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Synergistic effect of medicinal plant leaf extracts supplemented diet on non-specific immune responses in fresh water fish *Channa striatus*

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Abstract
Solanum trilobatum and *Ocimum sanctum* are the valuable medicinal plants. They are good immunostimulants. The purpose of this study to confirm the synergistic effect of aqueous methanolic extract of *Solanum trilobatum* and *Ocimum sanctum* individually and in combination on non-specific immune response of the fresh water fish *Channa striatus* through oral administration. *Solanum trilobatum, Ocimum sanctum* individually and in combination were mixed thoroughly with the artificial feeds at concentrations of 0.0%, 0.03%, 0.3%, 3.0% of dry diet. The prepared diets were fed to healthy *C.striatus* for 40 days.Non-specific immune response such as Total WBC count and Phagocytic activity were analysed. The oral administration of plant extract with their diet increased their total WBC count and Phagocytic activity significantly. The total WBC count and Phagocytic activity were significantly increased in the mixture plant extract treated fish in 1:1 ratio than the individual plant extract treated fish. The present study proved that the mixture of plant extracts in 1:1 ratio treated group increased nonspecific immune responses when compare with individual extract.

Keywords: *Solanum trilobatum, Ocimum sanctum, Channa striatus,* oral administration, Phagocytic activity

1. Introduction
Aquaculture is a fast developing industry contributing fish protein with minerals such as zinc,magnesium, sodium to the consumers (Ravenhalt, 1982; and Barlas, 1986) [32, 4]. Immunostimulants are substances which stimulate the specific and nonspecific defense systems of fish. It helps to enhance resistance to pathogens during stressful periods. (Masoud Haghighi., and Mostafa Sharif Rohani 2013) [22]. Due to intensive culture practices for the increased production, disease management continues to pose a serious threat to aquaculture industry. Some plants are rich sources of compounds like volatile oils, saponins, phenolic compounds, tannins, alkaloids, polypeptides and polysaccharides. These natural plant products have various activities like antistress, appetizer, antimicrobials and immunostimulants (Citarasu *et al.,* 2002, 2003) [8] the use of herbal immunostimulants through oral administration by the incorporation of it into aqua-feed is considered as a modern and promising alternative to antibiotics and vaccines. This study is being concentrated by many researchers. It can be used as prophylactic measure in intensive aqua-culture. Immunostimulants also have the ability to increase resistance to microbial infections and stress due handing, transport, grading and poor water quality in cultivated fish (Raa, 2000) [28].

Current trends in the use of medicinal plant extracts as an alternative to the drugs, chemicals and antibiotics in controlling fish diseases is growing because plant extracts, in contrast to vaccines, enhance the innate (or nonspecific) immune response (Sakai 1999) [7]. The best-known immunostimulants are components of the bacterial cell walls, such as lipopolysaccharides (Goetz *et al.,* 2004) [13] and glucans (Engstadt and Robertson, 1993) [12]. Similarly vitamins, animal and plant extracts are found to enhance the non-specific immune response as well as specific immune response (when immunized or infected) in fish (Anderson, 1992; Raa *et al.,* 1992; Jeney and Anderson, 1993; Sakai, 1999; and Raa *et al*. 2000) [2, 29, 15, 35, 28]. A similar immunomodulatory effect of probiotics have also been observed by various workers; bacteria on cultured *Oreochromis niloticus* (Marzouk *et al.,* 2008) [21]; Bacillus subtilis on Indian major carp *Labeo rohita* (Rajesh *et al.,* 2006) [30]; lactic acid bacteria on Atlantic salmon, Many herbal plants found to inhibit the bacterial pathogens and activate the immunity (Chansue *et al.,* 2000; and Dugenci *et al.,* 2003) [7, 10] at a low concentration and hence its use was very cost effective (Lipton, 2009) [18].

The application of herbal plant extracts as a potential therapeutic measure for modulating the immune response in fish was found out; Achyranthus aspera on *Labeo rohita* (Rao *et al.,* 2006) [31]; Withania somnifera on *Labeo rohita* (Sharma *et al.,* 2010) [37]; Toona sinensis on *O. mossambicus* (Wu *et al.,* 2010) [41]; neem formulation on *Cyprinus carpio* (Balasubramanian, 2006) [3]; *Cynodon dactylon* on *Catla catla* (Kaleeswaran *et al.,* 2010) [16]; plant extract supplemented diet on *Cyprinus carpio* (Mohamed and Abasali., 2010) [23]; The immunostimulatory effect of medicinal plants in fish were documented; *S.trilobatum* and *O.sanctum* in *M.keletius*., (Subeenabegum and Navaraj., 2016)*Ocimum sanctum* in *Cyprinus carpio* (Pavaraj *et al.,* 2011);*Solanum trilobatum* (Divyagnaneswari *et al.,* 2007); *Tinospora cordifolia* leaves (Sudhakaran *et al.,* 2006; and Alexander *et al.,* 2010); water, hexane and methanolic extracts of fresh leaf of *Ocimum gratissimum* against Aeromonas hydrophila (Harikrishnan *et al.,* 2003); and *Azadirachta indica* leaves (Wafaa *et al.,* 2007) in *C. carpio* and the extract of The measurement of total WBC count is the first signal to understand immune responses in fish administered with plant extracts. The total WBC counts increase by the plant extracts; *Allium sativum* on rainbow trout *Oncorhynchus mykiss* (Nya and Austin, 2009) were identified. Similar observations in other plant extracts; Fin

– immune TM (a preparation of Cordyceps mushroom derived β glucan vitamins and essential minerals) in *Onchorhynchus mykiss* (Barker and Holliday, 2009); lipopolysaccharide in *Cyprinus carpio* (Selvaraj *et al*., 2006).

Phagocytosis has been recognized as an important activity in the host's defense against invading microorganisms (MacArthur *et al*. 1985; and Olivier *et al.,* 1986). Fish were showing enhanced phagocytic activity due to various immunostimulants such as ginger (Dugenci *et al.*, 2003) [10] in rainbow trout; *Withania somnifera root* in *Labeo rohita* fingerlings (Sharma *et al.*, 2010) [37]; *Ocimum sanctum* in *Cyprinus carpio* (Pavaraj *et al.*, 2011); *Solanum trilobatum* in *Cyprinus carpio*(Durga devi and Balasubramanian, 2009) [11]; Hitherto, the understanding about the synergistic effect of *Solanum trilobatum* and *Ocimum sanctum* plant leaf extracts (aqueous extract and water soluble fractions) on non-specific immune responses via., total WBC counts, phagocytic activity, in the fresh water fish C.striatus is very limited and hence this study. The main objective of this study was to determine the synergism of the phytocomponents present in the mixture of plant extracts (*S.trilobatum & O.sanctum*) on the non-specific immune response in fish *Cstriatus*.

2. Methods and materials

2.1 Fish: Collection and maintenance

Clinically active fish purchased from local ponds from pudhuwayal near Karaikudi, Sivagangai District, India were acclimated to laboratory conditions in daily renewed fresh water for fifteen days. Fish were fed ad libitum with a balanced diet prepared in the laboratory (Table.1) were maintained before the commencement of the experiment. Fishes were provided with adequate aeration. All healthy fishes alone selected and were maintained in the rectangular plastic tank of 70 l capacity with conditioned parameters; temperature 28.5 ± 0.5º C, pH 7.8, dissolved oxygen 5.37 ppm. Fish weighing70 ± 5 gm were used in all experiments. Fishes were provided with adequate aeration.

2.2 Preparation of aqueous methanolic extract

Selected plants are authenticated by Dr. Jothibasu, Assistant professor in Botany (DDE) Alagappa University, Karaikudi. Freshly collected leaves are washed with distilled water shade dried and ground as a coarse powder. Coarse powder was soaked with aqueous methanol in 1:3 ratio for 72hrs. It is filtered with pure muslin and evaporated. After evaporation it was incorporated with the basal diet in various concentration.

2.3 Preparation of Diets

The balanced diet prepared by adding fishmeal, soyabean meal, ricebran, wheat flour, vitamins and minerals in appropriate proportion. The nine experimental diets namely E1, E2, E3, E4, E5, E6, E7, E8, & E9 were prepared by adding the appropriate concentration of chosen medicinal plant aqueous methanolic extracts as follows. The diet E1-E3 were supplemented with aqueous methanolic extract of *S. trilobatum*, the diet E4-E6 were supplemented with aqueous methanolic extract of *O. sanctum*, the diet E7-E9 were supplemented with aqueous methanolic extract *S. trilobatum* and *O. sanctum* mixture of 0.03%, 0.3%, 3.0% respectively.

2.4 Total WBC Count.

The total WBC was calculated using the formula (Larsen and Snieszko, 1964) [17].

$$\text{Number of cells (cu.mm}-1) = \frac{\text{Number of cells counted x dilution}}{\text{Area counted x Depth of fluid}}$$

2.5 Phagocytic activity

The phagocytic activity assay was performed by the following modified method of Sahoo & Mukherjee (2002). Blood (100 µl) was mixed with equal quantity of bacterial suspension (1:1) in eppendorff tubes. The density of the bacterial culture was maintained throughout the experiment at 10^4 cells/ml in PBS. The mixture was incubated for 20 min at room temperature. After incubation, a thin smear was prepared and fixed with absolute alcohol for 5 min. The smear was later stained with Giemsa stain for 5 min and the phagocytic cells that have engulfed bacteria were counted (under microscope) as positive (Seeley, Gillespie & Weeks, 1990).

$$\text{Phagocytic index (\%)} = \frac{\text{Phagocytic leukocyte number}}{\text{Observed total leukocyte number}} \times 100$$

2.6 Statistics Mean, Standard Deviation, ANOVA tests

Tukey's Multicomparison test were performed in this study by using the SPSS software package. Differences were stastically significant at $p<0.05$ for the all the experiments used in this study.

3. Result

3.1 Total WBC count

The Total WBC count in fish administered orally with the different concentration of plants extract mixture (*S. trilobatum* and *O. sanctum)* increased over that of the control. Thus the control value of 22.60 x 10^3/mm^3 increased to 25.36 x10^3/mm^3, 28.22 x10^3/mm^3 and 40.0 x 10^3/mm^3 in the firstweek;23.44x 10^3/mm^3 increased to 29.18 x10^3/mm^3, 30.14 x10^3/mm^3 and 43.33 x10^3/mm^3 in the second week; and 23.33 x 10^3/mm^3 to 32.24 x 10^3/mm^3, 32.26x10^3/mm^3 and 48.22x10^3/mm^3 in the third week;24.14 x 10^3/mm^3 increased to36.00× 10^3/mm^3, 36.66 ×10^3/mm^3 and 54.66×10^3/mm^3 in the fourth week after the fish administered orally 0.03%,0.3%,3.0% with the plant extracts mixture respectively. Thus, the Total WBC count increases in the fish were high in the fourth week than the increases in the first, second and third week of the experiment (Fig.1).Thus, this study highlights that the equal proportion 3% of plants extract mixture (*S. trilobatum* and *O. sanctum*) has enhanced the Total WBC count to a higher level when compared to individual plant extract *S.trilobatum* or *O.sanctum* in *C.striatus*. (Fig.2).Total WBC count was significantly ($p<0.05$) higher in all the fish administered orally with plant extracts at all the assay period as compared to the control group. Moreover, the significant increase of WBC count ($p<0.05$) was observed maximum in the fish administred orally with mixture of extract than individual extracts. (Fig, 1 & 2)

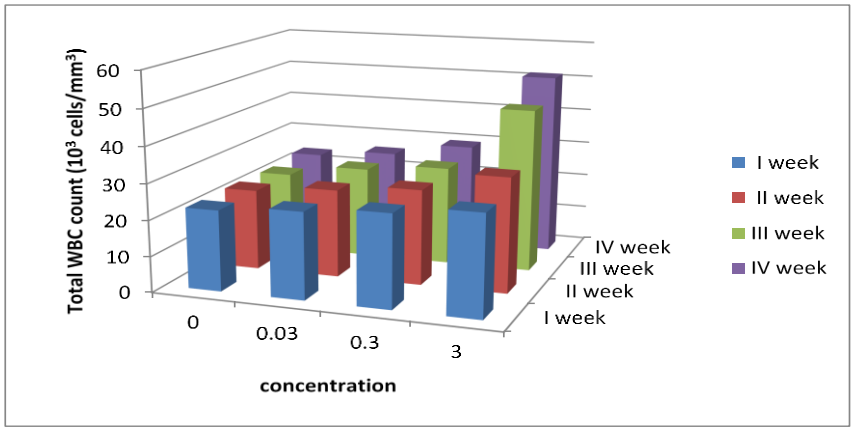

Fig 1: Graphical presentation showing Total WBC count in oral administration of *S.trilobatum* alone in *C.striatus* from I st to IV th week (Mean±S.D)

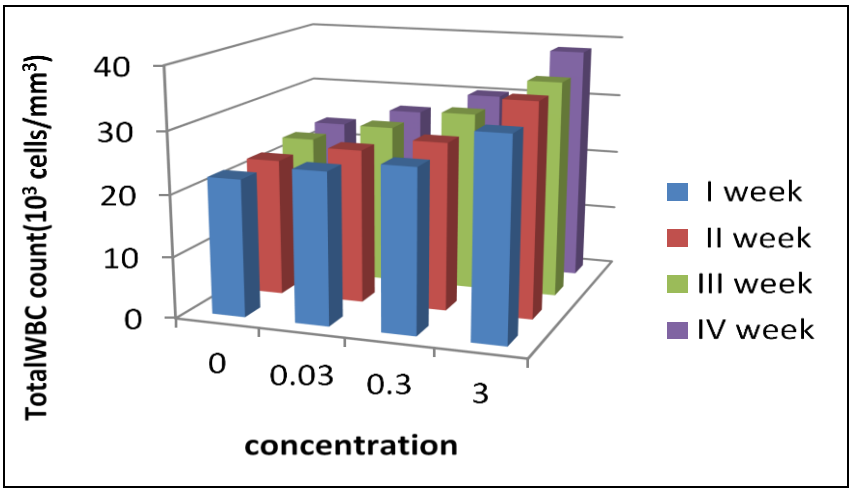

Fig 2: Graphical presentation showing Total WBC count in oral administration of *O. sanctum* alone in *C. striatus* from I st to IV th week (Mean±S.D)

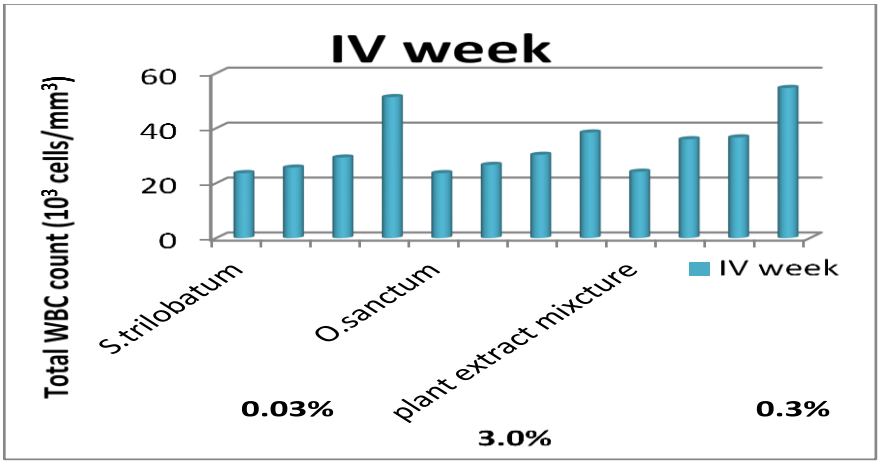

Fig 3: Effect of plant extracts on total WBC count in *C.striatus* after four weeks of the experiment (Differences between individual extracts and mixture) (Mean±S.D)

3.2 Phagocytic activity

The phagocytic activity in fish administered orally with different concentrations of aqueous methanolic extract of plants mixture (S. trilobatum and O. sanctum) increased over that of control. Thus the control value of 32.0 % increased to 34.16%, 36.22 % and 44.44 % in the first week; 32.44% increased to 39.22%, 42.66% and 50.16% in the second week; and 33.66% increased to49.26%, 56.44 % and 62.44 % in the third week; 33.66% increased to 53.64%,58.44%,66.56% in the fourth week after the fish administered orally with 0.03%,0.3%,3.0% of plants extract mixture respectively (Fig.3) Thus, the phagocytic activity increase was the maximum in the fourth week than the increase in the first, second and third week of experiment. Highest 3% of the plant extract showed a best result than other doses of aqueous

methanolic extract of plants. (*S. trilobatum and O. sanctum*).However, this increase is the maximum in the oral administration of fish with plants extract mixture (*S. trilobatum and O. sanctum*) than with individual *S. trilobatum or O.sanctum* extract in *C.sriatus* (Figs.4) The phagocytic activity significantly ($p<0.05$) increased in all experimental groups compared with the control group (Fig,3&4). The significant ($p<0.05$) elevation of phagocytic activity was recorded in the fish fed orally with mixture of plant extract than the individual extracts.

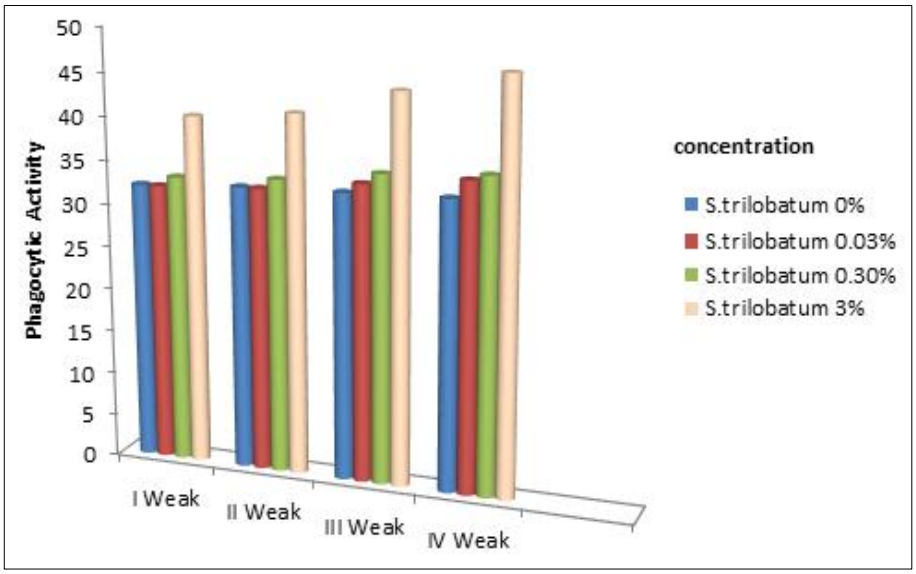

Fig 4: Graphical presentation showing Phagocytic activity in oral administration of *S.trilobatum* alone in *C.striatus* from I st to IV th week (Mean±S.D)

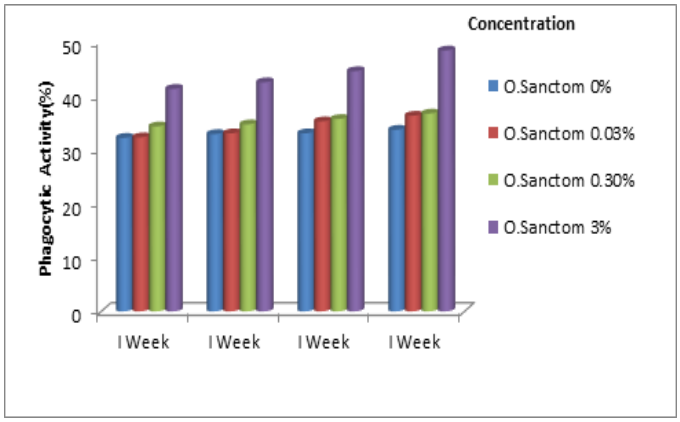

Fig 5: Graphical presentation showing Phagocytic activity in oral administration of *O.sanctum* alone in *C.striatus* from I st to IV th week (Mean±S.D)

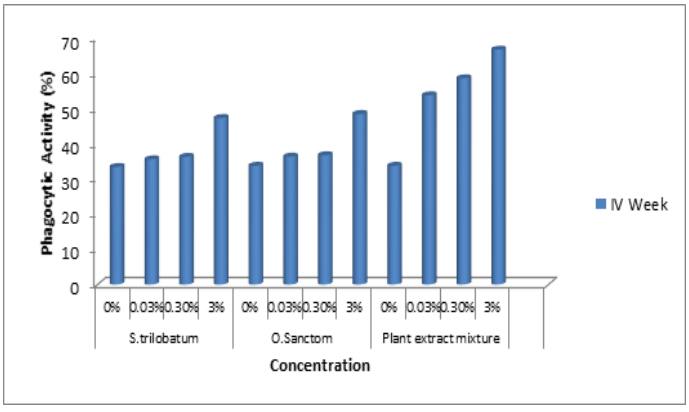

Fig 6: Effect of plant extracts on Phagocytic activity in *C.striatus* after four weeks of the experiment (Differences between individual extracts and mixture) (Mean±S.D)

4. Discussion

4.1 Total WBC count

The percentage of increase in the total WBC count of fish administered orally with the plant extract is 52.5% in *S.trilobatum*, 52.4% in *O.sanctum* and 72% in plants extract mixture when compared with the control group. This corroborates with the total WBC count increase in fish by the different plant extracts; 59%increase in *Cyprinus carpio* with *Euphorbia hirta* plant leaf extract (Pratheepa V and Sukumaran N, 2014) [27]. 76%increase with *Solanum trilobatum* and *Ocimum sanctum* in *Mystus keletius* (Subeenabegum and Navaraj PS, 2016); 65% in *Cyprinus carpio* fed with dietary levamisole (Maqsood, 2009); 56% in carp immunized with Ganaderma and Astragalus (Yin *et al.*, (2009); in rainbow trout, Oncorhynchus mykiss after 2 weeks feeding with garlic and Allium cepa (Nya and Austin, 2009) [24]; in *O. mossambicus* after 3 weeks feeding with *Tinospora cordifolia* leaf extracts (Alexander, 2007); in Labeo rohita after 3 weeks feeding with 0.5% (or) 1% of *Allium sativum* (Sahu *et al.*, 2006); Thus the Total WBC count increase in the fish may be due to an initial sign of non-specific immune response (Manjrekar *et al.*, 2000; Christybapita *et al.*, 2007) [9] or may be due to interdependent mechanism of an innate resistance and adaptive immunity (Mishra *et al.*, 2009) that may enhance the Total WBC count, as it is the first line of defense (Mydeen and Haniffa, 2011). This finding has gained the support from an enhancement in monocytes, granulocytes, neutrophils and macrophages in fish fed with Solanum trilobatum (Bouic *et al.*, 1996) [6].

4.2 Phagocytic activity

The percentage of increase in the phagocytic activity of fish administered orally with the plant extract is 35.3% in *S.trilobatum*, 35.2% in *O.sanctum* and 86.05% in plants extract mixture when compared with the control group. The oral

administration 3% of the plant extract elevates the phagocytic activity in the fish in the fourth week of the experiment. This finding corroborates with the findings of; 64% *Cyprinus carpio* with *Euphorbia hirta* plant leaf extract (Vijayakumari Pratheepa and Nataraja Pillai Sukumaran, 2014) [27]., in hybrid tilapia fed with a diet supplemented with garlic for 2 weeks (Ndong and Fall, 2011); in tilapia fed with a diet supplemented with Allium sativum for 4 weeks (Govind *et al.*, 2012); in rainbow trout fed with 1% aqueous extract of powdered ginger root for 3 weeks (Dugenci *et al.*, 2003) [10]; in parrot fish Oplegnathus fasciatus fed with diet formulated with DL α tocopheryl acetate diet for 12 weeks (Galaz *et al.* 2010); 70.33% increase *O.sanctum* in fingerlings of *Cyprinus carpio* with extract of *Andrographis paniculata* and *Acalypha indica* (Muthumurugan *et al.*, 2007); 58% in *Solanum trilobatum* and *Ocimum sanctum* in *Mystus keletius* (Subeenabegum and Navaraj PS 2016) [38]; 65.0 % increase in *C.carpio* with leaf extract of *Ocimum sanctum* (Pavaraj *et al.*, 2011) [33]; 68.4% increase in *C.carpio* with the leaf extract of *S.trilobatum* (Durgadevi and Balasubramanian, 2009) [11]. Moreover, the enhancement of the phagocytic activity in fish may be due to the first level defense against pathogen (Manjrekar *et al.*, 2000) or may be due to the proliferation of lymphocytes which is under the influence of gene expression of cytokine (Wang *et al.*, 1997) or may be due to the stimulation by macrophages (Tang *et al.*, 2010) or due to an increase in lysozyme activity and other humoral factors (Chung and Secombes, 1987) or due to an increase in neutrophilic granulocytes (Secombes and Fletcher 1992); and Vankemenade *et al.*, 1995 Thus, the maximum phagocytic activity in the fish was elevated by the plants extract mixture when compared to the individual plant extract of *S.trilobatum or O.sanctum*. Hence, this trend may be due to the synergism of the phytocomponents in the plants extracts mixture**.**

5. Conclusion

Plant leaf extracts in combination rather than alone used in this study considerably has enhanced the non- specific immune response in *C.striatus*. Anyhow in addition, the underlying molecular mechanism beside the isolation and characterization of the active compounds from these plants require more studies.

6. Acknowledgement

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