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Effect of aqueous extract of *Polygonum minus* leaf on the immunity and survival of African catfish (*Clarias gariepinus*)

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ABSTRACT

Objective: To establish immunomodulatory potential of aqueous extract of *Polygonum minus* (*P. minus*) leaves, the haematological effects and lysozyme activity of aqueous extract of *P. minus* leaf on *Clarias gariepinus* was studied.
Methods: The fish were grouped and administered with the *P. minus* aqueous leaf extract intraperitoneally. Blood and serum samples were collected from each group and examined for various blood parameters. The turbidimetric assay for lysozyme activity using *M. luteus* and the survival rate of fish against *A. hydrophila* was carried out.
Results: There was no significant impact (P>0.05) on white blood cell count, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and lysozyme activity was significantly (P<0.05) higher than control. The serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase levels were significantly lower than control (P<0.05). The highest dose group showed longest survival.
Conclusions: From this study, it is considered that the aqueous extract of *P. minus* can be used as an immunostimulant in African catfish, but continuous administration may require maintaining the protection.

KEYWORDS
Haematological effect, African catfish, *Polygonum minus*, Aqueous extract, *Clarias gariepinus*, Lysozyme activity, Immunomodulatory activity

PEER REVIEW

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Comments
This is a research work in which authors have illustrated haemato-immunological effects of *P. minus* on African catfish (*C. gariepinus*). They indicated that dose of 15 or 10 mg/kg of *P. minus* aqueous leaf extract had significant stimulatory effects on the non-specific immune mechanisms and disease resistance in *A. hydrophilia*.
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1. Introduction

Polygonum s.l. (Polygonaceae) is cosmopolitan genus of about 170 species, which are mainly distributed in the temperate northern regions with few species in the tropical and subtropical regions[1]. It is originated from Southeast Asia countries namely Malaysia, Thailand, Vietnam and Indonesia. It grows wild in damp areas near the river banks, ditches and lakes. It survives well on cool and hilly area[2]. This plant is locally named as 'kesum' in Malay and often consumed as ulam (raw medicinal plants consumed with daily meals) for preventive health care. In the Malaysian Traditional Medicine System, the decoction of kesum leave is recommended for digestive disorders and stomach pain[3]. *Polygonum minus* (*P. minus*) has a kind of sweet and pleasant aroma and is commonly used amongst the Malaysian population as a flavouring ingredient in Malaysian food, Laksa[2]. *P. minus* was chosen for our research purpose mainly because of its enormous use in traditional medicine. Studies have reported that *P. minus* possess antioxidant activity[3,4], antimicrobial activity[5], antiulcer activity[6], cytotoxicity and antiviral activity[7,8].

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There is no published report on immunomodulatory effect of *P. minus* leaf extract on catfish.

Use of expensive chemotherapeutic agents and antibiotics for managing various infections has been widely criticized for their pessimistic impact like residual accumulation in the tissue, development of resistance and immune-suppression, thus resulting in reduced consumer preference for fish as a food treated with antibiotics[9]. Prevention of disease is much more desirable than intervention to stop and reverse the disease process once it begins.

This approach could be achieved with application of vaccines, which can enhance the specific immune response of the fish and are considered to be the most effective agents. The available commercial vaccines are highly expensive for fish farmers and they are specific against particular species[10]. In contrast to vaccines, immunostimulants enhance the non-specific immune response of fish[11,12]. The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, like lysozyme[13-15]. Several biological and synthetic compounds have been shown to enhance non-specific immune system of cultivated fish[12,16-23].

Hence, instead of chemotherapeutic agents and vaccines, increasing attention is being paid to the use of immunostimulants as a disease control measure in aquaculture. *Aeromonas hydrophila* (*A. hydrophila*) has been reported to cause fin rot disease in hatchery reared *Cyprinus carpio* fingerlings under temperate climatic conditions of Kashmir valley and had recommended the use of multiple antibiotics as a bath for controlling fin rot disease[24]. However, as mentioned above, controlling diseases with antibiotics is not a safe procedure in aquaculture practices. There was no published report on immunomodulatory effect of *P. minus* leaf extract on catfish. Based on the observations from literature survey, it was envisaged to study the various haematological and immunomodulatory effect of the water extract of *P. minus* leaf in African catfish, *Clarias gariepinus* (*C. gariepinus*).

2. Materials and methods

2.1. Extraction of plant materials

Fresh leaves of *P. minus* were collected from the Penang region of Malaysia, air-dried at room temperature (25±2) °C for 2 weeks, then grinded and sieved through sieve No. 20 to get uniform powder. Then 150 g of the powder was extracted (agitated in an orbital shaker at 185 r/min for 72 h) with 600 mL of distilled water in six separate conical flasks with each conical flask contains 25 g of powder. The water extracts were then filtered using muslin cloth and the filtrate was concentrated using rotary vacuum evaporator. Phytochemical screening was performed using standard procedures.

2.2. Experimental fish and husbandry

Healthy African catfish with average weight (200±25) g, were procured from fish market, Sungai Petani, Kedah Darul Aman, Malaysia. The fish were kept acclimatized for a period of 5 d at (25±2) °C in circular cement tanks (400 L) and fed with formulated catfish diet pellet once daily. Water exchange was done daily by replacing 80% of the tank water and water quality was monitored throughout the experiment.

2.3. Preparation of injection dose

The extract powder was properly weighed in two quantities such as 375 mg and 250 mg, dissolved separately in 25 mL of distilled water, filtered through 0.45 μm membrane filter and labeled as high dose (15 mg/kg) and low dose (10 mg/kg) respectively.

2.4. Experimental design

African catfish (*n*=108) were selected for the study and divided into 3 groups (A, B and C). Each group is consisting of 36 fish, which was again divided into three equal subgroups. Group A (control) was injected with distilled water, Group B and C were injected with low and high dose of plant extract respectively. Feed was withheld before blood sample were collected. Eighteen fish from each group (6 fish from each subgroup) was randomly picked for blood withdrawal at two days intervals. Blood was collected from caudal vein of catfish with the help of needle. Blood and serum samples were examined for the following parameters: total protein, albumin, globulin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), packed cell volume (PCV), white blood cells (WBC), serum alkaline phosphatase, haemoglobin and serum lysozyme activity.

2.5. Lysozyme activity and challenge on survival of fish

A virulent strain of *A. hydrophila* and *Micrococcus luteus* received from Faculty of Biotechnology, AIMST University was used in this study. The bacterial suspension (1×10^7 CFU/mL) was prepared by using the method reported by Sahu *et al*.[25] and used for the challenge experiment and lysozyme activity. The turbidimetric assay for lysozyme was carried out according to Parry *et al*[26].

Thirty African catfish were taken for this study and divided into three groups containing equal number of fish. After the administration of plant extract, fish were challenged intraperitoneally with virulent *A. hydrophila* (1×10^7 CFU/mL, 0.2 mL) on Day 2 and observed for a 10-day period for mortality.

3. Results

WBC count was higher in both the treated groups than the control but not significant ($P>0.05$) (Table 1). The RBC count increased with the administration of *P. minus*. Haemoglobin level in group C was significantly ($P<0.05$) higher as compared to group A on both days, but non-significantly higher in group B on both days. MCV and MCH content was significantly ($P<0.05$) higher in group B and C as compared to group A on both days (Table 1). MCHC count was significantly ($P<0.05$) higher in group C on day 2 and 4 as compared to group A.

There was no significant (P>0.05) difference on both days in group B when compared to group A. There was significant (P<0.05) level of increment in PCV level in both treatment groups on both days compared to control.

The serum total protein, albumin and globulin level in treated groups was not significantly (P>0.05) different from control, but the amount is higher than the control. Alkaline phosphatase level was significantly (P<0.05) higher in both treated groups compared to control. SGOT and SGPT level was significantly (P<0.05) lower in both treated groups compared to control (Table 1).

Table 1

Haematological and immunomodulatory parameters observed on different assay days after injection of *P. minus* aqueous leaf extract.

Parameters	Group A		Group B		Group C	
	2nd day	4th day	2nd day	4th day	2nd day	4th day
WBC (1 000 cells/mm)	2.00±0.24	2.00±0.25	2.07±0.20	2.23±0.15	2.27±0.06	2.37±0.23
RBC (1000000 cells/mm)	2.20±0.10	2.20±0.10	2.37±0.21	2.40±0.26	2.50±0.10	2.60±0.20
Hb (g/L)	72.00±8.53	75.33±7.37	80.00±2.65	86.67±3.79	98.67±8.06	101.33±3.05
MCV (fL)	116.33±3.51	115.67±4.73	123.00±2.00	124.33±1.53	125.67±4.04	128.67±3.51
MCH (pg)	36.33±1.53	37.00±2.00	42.00±1.73	44.33±1.53	43.33±1.53	45.67±2.52
Tp (g/L)	40.33±3.51	39.00±3.36	43.33±2.08	42.33±2.08	45.00±3.57	40.67±3.13
Ap (U/L)	12.33±0.57	13.67±1.55	16.00±1.73	20.33±1.53	17.67±1.53	24.33±1.51
SGOT (U/L)	247.67±11.37	383.67±5.31	185.67±8.96	321.67±10.50	131.67±5.01	136.67±2.52
SGPT (U/L)	47.33±3.51	46.33±2.08	37.33±3.51	33.00±3.61	34.00±3.61	25.67±3.78
LA	58.33±14.43	58.33±14.43	125.00±25.00	91.67±14.43	166.67±14.43	133.33±14.43

Values are means of triplicate±SE, n=6 per group. Group A: control; Group B: received 10 mg/kg of *P. minus* aqueous leaf extract; Group C: received 15 mg/kg of *P. minus* aqueous leaf extract. Ap: Alkaline phosphatase; Tp: Total protein. The values given in the parenthesis are standard error of three determinations. These values are within 90% confidence interval.

Lysozyme activity in the serum of *P. minus* aqueous leaf extract injected groups was significantly (P<0.05) higher at all sampling times, when compared with the control group. Highest lysozyme activity [(166.67±14.43) U/mL] was observed in group C fish on Day 2.

After administration of fish with *A. hydrophila*, the mortality was recorded for 10 d. There was no mortality of fish up to 24 h. The group of fish injected with different concentration of *P. minus* leaf extract (group B and C) showed higher survival percentage when compared with control (group A). Mortalities due to *A. hydrophila* first occurred in control fish reaching almost 50% after 3 days of post–infection. Fish treated with *P. minus* leaf extract only started to die after 2 days of post–infection. The highest survival (90.48±2.749)% was shown in group C (Figure 1). The relative percentage survivability of group B was 36.36 and group C was 81.82.

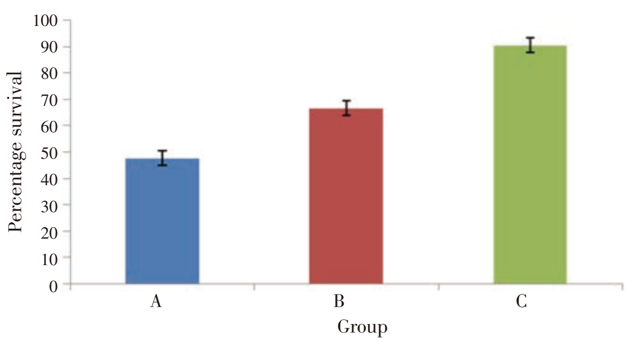

Figure 1. Effect of water extract of *P. minus* on survivability (percentage) of African catfish against bacteria.

Values are means of triplicate±SE, n=6 per group). Group A: Control; Group B: 10 mg/kg of water extract of *P. minus* leaf; Group C: 15 mg/kg of water extract of *P. minus* leaf.

4. Discussion

P. minus has primarily been described as antioxidant, antimicrobial, antiviral, antiulcer and cytotoxic. Hence, the present work focused on the study of immunomodulatory activity of water extract of *P. minus* leaf in African catfish. The percentage yield of crude water extract of *P. minus* leaf was 15%. The phytochemical screening of the water extract of *P. minus* showed the presence of reducing sugar, terpenoids, flavonoids, saponins and tannins. These phytochemical constituents may play a crucial role in the immune system stimulation and in the function of organs related to blood cell formation such as thymus, spleen, and bone marrow as reported by Jeorg and Lee[27].

Even though, the significant increase (P<0.05) in RBC and haemoglobin level, non–significant increase in WBC count observed in this present study agree with the works of Iranloye (2002)[28] and Martins *et al.* (2002)[29] who reported increases in total leucocyte count, neutrophils, lymphocytes, monocytes, RBC and haemoglobin concentration in rats when fed with garlic containing diet. Sahu (2004)[30], Duncan and Klesius (1996)[31] and Salah *et al.* (2008)[32] observed significant (P<0.05) increase in erythrocytic count in fish fed with a diet containing β–glucan and garlic. WBCs play an important role in non specific immunity and their count can be considered as an indicator of the health status of fish.

The significant (P<0.05) increase observed in the PCV of fishes in this present study, concur with that of Martins *et al.* (2002)[29] and Salah *et al.* (2008)[32], however, contradicts the results of Banerjee and Maulic (2002)[33], Adebolu *et al.* (2011)[34] and Ugwu and Omale (2011)[35], observed significantly decreased PCV values in the treated rats when compared with that of the control. Meanwhile, red cell indices (MCV, MCH and MCHC) on the other hand, are particularly important for the diagnosis of anemia in humans and most animals. Significant increases (P<0.05) in these red cell indices as observed in the treated fishes in this current study is not agree with the findings of Martinez *et al.* (2007[36]. It is assumed that the decrease or increase of blood indices may be attributed to a defense reaction against *P. minus*, which occurs by stimulation of erythropoiesis.

Increase in serum protein, albumin and globulin levels are thought to be associated with a stronger immune response of fish[37]. The change in level of alkaline phosphatase, SGOT and SGPT may be due to the hepatoprotective effect of the plant.

Lysozyme is an important fish defense factor, which prevents biofilm formation by adherence and colonization of microorganisms. The total lysozyme level is a measurable humoral component of the non–specific defense mechanism[38]. Taoka *et al.* (2006)[39] found that the probiotics treatment enhanced the non–specific immune parameters such as the lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in the improvement of fish resistance against *Edwardsiella tarda* infection. However, the levels of lysozyme activity in trout fed with different levels of glucan did not differ[40]. In the present study, the lysozyme activity of African catfish that received *P. minus* aqueous leaf extract at 10 and 15 mg/kg increased within 2 d (highest lysozyme activity [(166.67±14.43) U/mL] was observed in group C fish on day 2), but decreased significantly after 3 days of treatment. Robertson *et al.* (1990)

[41] reported an increased protection against fish bacterial infection, which correlated with an increment in serum lysozyme levels, phagocytic activity and bactericidal activity of head kidney leucocytes. So this results concerning lysozyme activity support the observations that humoral factors may enhance phagocytosis in fish[42].

Administration of medicinal plant extracts has been reported to enhance the immune ability of the fishes. In the present study, African catfish injected with water extract of *P. minus* at 10 and 15 mg/kg displayed an increase in resistance to *A. hydrophila* after 2 to10 days of challenge. The best survival rate [(90.48±2.749)%] was observed in group C. The relative percentage survivability of group B was 36.36 and group C was 81.82. Therefore, the water extracts of *P. minus* at 15 mg/kg or less showed positive effects on preventing *A. hydrophila* infections.

In conclusion, the present results suggest that African catfish which received aqueous extract of *P. minus* at a dose of 15 mg/kg or less exhibited a significant stimulatory effect on the non–specific immune mechanisms tested, and disease resistance against *A. hydrophila*. Further purification of the active compounds and their evaluation may substantially improve quality as well as their usage in the culture system. It is considered that aqueous extract of *P. minus* can be used as an immunostimulant in African catfish, but continuous administration may be necessary to maintain the protective response. Also further investigation on the immunostimulatory effect of this plant preparation when administered along with feed (which is the preferred route of administration in the field) for disease prevention in aquaculture is warranted.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Herbal immunostimulants are substances which may render fishes more resistant to infectious diseases, by stimulating phagocytic cells as well as complement lysozyme and antibody responses of fish. In this research, the effects of *P. minus* aqueous leaf extract was studied on it effects on the haematoimmunological indices and survival rate of African catfish (*C. gariepinus*).

Research frontiers

The present study illustrates the effects of various doses of aqueous extract of *P. minus* leaf on the immunity responses, haematology indices and survival rate of African catfish (*C. gariepinus*).

Related reports

P. minus is a perennial herb of the Polyconaceae family. There is a lack of evidence in support of *P. minus* for any clinical indication. *P. minus* has been studied as antioxidant and antimicrobial in refrigerated duck meatballs. Antioxidant effect of *P. minus* may help to improve the immune system in the body.

Innovations and breakthroughs

P. minus is locally known as kesum, has antioxidant effects thus may be a potential source of natural antioxidants. This study investigated the potency of Polygonum minus as immunostimulant in fish. The objective of this study was to determine whether *P. minus* is sufficiently potent to protect immune system of fish.

Applications

P. minus has been studied as antioxidant and antimicrobial in refrigerated duck meatballs. *P. minus* is likely safe when eaten in normal amounts in the diet. It must be cautious when consuming the leaves of *Polygonum* plants, due to their oxalic acid content which may cause kidney stone formation.

Peer review

This is a research work in which authors have illustrated haemato–immunological effects *P. minus* on African catfish (*C. gariepinus*). They indicated that dose of 15 or 10 mg/kg of *P. minus* aqueous leaf extract had significant stimulatory effects on the non–specific immune mechanisms and disease resistance in *A. hydrophilia*.

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