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Original Article

EFFECTS OF EXTRACTS OF *STOCHEOSPERMUM MARGINATUM* AND *ULVA LACTUCA* ON THE HAEMATOLOGICAL AND IMMUNOLOGICAL PARAMETERS ON *AEROMONAS HYDROPHILA* INFECTED *CYPRINUS CARPIO*

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ABSTRACT

Objective: Marine algae are the rich source of natural products with pharmacological and biological activities. In the present study the seaweeds *Stocheospermum marginatum* and *Ulva lactuca* extracts were used as a immuno stimulants of non-specific immune response in common carp were studied.

Methods: The seaweeds *Stocheospermum marginatum* and *Ulva lactuca* chosen for the study and were extracted with ethanol. The fishes common carp (*Cyprinus carpio)* (weight 10±5g)] were divided into five groups. They were infected with *A. hydrophila* (1.6×10^4 CFU/fish). The infected fishes were injected with seaweeds extracts (0.30 mg) suspended in saline solution. One group was injected only saline (control) and one group was injected with ciprofloxacin (0.30 mg) (standard) suspended in saline. The experiment was carried out for 28 d. In every seven days interval the fishes were injected with seaweed extract and blood parameters of RBC, WBC, HB, Ht, MCV, MCH, and MCHC were recorded.

Results: RBC was recorded on 28th day in group 3 ($2.86\pm0.11\times10^6$ cells/mm^3) followed by group 4 ($2.13\pm0.04\times10^6$ cells/mm^3). Similarly the WBC values also increase from initial day to final day.

The phagocytic assay was same on 28th day in group 3 and group 4 (58.89±1.68). The lysozyme activity was more in group 3 on 28th day 1285±90.92 but the lowest activity recorded in group 4on 28th day 1205±34.35. The respiratory burst activity was higher in group 3 on 28th day 0.184±0.012. Overall results group 3 had the best activity when compared to others.

Conclusion: From this study we can understand that the fish injected with seaweed extracts *Stocheospermum marginatum* and *Ulva lactuca* seweed as a good immunostimulant. The seaweeds *Stoechospermum marginatum* had alkaloids, phenol, quinone, saponin, steroid and terpenoid. *Ulva lactuca* containedalkaloids, sugar, flavenoid, phenol, quinone, steroid and terpenoid. These secondary metabolites were responsible for the immunostumilant activity.

Keywords: *Stocheospermum marginatum*, *Ulva lactuca*, *Aeromonas hydrophila,* Haemotology, Immunostimulant assay

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INTRODUCTION

The increasing demand for fish and other aquatic food organisms is the main factor behind growing aquatic animal husbandry or aquaculture [1]. Aquaculture is also called 'underwater agriculture' [2]. *A. hydrophila* is widely distributed in aquatic environments [3]. *Aeromonas hydrophila* infection causes a serious damage in pond and aquarium culture. The pathogenesis and histopathology of red-sore disease has been extensively studied in common carp (*Cyprinus carpio*) [4]. Different approaches have been applied by the farmers mainly Chemotherapies are widely used to control and prevent diseases, which have several drawbacks such as environmental risks, development of resistant pathogens and bioaccumulation [5]. Nowadays, several alternative strategies such as immunostimulants, probiotics, green water technique, vaccination and quorum sensing have been introduced in aquaculture to improve fish resistance to pathogen and improve growth performance [6]. Marine algae are source natural products with pharmacological and biological activities [7]. Especially seaweeds are the source of biomedical compounds [8]. Seaweeds are the potential source of antibiotics, antioxidant and anti-inflammatory [9]. Some seaweed has bio-active components which affected the germination of some pathogenic bacteria [10]. Seaweeds extracts were used as therapeutic agents, new compounds were present in oceans and have commercial value [11]. Numerous compounds with cytostatic, antiviral and antibacterial activities have been detected in green, brown and red algae [12]. Marine organisms are potential sources of bioactive secondary metabolites with potential for use in the development of new pharmaceutical agents [13, 14] Immunostimulant can be administered to fish is by injection [15]. In the present study the seaweeds *Stocheospermum marginatum* and *Ulva lactuca* extracts were used as a immuno stimulants of non-specific immune response in common carp were studied.

MATERIALS AND METHODS

Collection of fishes

Common carp (weight 10±5g) were collected from a local fish farm in Vellanguli Tirunelveli District. The fish were acclimatized in 100 litre tanks (10 fish/tank) and provided with continuous aeration. During the holding period the fish were fed a control feed at 5% of their body weight, once a day.

Collection of seaweed

Live samples of the seaweeds were collected by handpicking during low tide from Hare Island in the Gulf of Mannar of Tuticorin coast (08 46' 2.15"N lat; 78 11' 16.05" E long). The seaweeds were identified with the help of Botany expert in our college. After identification the seaweeds were shade dried and powdered.

Preparation of extracts by soxhlet extraction method

The powdered samples were extracted by using soxhlet apparatus. Ethanol was taken as the solvent for extraction. 25g of the sample and 250 ml of the solvent were taken for extraction. The apparatus was run for 4hr and syrupy extracts were collected. The extract obtained were concentrated using rotary vacuum evaporator. Then the extract was stored in cold storage for further study.

Test for secondary metabolites

The secondary metabolites like Alkaloids, Anthroquinones, Catachins, Sugar, Glycoside, Coumarin, Flavonoid, Phenol, Quinone, Saponin, Steroid, Terpenoid were tested.

Infection of fishes

Bacterial strain, *Aeromonas hydrophila* (MTCC No1739) was obtained from Microbial Type Culture Collection and Gene Bank (MTCC) Institute of Microbial Technology, Chandigarh, India. After obtaining bacteria, it was cultured in tryptone soya broth (Himedia) for 24 h at 37^0 C. After incubation period, the culture was centrifuged at 800g for 15 min at 4^0 C. The packed cells were washed with phosphate buffered saline (PBS; pH 7.2) twice and then the required dose was prepared in PBS. The LD_{50} value was calculated based on the mortality rate, with the help stat plus software. After the LD_{50} calculation the standard infection dosage was analysed. The bacterial suspension was prepared to 1.6×10^4CFU/ml^{-1}fish as determined using an infectiual dosage for *Aeromonas hydrophila*.

Intra peritoneal injection

After infection the fish were divided into four groups and injected intraperitonially in seven days interval, namely control group (group 1) injected only saline second one was standard group (group 2) injected with 0.30 mg cyprofloxacin suspended in saline and the third and fourth test groups ethanol extracted seaweeds like *Stocheospermum marginatum* and *Ulva lactuca* injected at 0.30 mg of each extracts suspended in saline solution (group 3 and group 4) after determining the LD_{50} values. Then immunological parameters were analyzed for every 7 d interval. In the experimental period all group of fishes were fed with control diet.

Haematological studies

a. Blood collection

Blood sample was collected from fish of each group. The fishes were collected and gently wiped with a dry cloth to remove water. Caudal peduncle was cut with a sharp blade and the blood was collected in a watch glass containing EDTA, an anticoagulant (6% Ethylene Diamine Tetra Acetic Acid). The blood was mixed well with the EDTA solution by using a needle and this sample was used for determining Hematological studies.

Immune response in common carp was studied by analysing various parameters like RBC, WBC, HB, Hematocrit values, MCV, MCH, MCHC. All these values were determined using a method originally derived by Yokoyama [16] and later on modified by Christensen *et al.,* [17].

b. Immunological assay

Phagocytic assay

Phagocytic activity of neutrophils and monocytes in blood was determined as described by Anderson and Siwicki [18]

Lysozyme assay

Lysozyme activity of blood serum was determined as described by Anderson and Siwicki [18]

NBT assay

Production of Oxygen radicals from Phagocytes in the blood was measured using Nitrobluetetrazolium (NBT) due as described by Anderson and Siwicki [18].

Data analysis

Results are expressed as mean±SD. The statistical analysis was performed by using one way ANOVA using SPSS package version 19.

RESULTS

The seaweeds *Stoechospermum marginatum* had alkaloids, phenol, quinone, saponin, steroid and terpenoid. *Ulva lactuca* obtained alkaloids, sugar, flavenoid, phenol, quinone, steroid and terpenoid (table 1).

Table 1: Secondary metabolites in ethanolic extracts of seaweeds

S. No.	Secondary metabolites	*Stoechospermum marginatum*	*Ulva lactuca*
1	Alkaloids	+	+
2	Anthroquinones	-	-
3	Catachins	-	-
4	Sugar	-	+
5	Glycoside	-	-
6	Coumarin	-	-
7	Flavonoid	-	+
8	Phenol	+	+
9	Quinone	+	+
10	Saponin	+	-
11	Steroid	+	+
12	Terpenoid	+	+

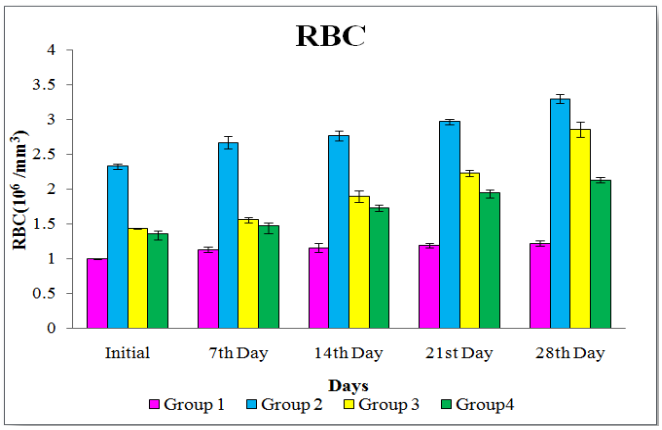

Fig. 1: The RBC value of *Cyprinus carpio* treated with seaweed extracts

After *Aeromonas hydrophila* infected fishes were treated with seaweeds extracts there was a gradual increase in the RBC count (fig. 1) from the initial day to the 28th day the highest content of RBC was recorded on 28th day in group 3 ($2.86\pm0.11\times10^6$ cells/mm^3) followed by group 4 ($2.13\pm0.04\times10^6$ cells/mm^3). Similarly, the WBC values also increase from initial day to final day. The WBC value of

group 3 was 20.73±0.08×10³cells/mm³ and group 4 recorded 19.7±0.11×10³cells/mm³ on 28th day. When compared to brown and green seaweeds the brown seaweed (group 3) containing *Stocheospermum marginatum* was more active. The haemoglobin value and hematocrit value also increased from initial day to final day. The maximum haemoglobin was noted in group 4 on 28th day (5.6±0.24g %) and minimum haemoglobin observed in group 3 (5.33±0.24g %). The highest hematocrit was observed in group 3(19.56±1.11%) and 19.33±0.31% for group 4 on 28th day. The MCV and MCH value was decreased from initial to final day. Final day the MCV value of 68.39±3.15fl recorded in group 3 and 90.75±2.07fl found in group 4. The best activity was found in group 3 and the MCH value of group 3 was 18.64±0.40pg and 26.29±1.15pg observed in group 4 on 28th day. The MCHC value also increased from initial day to final day the maximum MCHC was recorded in group 4 on 28th day 28.97±0.31g/l and minimum recorded 27.25±1.28g/lin group 3. The phagocytic assay was same on 28th day in group 3 and group 4 (58.89±1.68). The lysozyme activity was more in group 3 on 28th day 1285±90.92 but the lowest activity recorded in group 4on 28th day 1205±34.35. The respiratory burst activity was higher in group 3 on 28th day 0.184±0.012. Overall results group 3 had the best activity when compared to others.

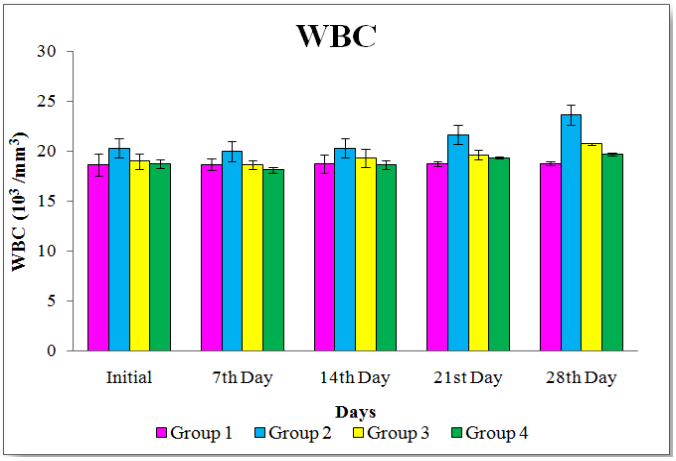

Fig. 2: The WBC value of *Cyprinus carpio* treated with seaweed extracts

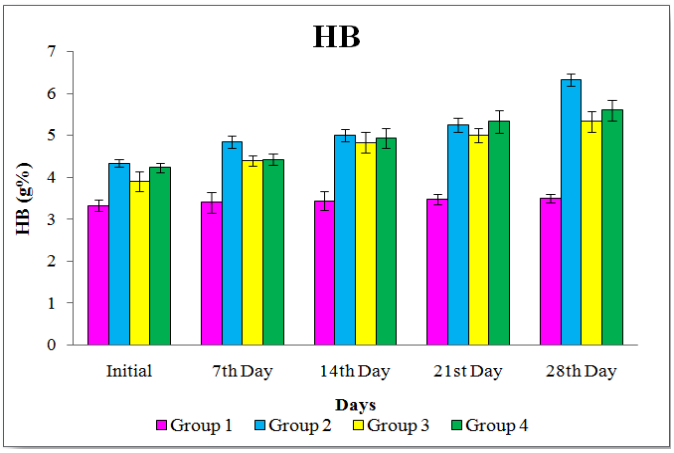

Fig. 3: The Hb value of *Cyprinus carpio* treated with seaweed extracts

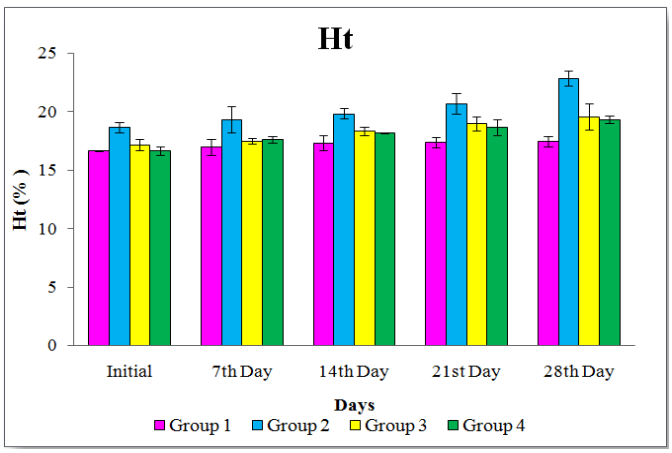

Fig. 4: The Ht value of *Cyprinus carpio* treated with seaweed extracts

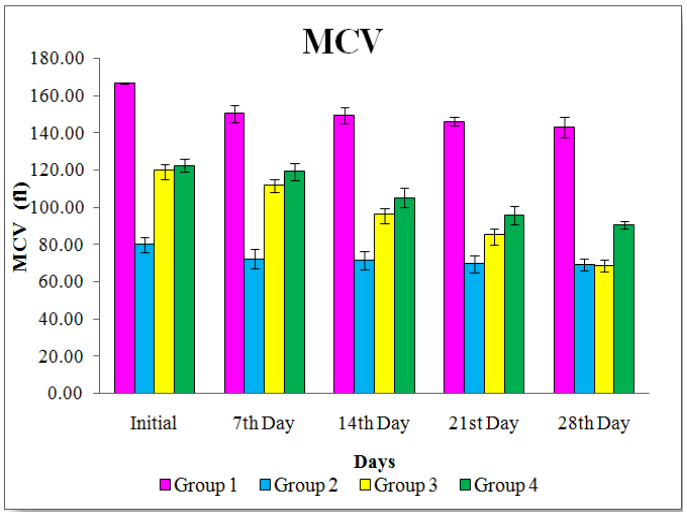

Fig. 5: The MCV value of *Cyprinus carpio* treated with seaweed extracts

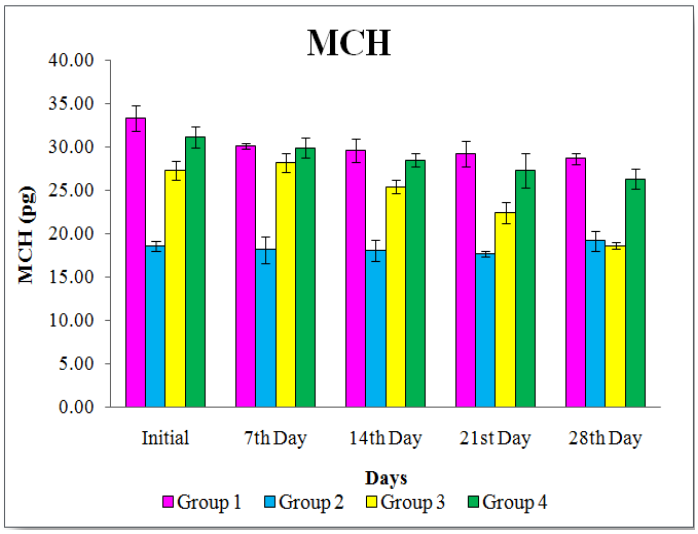

Fig. 6: The MCH value of *Cyprinus carpio* treated with seaweed extracts

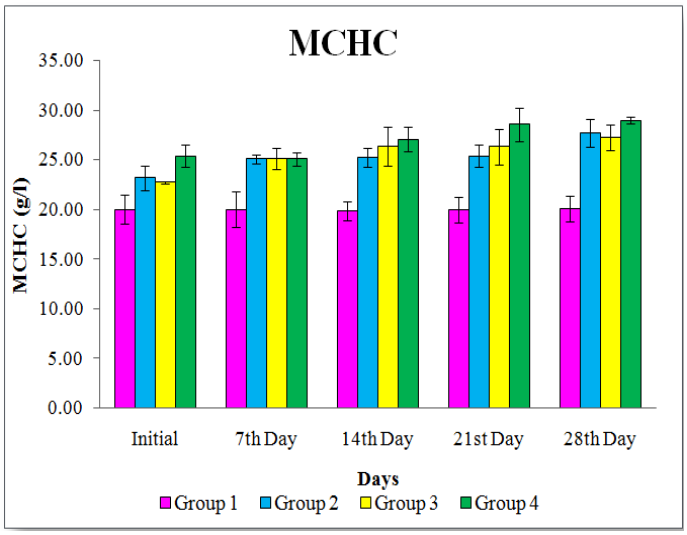

Fig. 7: The MCHC value of *Cyprinus carpio* treated with seaweed extracts

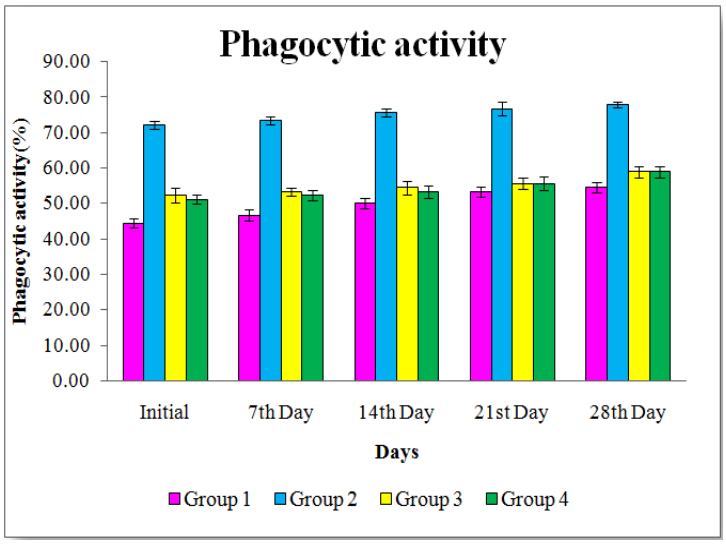

Fig. 8: The phagocytic activity of *Cyprinus carpio* treated with seaweed extracts

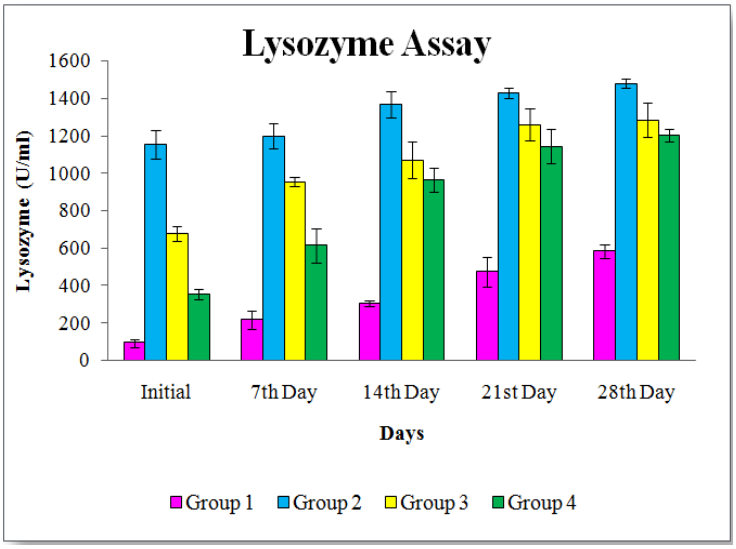

Fig. 9: The lysozyme activity of *Cyprinus carpio* treated with seaweed extracts

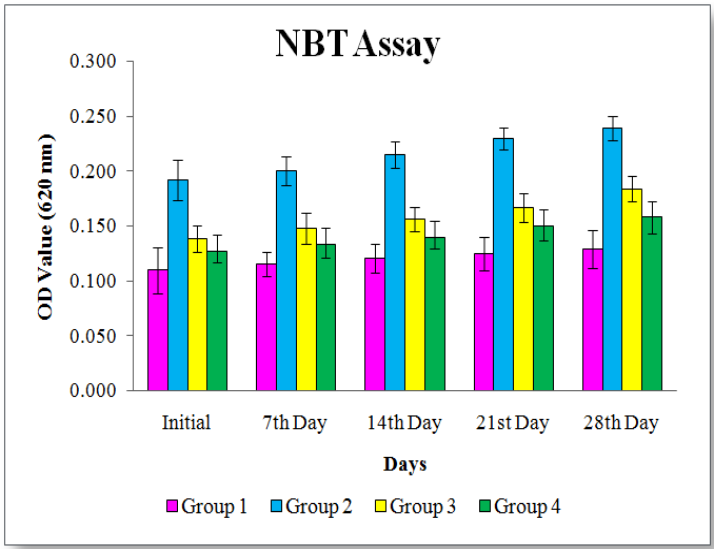

Fig. 10: The NBT Assay of *Cyprinus carpio* treated with seaweed extracts

DISCUSSION

In the present study two types of extracts namely extracts of *Stocheosapermum marginatum* and *Ulva lactua* were chosen to study their immunostimulating properties in *A. hydrophila* infection. The haematological parameters like RBC, WBC, Hb, Ht, MCV, MCH and MCHC were recorded. The major components of the innate immune system (Non-specific) are macrophages, monocytes, granulocytes and humoral elements [19]. Herbal based immunostimulants are capable of enhancing immune responses and reducing losses from viruses, bacteria and parasitic infections in carp [20]. In the present study, immunostimulant effect of seaweed extract was performed in *Cyprinus carpio* at the dose of 0.30 mg/ml through intraperitoneal injection and the immunological parameters were measured at 0, 7th,14th, 21st and 28th day.

The WBC plays an important role in the immune response of fish particularly in inflammation [21]. The WBC content increased from the initial day to the final day after injection they were higher in both group 4 and group5 when compared to the control. This corroborates with the total WBC count increase in fish by the different plant extracts 65% in *Cyprinus carpio* [22]; 56% in carp immunized with Ganaderma and Aastrapulus [23]. Thus the total WBC count increase in the fish may be due to an initial sign of non-specific immune response [24]. They may be enhanced as it is the first line of defense [25].

In fish phagocytosis has been recognised as one of the important elements in the host's defense against invading micro-organisms [26]. Harikrishnan *et al.,* [27] has reported that administration of 50 and 100 mg/kg doses of all triherbal TKM solvent extracts significantly enhanced phagocytic activity of leucocytes isolated from the olive flounder in 30 d. Subeenabegum and navaraj [28] has observed percentage increase in the phagocytic activity of fish administered intraperitoneally with the plant extract is 32.36% in *S. trilobatum*, 35.2 % in *0. Sanctum* and 96.05% in plant extract mixture compared with the control 68.4% increase was reported by Durgadevi and Balasubramanian [29] in C. *carpio* injected with the leaf extract of *S. trilobatum*. In the present study the phalocytic activity was siginificant enhanced in the common carp after administration of both medium and high doses of plant extract [30].

Lysozyme plays an important role in innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria. In this work the highest lysozyme activity was observed in group 3on 28thday1285±90.92. In another study, among various doses of an aqueous extract of polygonum mines leaf, intraperitoneally injected into African catfish, *Clarias gariepinus*, only the dose of 15 mg/kg BW of plant extract could significantly improve lysozyme activity 2 d post-treatment [31]. Fish lysozymes possess a high potential for bacteriocidal or bacteriolytic activity, anti-viral and anti–inflamatory properties and also play an important role in the bio-defense system against gram-positive and gram–negative bacteria [32]. In the jian carp, the lysozyme activity was observed on 20th, 25thand 30th day after feeding [33].

In the present study the respiratory bust activity was found to be significantly higher in the treated groups compared to control ($p<0.05$) of this group3 showed a higher activity. Phagocytes produce toxic oxygen forms during a process called respiratory burst [34] since superoxide anion is the first product to be released from the respiratory burst, the measurement of O_2 has been accepted as a precise way of measuring respiratory burst [35]. The present results showed that experimental fish treated with 0.30 mg/ml doses of *Stocheospermum marginatum* showed enhanced respiratory burst after 28th days. Jang*et al.,* [36] reported that *in vitro* treatment with glycyslizine enhanced the respiratory burst activity of macrophages and the proliferative responses of lymphocytes from rainbow trout. Similarly, in the present study the groups treated with *Ulva lactuca* also showed an improvement in the respiratory burst activity in the second week.

CONCLUSION

Collectively, it can be concluded that the ability of the *Stocheospermum marginatum* and *Ulva lactuca* to mediate nonspecific immune mechanisms is evident from the enhanced WBC, RBC, Hb number and as well as elevated phagocytic, lysozyme and NBT activities. Due to its effectiveness seaweeds can be used as potential drug as an immunostimulant. However appropriate field trials are necessary before using them in aquaculture.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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