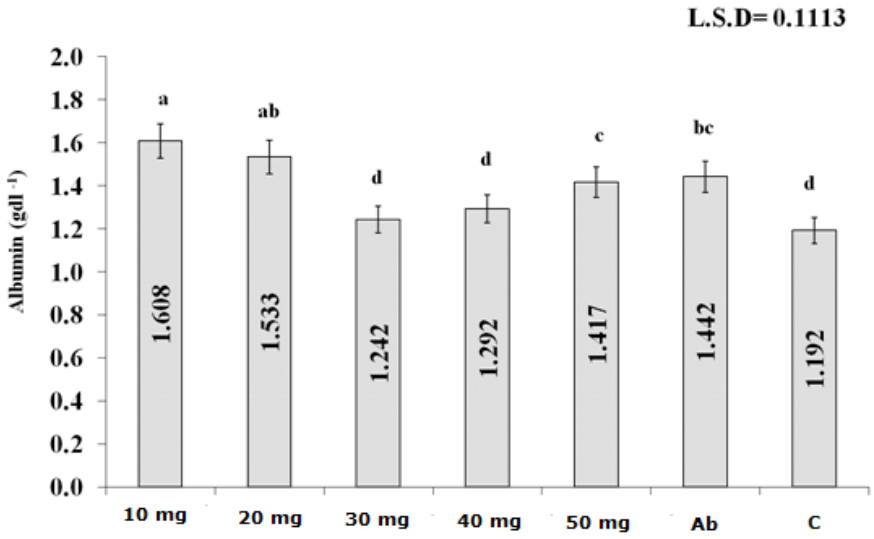


Figure 4: The effect of different concentrations of *Excoecaria agallocha* on albumin levels (gdl^{-1}). Columns with different letters are considered a significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

On the other hand, there is a significant decrease in Hb and PCV levels by about 5.78%, 6.04% and 9.06%, 9.73% as compared to the control and antibiotic groups, respectively (Figs. 5, 6).

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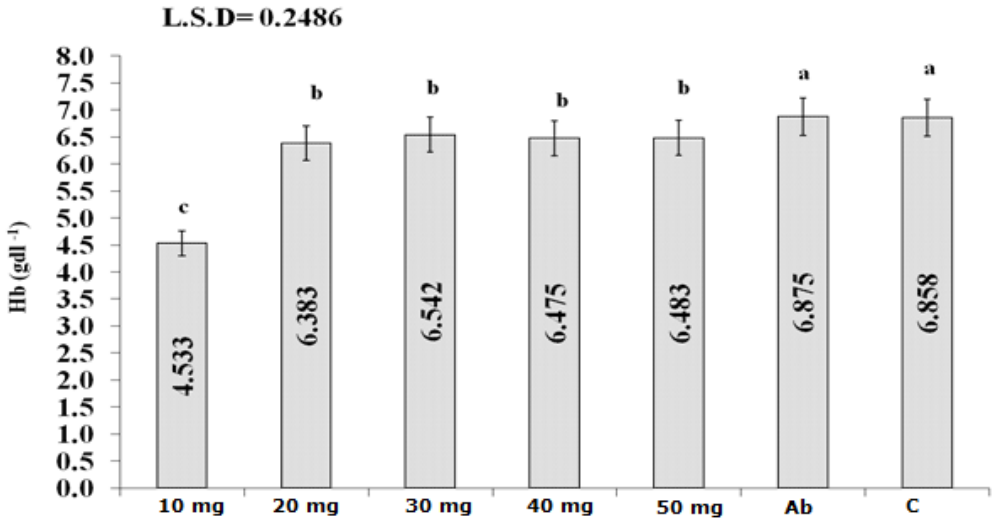


Figure 5: The effect of different concentrations of *Excoecaria agallocha* on the hemoglobin (g dl^{-1}) levels. Columns with the different alphabets are considered a significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

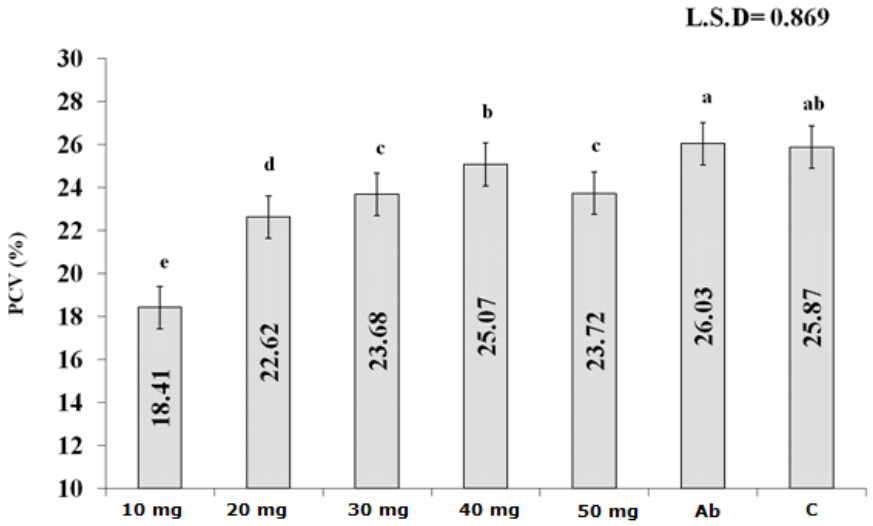


Figure 6: The effect of different concentrations of *Excoecaria agallocha* on the percentage of Packed Cell Volume (PCV %). Columns with the different alphabets are considered a significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

In addition, there was no difference between application of 50 mg kg^{-1} *Excoecaria agallocha* with antibiotic and control groups in the number of RBC (Fig. 7).

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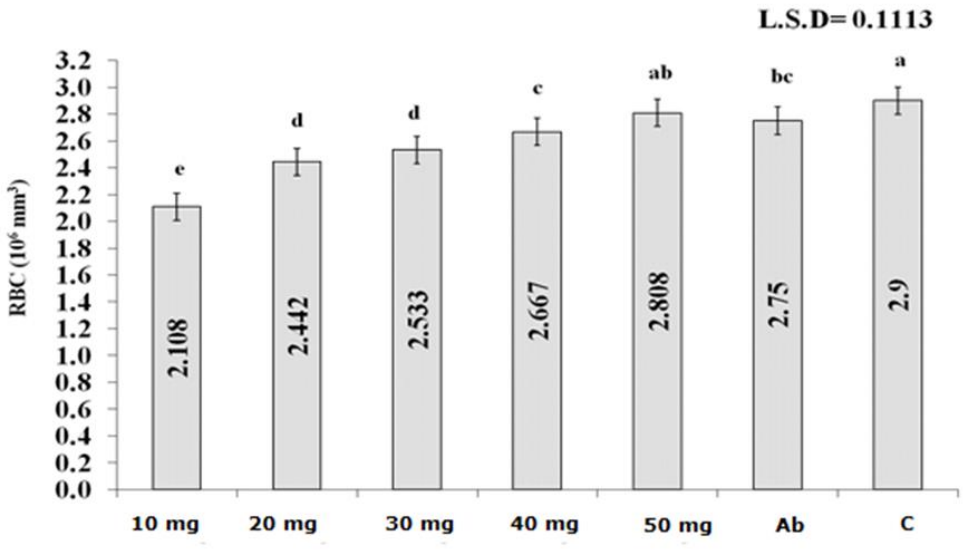


Figure 7: The effect of different concentrations of *Excoecaria agallocha* on number of red blood cells (RBC 10^6 mm^3). Columns with the different alphabets are considered a significant difference (p<0.05).
Ab-: antibiotic Group C-: Control

Application of 50 mg kg^{-1} *Excoecaria agallocha* significantly decreased the value of MCV (u^3) by about 4.81% and 11.30% as compared to the control and antibiotic groups, respectively (Fig. 8).

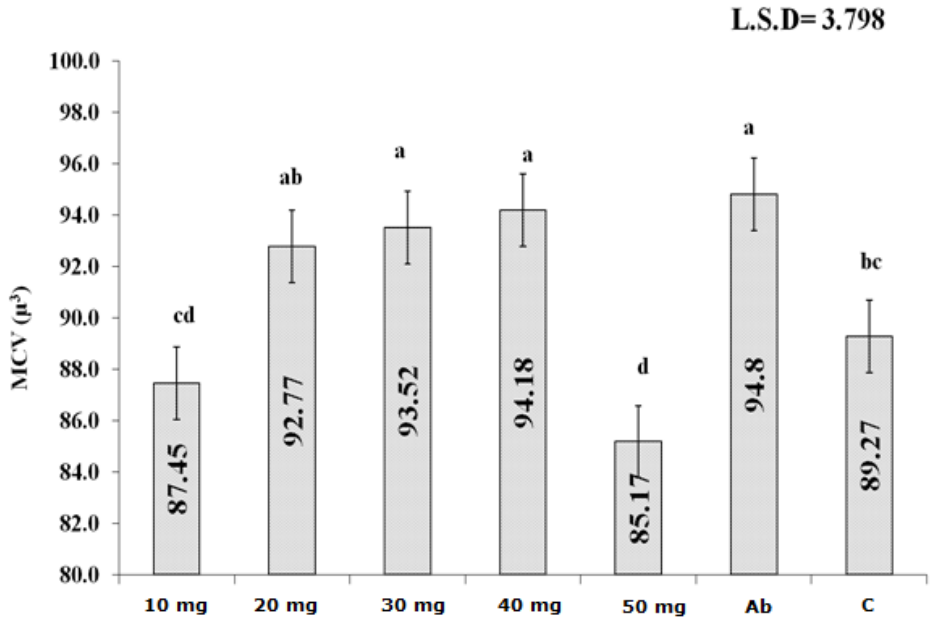


Figure 8: The effect of different concentrations of *Excoecaria agallocha* on mean corpuscular volume (MCV). Columns with different alphabets are considered as significant difference (p<0.05).
Ab-: Antibiotic group C-: Control group

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Application of all the treatment groups, including antibiotic group, showed a significant increase in the A/G ratio as compared to the control group (Fig. 9).

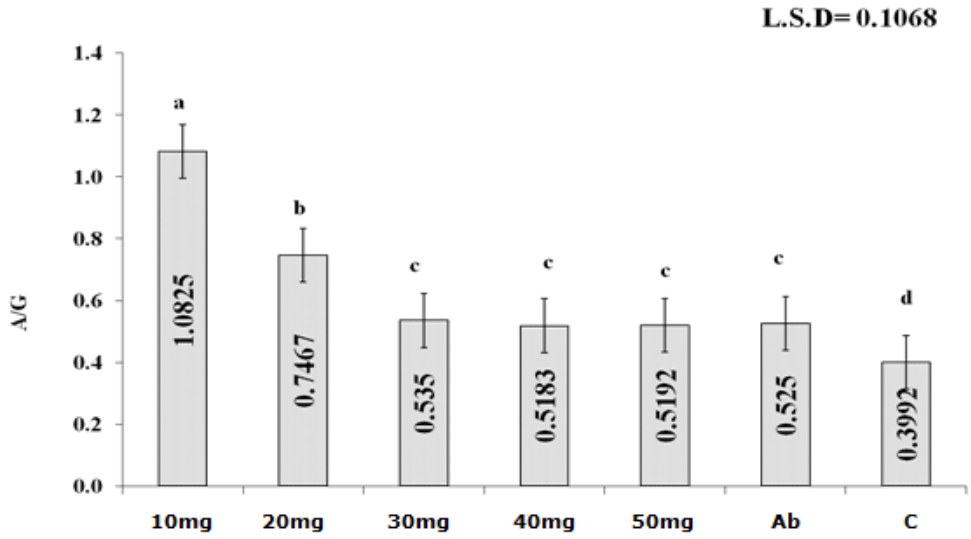


Figure 9: The effect of different concentrations of *Excoecaria agallocha* on the A/G ratio. Column with the different alphabets considered as significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

Survival rate

Survival rate was the highest in fish fed with 50 mg kg^{-1} *Excoecaria agallocha* (p<0.05) compared with the experimental groups of 10, 20, 30, and 40 mg kg^{-1} after challenge, but there were no significant differences among the control group (C) and antibiotic group (AB). However, fish fed with 50 mg kg^{-1} *Excoecaria agallocha* showed high value on survival rate against pathogenic bacteria during the study at 99.67% (Fig.10).

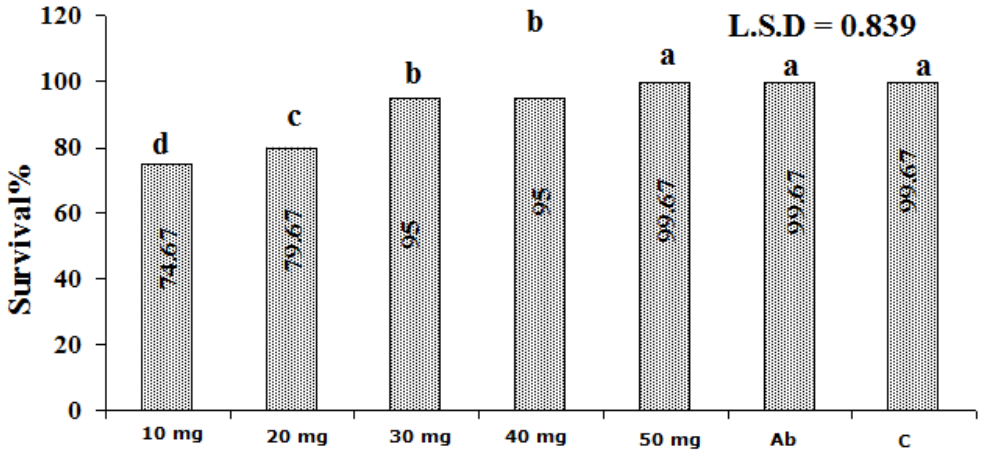


Figure 10: The effect of different concentrations of *Excoecaria agallocha* on survival rate (%). Columns with the different alphabets are considered as significant difference (p<0.05). Ab-: Antibiotic group C-: Control group

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Discussion

The results of this study suggest that treatment with crude extract of *E. agallocha* as medicated feed increases disease resistance in hybrid tilapia. In the present study, it was revealed that the higher dose (50 mg kg^{-1}) of crude extract of *E. agallocha* is beneficial to improve the overall performance of fish; however, the lower dose (10 mg kg^{-1}) do not influence *Streptococcus agalactiae* count after infection. In addition, the results of present work indicate a significant difference between the treatment groups; this may be attributed to the mangrove plant extracts and their antimicrobial activity against pathogenic bacterial strains. The observation of such antibacterial activity is due to the active components which are present in plant extracts. Our results were in accordance with the previous study of Rattanachaikunsopan *et al*. (2009) who investigated using feed supplemented with the herb *Andrographis paniculata* as a treatment regimen against *S. agalactiae* infections in Nile tilapia (*Oreochromis niloticus*). However, none of the previous research in this field revealed the potential of *E. agallocha* leaf extract as a blood enhancement agent in the aquaculture industry. Therefore, the present work could possibly be the first report on the potential of *E. agallocha* leaf extract to improve blood parameters in diseased fish.

Blood operates as a pathological marker for organisms (Omotoyin *et al*., 2006). Hence, the haematological parameters in this study were used to evaluate the effect of different concentrations of methanolic crude extraction of *E. agallocha* on hybrid tilapia. The results revealed different degrees of effectiveness of *E. agallocha* on hematological values of hybrid tilapia after the period feeding for 28 days. The reduction was observed in the values of RBC, Hb, and PCV may be due to the pathogenicity of *S. agalactia*, in agreement with the result of study by Suwannasang *et al.* (2014) which reported that RBC, Hb, and PCV values were lower in Nile tilapia infected with *S. agalactiae* compared to control group. The crucial role of WBC in defending the body against infection and tissue damage is well known. Moreover, the production of more WBC indicates an improvement of the health status of the acutely infected fish (Adewaye *et al*., 2005; Gabriel *et al.*, 2007). This study revealed that WBC count increased significantly in the group administered 50 mg kg^{-1} of *E. agallocha* leaf extract, which would be attributed to the organisms body defence against infection and tissue damage. Furthermore, PCV is an important tool for detecting the amount of plasma and corpuscles in the blood and is utilized to determine the oxygen-carrying capacity of blood cells (Larsson *et al.*, 1982). Hence, reductions PCV and RBC were also observed during the exposure period and believed to be as a result of the destruction of erythrocytes by *S. agalactiae* toxin. The group administered 50 mg kg^{-1} leaf extract showed a significant increase in RBC and may be influenced by dietary treatments (Aleter *et al.*, 1998). There

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was a decrease in hemoglobin levels and this could possibly be linked to depression and exhaustion of the hemopoietic capability of the fish (Sawhney *et al*., 2000) or could be attributed to the inadequate intake or absorption of iron, as a result of acute hemorrhage induced by *S. agalactiae* infections in fish. There were no significant changes in MCV, MCH and MCHC values in the treatment group when compared with control, which could be due to either hypoxia or microcytic anemia, given that the diagnosis of anemia in animals relies on blood indices such as MCV, MCH and MCHC (Coles, 1986). Furthermore, our results indicate that the experimental fish in the group administered 50 mg kg^{-1} feed extract have been normocytic anemia, which is in agreement with Ford *et al*. (2013) who reported the anemia may be normocytic normochromic as a result of the acute loss of blood cell mass as seen in hemorrhage and hemolysis. Therefore, this condition may be related to the beneficial properties of *E. agallocha* due to one or more phytochemical constituents (Laith *et al*., 2015). Moreover, the highest survival rate (99.67%) was observed in the group administered 50 mg kg^{-1} feed extract. This is in agreement with the findings by previous studies that tilapias fed a diet including *Cinnamomum verum*, *Andrographis paniculata* (Rattanachaikunsopan *et al.*, 2009), *Rosmarinus officinalis* (Zilberg *et al.*, 2010) and herbs and spice increased the survival rate against streptococcal challenge.

The concentration of 50 mg kg^{-1} of crude extract of *E. agallocha* could be used to stimulate blood parameters and impact the infections caused by *S. agalactiae* of hybrid tailapia. This suggests that the use of this extract, especially at high doses, could suppress the hemopoetic system.

It can be concluded that *E. agallocha* leaf extract causes significant changes in hematological characteristics of infected tilapia, having demonstrated the ability to combat pathogens and improve overall health during an infectious state in fish.

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