
**ISSN: 2347-5129**
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.352
IJFAS 2016; 4(2): 376-381

www.fisheriesjournal.com
Received: 05-02-2016
Accepted: 08-03-2016

**Dr S Subeenabegum**
Assistant Professor in Zoology,Alagappa University, Karaikudi, Tamil Nadu, India.

**Dr PS Navaraj**
Principal,Annai Fatima College of Arts and Science,Madurai, Tamil Nadu, India.

**Correspondence**
**Dr S Subeenabegum**
Assistant Professor in Zoology,Alagappa University, Karaikudi, Tamil Nadu, India.

# Studies on the immunostimulatory effect of extract of *Solanum trilobatum* and *Ocimum sanctum* in *Mystus keletius*

**Dr S Subeenabegum, Dr PS Navaraj**

**Abstract**
The fresh water cat fish *Mystus keletius* was injected with the methanolic extract (water soluble fraction) of *Solanum trilobatum* and *Ocimum sanctum* alone and in combination in 3mg, 30mg.300mg/Kg body weight. Serum was collected every 7 days interval. Fishes were fed with normal diet for the entire period of the experiment. The nonspecific immune response such as Total WBC count, phagocytic activity and serum antiprotease activity were observed. They were enhanced ($p$<0.05) in fish injected with methanolic extract (water soluble fraction) *S.trilobatum* and *O. sanctum* alone and in combination than control group. Highest level of WBC count, phagocytic activity and serum antiprotease activity were confirmed when the fish injected with the mixed extract of 1:1 ratio of *S.trilobatum* and *O.sanctum* in 30mg/kg (b.w).The effect of mixture of methanolic extract of medicinal plant in *M. keletius* is highlighted.

**Keywords:** Intraperitoneal injection, Methanolic extract, *Solanum trilobatum, Ocimum sanctum, Mystus keletius.*

## 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) [34, 4]. Intensive fish farming creates a highly stressful environment for the fish with consequent suppression of the immune response resulting in increased susceptibility to diseases (Sakai *et al*., 1991; Yano *et al*., 1991; Austin and Austin, 1999) [36, 46, 3]. Different approaches have been applied to address this problem including sanitary prophylaxis and vaccination, which offer a wide range of attractive methods for inducing protection against fish diseases of both bacterial and viral origins. Vaccines as immunostimulant elevate nonspecific defense mechanisms when the organism is immunized or infected (Anderson, 1992) [2]. However, the non-availability of commercial vaccines for many diseases, particularly in developing countries, poses a threat for the prevention of diseases. 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) [37]. Many herbal plants found to inhibit the bacterial pathogens and activate the immunity (Chansue *et al*., 2000; and Dugenci *et al*., 2003) [5, 7] at a low concentration and hence its use was very cost effective (Lipton, 2009).The application of herbal plant extracts as a potential therapeutic measure for modulating the immune response in fish was found out; Chinese medicine on large yellow croaker, *Peseudosciaena crocea* (Jian and Wu, 2003) [16]; *Achyranthus aspera* on *Labeo rohita* (Rao *et al.,* 2006); *Withania somnifera* on *Labeo rohita* (Sharma *et al*., 2010) [39]; *Toona sinensis* on *O. mossambicus* ( Wu *et al*., 2010) [44]; neem formulation on *Cyprinus carpio* (Balasubramanian, 2006); *Cynodon dactylon Catla catla* on (Kaleeswaran *et al*., 2010). water soluble extraction of *Solanum torvam* in *Cyprinus carpio* (Udhaya kumar *et al*., 2012) [41].Water soluble fractions of *S.trilobatum* leaves in *O. mossambicus* (Divyaganeswari *et al*., 2007). The immunostimulatory activity of indigenous garden plant extracts of *Hypericum triquetrifolium, Ballota undulate, Ruta chalepensis, Ononis Natrix, Paronychia argent and Marrubium vulgare* against pathogenic bacteria, *Staphylococcus aureus*, *E.coli* and *Pseudomonas* sp in fish was also confirmed (Amal *et al.,* 2007) Immunostimulant can be administered to fish is by injection(Jenny and Anderson.1993; Sakai1999; Yin *et al*., 2006) [15, 37, 47].The measurement of

of total WBC count is the first signal to understand immune responses in fish administered with plant extracts; *Nyctanthus arbor tristis* on *O.mossambicus* (Kirubakaran, 2009) [18]; and *Allium sativum* on *Labeo rohita* (Sahu *et al*., 2006) [35]; *Allium sativum* on rainbow trout *Oncorhynchus mykiss* (Nya and Austin, 2009) [31] water,hexane methanolic extracts of fresh leaf of *Ocimum gratissimum* against *Aeromonas hydrophila* (Harikrishnan *et al.*,2003) [11] The plant extracts administered intraperitoneal has enhanced total WBC count was reported; in *O. mossambicus* injected with *azadirachtin* (Logambal and Michael, 2001) [23]; and with hot water extracts of *Toona sinensis* (Wu *et al*., 2010) [44]. 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) [24, 32]. The Intraperitoneal administration of *Andrographis paniculata* and *Acalypha indica* in the fingerlings of *Cyprinus carpio* (Muthumurugan *et al*., 2007) Antiproteases are the one of the components of non-specific immunity of the vertebrates. The enhancement of anti-protease is an advantage to fish to overcome the diseases caused by pathogens (James *et al*., 1999) [14] and explained that protease inhibitors could selectively arrest replication of bacterial pathogen without untoward toxicity to the host. Hitherto, the understanding about the synergistic effect of methanolic extract (water soluble fractions) of *S. trilobatum* and *O. sanctum* on non-specific immune responses via, total WBC counts, phagocytic activity, antiprotease activity, in *M. keletius* is very limited. These findings suggest that herbs and their derivatives could be an alternative to the chemotherapeutics in aquaculture. 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 *M.keletius*.

## 2. Materials and Methods

### 2.1. Collection and Maintenance

Clinically active fish purchased from local ponds around Madurai city were acclimated to laboratory conditions in daily renewed fresh water for fifteen days. Fish fed *ad libitum* with a normal diet prepared in the laboratory. They were maintained before the commencement of the experiment. Fishes were provided with adequate aeration.

### 2.2. Preparation of methanolic extract of water soluble fraction.

The plant leaf extraction was done by following the methods of Lee *et al.,* (2000) [20] and Xu *et al.,* (2000) [45]. The mixture of the plant extracts of *S. trilobatum and O. sanctum* were prepared in equal proportion (1:1) of both. This mixture was compared with the immunostimulatory efficacy of individual extract of *S.trilobatum* and *O.sanctum* in intraperitoneal administration to the fish, *M. keletius*.

### 2.3. Intraperitoneal Injection

Fish were injected intraperitoneally with methanolic extract of water soluble fractions of *S.trilobatum and O. sanctum* alone and in combination in different proportions via, 3mg/Kg,30 mg.Kg,300 mg.Kg (b.w) whereas the corresponding control fish received 0.1ml of distilled water. Intraperitoneal administration of the preparation was carried out using one ml tuberculin syringe fitted with a 28 gauge needle. After administration, blood samples were collected through a bleeding technique for every seven days interval. The blood was collected on 7th day, 14th day and 21st day The entire duration of the experiment is twenty one days

### 2.4. Blood collection

A day after the final feeding, blood samples were obtained from the common cardinal vein of randomly chosen five fish anesthetized with 100 mg tricaein methane sulfate (MS-222) by using a 1 ml heparinized syringe for every 7 days of the experiment in each tank for three weeks.

### 2.5. Serum Collection

Blood samples collected from an another anesthetized five fish in each tank were stored without heparin solution and subsequently allowed to clot in 2hrs at room temperature. After centrifugation the serum was collected and stored at -20 ºC for further analysis.

### 2.6. Total WBC Count.

The total WBC was calculated using the formula (Larsen and Snieszko, 1964) [19]

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

### 2.7. Phagocytic activity

The phagocytic activity assay was performed by the following modified method of Michael, *et al.* (1998) [27]

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

### 2.8. Antiprotease activity

The percentage of trypsin inhibition was calculated as described by Rao and Chakrabarti (2004).

$$\text{\% of Trypsin inhibition} = \frac{\text{Trypsin blank OD (A1)} - \text{Sample OD (A2)}}{\text{Trypsin blank OD (A1)}}$$

### 2.9. 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 intraperitoneally with the different concentration of plants extract mixture *(S. trilobatum* and *O. sanctum)* increased over that of the control Thus the control value of 24.66 x 103/mm3 increased to 29.66 x103/mm3), 55.0 x 103/mm3 and 32.0 x 103/mm3 in the firstweek; 24.0 x 103/mm3 increased to 35.66 x 103/mm3, 64.65 x 103/mm3 and 40.0 x 103/mm3 in the second week; and 25.33 x 103/mm3 to 34.33 x 103/mm3, 62.66 x103/mm3 and 37.0x 103/mm3 in the third week after the fish administered with 3 mg/Kg,30 mg/Kg and 300mg/Kg(b.w)methanolic(water soluble fraction)plant extracts mixture respectively. Thus, the Total WBC count increases in the fish were high in the second week than the increases in the first and third week of the experiment (Fig.1).Thus, this study highlights that the 30 mg/Kg (b.w)

concentration 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 *M. keletius*. (Fig.2). Total WBC count was significantly ($p$<0.05) higher in all the fish injected 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 injected with mixture of extract than individual extracts. (Fig, 1 &2)

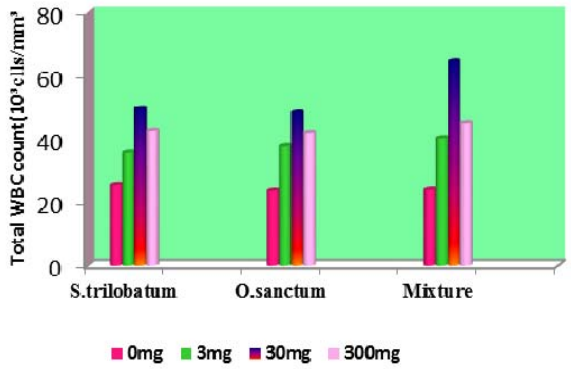
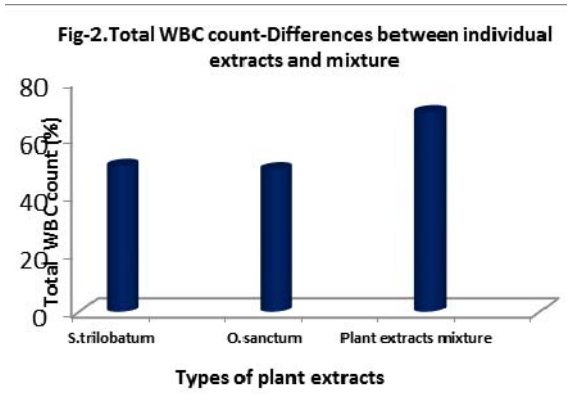



**Fig 1:** Effect of plant extracts on total WBC count in *M.keletius* after two weeks of the experiment (Mean±S.D)




### 3.2. Phagocytic activity

The phagocytic activity in fish administered intraperitoneally with different concentrations of methanolic extract (water soluble fraction) of plants mixture *(S. trilobatum* and *O. sanctum)* increased over that of control. Thus the control value of 34.0 % increased to 41.0%, 48.33% and 47.0% in the first week; 34.0% increased to 41.0%, 66.66% and 50.66% in the second week; and 34.33% increased to38.0%, 61.0% and 49.0% in the third week after the fish injected with 3 mg/Kg,30mg/Kg and300mg/Kg (b.w) of plants extract mixture respectively (Fig.3) Thus, the phagocytic activity increase was the maximum in the second week than the increase in the first and third week of experiment. Similarly, the mid dose, 30 mg/ Kg (b.w) of the plant extract showed a better effect than other doses of methanolic extract of plants mixture *(S. trilobatum and O. sanctum).*However, this increase is the maximum in the fish injected with plants extract mixture *(S. trilobatum and O. sanctum)* than with individual *S. trilobatum or O.sanctum* extract in *M. keletius* (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 injected with mixture of plant extract than the individual extracts.

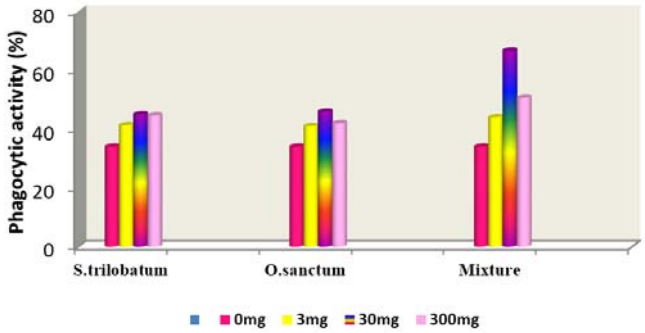
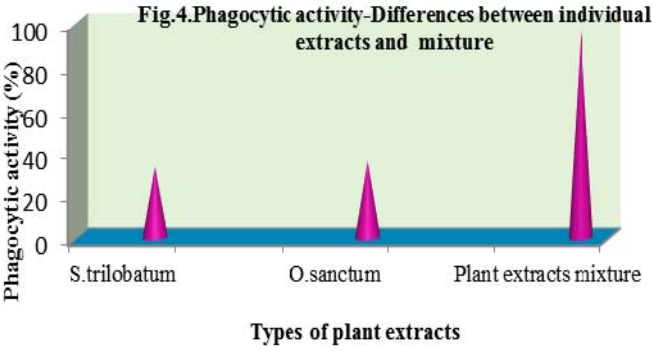



**Fig 3**: Effect of plant extracts on phagocytic activity in *M.keletius* after two weeks of the experiment (Mean±S.D).




### 3.3. Serum antiprotease activity

The serum antiprotease activity in *M.keletius* administered intraperitoneally with the different concentrations of plants mixture *(S.trilobatum* and *O.sanctum)* increased over that of the control. Thus the control value of 55.0% increased to 59.66%, 74.0 % and 60.66% in the first week; 56.66 % increased to 64.66%, 90.0% and 66.66% in the second week; and 56.0% increased to 63.33%, 87.66% and 65.0% in the third week after the fish administered intraperitoneally with 3mg/Kg, 30 mg/Kg and 300mg/Kg(b.w) methanolic extract (water soluble fraction) of plants mixture respectively(Fig.5).Thus, the serum antiprotease activity increase in the fish was maximum in the second week than in the first and third week of the experiment This study confirms that the plants mixture extract has elevated more serum antiprotease activity at the 30 mg/Kg (b.w) concentration. Moreover, this elevation was high in fish administered with plants extract mixture *(S.trilobatum* and *O.sanctum*) when compared to those of individual plant extract, *S.trilobatum* and *O.sanctum* in *M.keletius* (Fig.6) The results of the antiprotease activity are shown in (Fig.5 &6).Antiprotease activity in all the treated groups was significantly ($p$<0.05) higher than control group at all the assay periods. In addition, the highest level of antiprotease activity was observed in group of fish injected with mixture of extracts than the individual extracts. They were stastically significant.

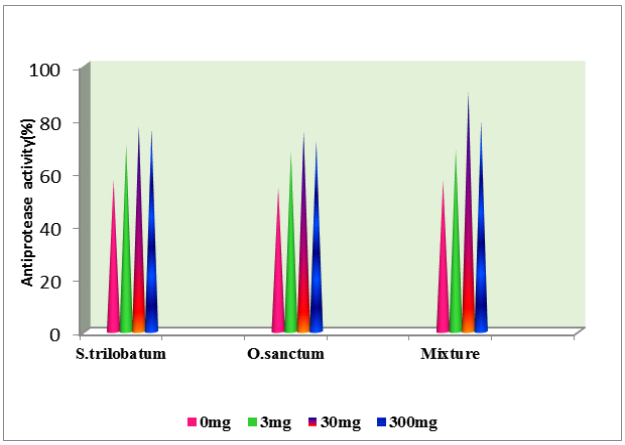
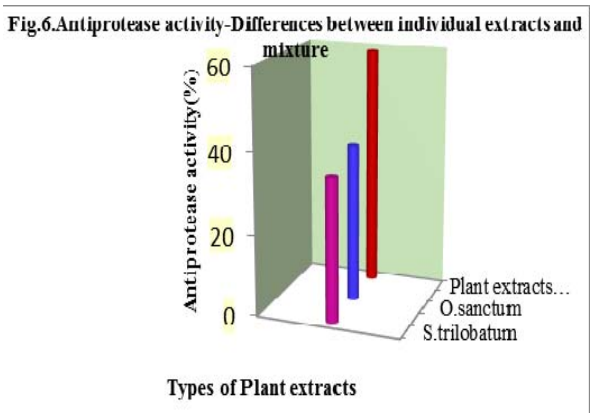



**Fig.5**: Effect of plant extracts on total WBC count in *M.keletius* after two weeks of the experiment (Mean±S.D).




## 4. Discussion

### 4.1. Total WBC count

The percentage of increase in the total WBC count of fish administered intraperitoneally with the plant extract is 50.5% in *S.trilobatum*, 49.3% in *O.sanctum* and 69% 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; 65% in *Cyprinus carpio* (Maqsood, 2009) [26]; 56% in carp immunized with *Ganaderma* and *Astragalus* (Yin *et al*., (2009) [48]. This study also exhibits a high elevation of the Total WBC count in fish administered intraperitoneally with mixture of plant extracts, that agrees with the findings in *Paralicthys olivaceus* injected with *Hericium erinaceum* (Harikrishnan *et al*., 2010) [12]; in cultured fin and shell fish injected with *Punica granatum* (Harikrishnan *et al*., 2011b) [13]; in Jian carp injected with chinese medicinal plants (Jian and Wu, 2004) [17]; in *O.mossambicus* administered with *O. sanctum* (Logammal *et al*., 2000); in tilapia injected with 8 µg. g-1 hot-water extract of *T.sinensis* (Wu *et al*., 2010) [44]. 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) [25] or may be due to interdependent mechanism of an innate resistance and adaptive immunity (Mishra *et al*., 2009).They may be enhanced as it is the first line of defense (Mydeen and Haniffa, 2011) [30]

### 4.2. Phagocytic activity

The percentage of increase in the phagocytic activity of fish administered intraperitoneally with the plant extract is 32.36% in *S.trilobatum*, 35.2% in *O.sanctum* and 96.05% in plants extract mixture when compared with the control group. This finding corroborates with the findings of 70.33% increase *O.sanctum* in fingerlings of *Cyprinus carpio* injected with extract of *Andrographis paniculata* and *Acalypha indica* (Muthumurugan *et al.,* 2007); 65.0 % increase in *C.carpio* injected with leaf extract of *Ocimum sanctum (*Pavaraj *et al*., 2011) [33]; 68.4% increase in *C.carpio* injected with the leaf extract of *S.trilobatum* (Durgadevi and Balasubramanian, 2009) [8]; 68.25 % increase in *O.mossambicus* injected with hot water extract of *Toona sinensis* (Wu *et al*., 2010) [44]; Thus, injection of the extract is found to be the most effective method of immunostimulation (Anderson and Siwicki, 1994a) [1] and that is confirmed in this study. Moreover, the enhancement of the phagocytic activity in fish may be due to the first level defense against pathogen (Manjrekar *et al.,* 2000) [25] or may be due to the proliferation of lymphocytes which is under the influence of gene expression of cytokine (Wang *et al*., 1997) [43] or may be due to the stimulation by macrophages (Tang *et al.,* 2010) [40] 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 Fletcher1992) [38]; 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 extract mixture.

### 4.3. Serum antiprotease

The present study also highlights the serum antiprotease activity in *M.keletius* administered intraperitoneally with 30mg.Kg-1 (b.w) plant extracts have shown significant increases via. 35% in *S. trilobatum*, 38.88% in *O.sanctum* and 58.84% in plant extract mixture over the control group in the second week of the experiment. A significant elevation of the serum antiprotease activity in fish administered intraperitoneally with 30mg/Kg (b.w) plants extract mixture *(S.trilobatum* and *O.sanctum)* was observed in this study. This finding gets the support from the works in rainbow trout *(Oncorhynchus mykiss)* administered with 1.0 % of lupin *(Lupinus perennis)* mango *(Mangifera indica)* and stinging nettle *(Urtica dioica)* (Elham, 2010) [9] 38.0% increase in 20mg.Kg-1 *Nyctanthus arbor tristis* seed extracts in *O.mossambicus* (Kirubakaran, 2009) [18]; 46.67% increase in the 50mg Kg-1 water soluble fraction of *Solanum trilobatum* (Divyaganeswari *et al*., 2007). The enhancement of the serum antiprotease activity observed in this study may be due to the potent bioactive substances influence over pathogen by enhancing the enzyme activity (Lin *et al*., 2006) [21] by acting against proteases from pathogenic organisms (Ellis, 2001) [10] and by inhibiting the function of extracellular enzymes in fish and by restricting the replication of microbial pathogens without untoward toxicity to the host (James *et al*., 1999) [14]. Thus an increase in Total WBC count, phagocytic activity, antiprotease activity in the fish was maximum in the fish administered intraperitoneally with plants extract mixture than with an individual extract, *S.trilobatum* or *O.sanctum* were confirmed. 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 methanolic extract of water soluble fraction of *S.trilobatum* or *O.sanctum* in *M.keletius*.

## 5. Conclusion

The plant leaf extract in combination rather than alone used in this study considerably has enhanced the non-specific immunity in *M.keletius*. In addition the underlying molecular mechanism beside the isolation and characterization of the active compounds from those medicinal plants require more study. It also requires well training.

## 6. Acknowledgement

The researchers are thankful to the management of both colleges, Yadava College, Madurai and M.S.S. Wakf Board College, Madurai for their strong support in carrying out this research.

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