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Research Article

EFFECT OF *ALLIUM SATIVUM* BULB EXTRACTS AS FEED ADDITIVES ON THE IMMUNOLOGICAL, ENZYMOLOGICAL, BIOCHEMICAL AND GROWTH PARAMETERS OF *CIRRHINUS MRIGALA*

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ABSTRACT

The present work was done to test the effect of (*A. sativum Linn*.) garlic bulb extracts on the immunological, enzymological, biochemical and growth parameters of (*C. mrigala Ham.*) Indian major carp fingerlings. Five experimental diets namely control, petroleum ether, toluene, ethyl acetate and methanol extract mixed feeds (0.10 %) were prepared and the fishes were fed with them for two months. The results indicated that the oral administration of the extracts led to desirable changes in immunological (superoxide anion production, bactericidal activity and lysozyme activity), enzymological(aspartate transaminase, alanine transaminase and alkaline phosphatise), biochemical (blood glucose, total protein, albumin, globulin, cholesterol and triglycerides)and growth **(**weight gain%, specific growth rate (SGR) and feed conversion ratio (FCR) parameters. Based on the findings, it could be derived that *A. sativum* bulb extracts prepared using different solvents; especially the 0.10 %/kg supplemented feed additives enhance the immunity and growth of Indian major carp fingerlings.

Keywords: *Cirrhinus mrigala*, *Allium sativum*, *Pseudomonas fluorescens*, Immunological parameters.

INTRODUCTION

Plant products have been recorded to play a significant role in numerous activities such as growth promotion, antistress, immunostimulation and appetite stimulation in aquaculture practices (Sivaram, *et al*., 2004). Garlic (*A. sativum Linn*.) is one of the bulb-forming perennial herbs that belong to the family *Liliaceae* in the genus *Allium*, and it has been practiced since time immemorial as a medicine, flavouring agent, and functional food to enhance physical and mental health. *A. sativum* has been studied in various forms such as ethanol extract, dried powder and aqueous extract (Shin and Kim, 2004). The herb is called Lasan in India, and it has been utilized for many years as an ayurvedic medicine in the country. Many of its useful health properties are attributed to the presence of organosulphur compounds, mainly thiosulfinates (R-S-S (O)-R) (Block, 1992). Previous investigations on the dietary applications of garlic in fishes have established that improves growth, survival, feed efficiency, and offers protection against pathogenic bacteria (Aly, *et al*., 2008; Lee, *et al*., 2012; Farahi, *et al*., 2010; Mahbubeh, *et al*., 2016).

Plant extracts have been proven to reduce the rate of mortality resulting from pathogenic organism challenges (Ardo, *et al*., 2008). *Allium* spp. also exerts immunological activities such as promotion of cytokine release, natural killer cell activity, lymphocyte synthesis and phagocytosis (Kyo, *et al*., 1998).Moreover, garlic enhances the immunity in fishes even at a low dose when administered as a feed supplement (Sahu *et al*., 2007). Besides, the application of herb as an immunostimulant in fish culture is gaining

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importance because of its important role as a therapeutic and prophylactic agent (Amagase, *et al*., 2001). Garlic can be employed to control the pathogenic organisms, especially fungi and bacteria and improve the health of fishes (Corzo Martinez, *et al*., 2007). The plant holds immense potential as an antimicrobial agent against pathogens attacking the fishes (Wei and Najiah, 2008). The application of *A. sativum* in aqua farming has become popular for developing the defense systems, conferring protection against infection causing pathogens and promoting growth (Nya and Austin, 2009; Diab *et al*., 2002; Metwally, 2009).

The antibacterial activities of *A.sativum*, have been generally studied. Indeed, allicin and garlic preparations (fresh, freeze and oven -dried garlic, etc.) have been shown to possess a wide spectrum of antibacterial activity, including effects on *Escherichia, Bacillus, Clostridium, Cryptocaryon, Aeromonas, Klebsiella, Mycobacterium, Helicobacter, Proteus, Salmonella, Photobacterium, Pseudomonas, Vibrio Staphylococcus,* and *Streptococcus spp*, Besides the herb it's preparations are also effective against common plant pathogenic organism such as *Pseudomonas syringae, Xanthomonas campestris, Agrobacterium tumefaciens* and *Erwinia carotovora* (Curtis, *et al.,* 2004; Bakri and Douglas, 2005). The value of Garlic extract in bacterial disease control and immunostimulation activity have previously been demonstrated in several cultured fishes (Talpur and Ikhwanuddin, 2012; Aly and Mohamed, 2010; Thane, *et al*., 2013). *A. sativum* can help in controlling the bacterial and fungal pathogens, and enhancing the wellbeing of fishes (Corzo-Martinez, *et al*., 2007; Ress, *et al.*, 1993; Salah Mesalhy, *et al*., 2008).

Indian major carps, namely *Cirrhinus mrigala, Labeo rohita* and *Catla catla* contribute about 70 to 75% the total fresh water aquaculture production. Major carp culture is the backbone of freshwater aquaculture system in India. The chief Indian states involved in this activity are West Bengal, Uttar Pradesh, Orissa, Haryana, Kerala, Andhra Pradesh, Tamil Nadu, Maharashtra, Karnataka, Gujarat and Goa (CIFA, 2004). Moreover, the Indian major carps such as mrigal, rohu and catla are the fast-growing fishes capable of gaining a marketable size of 800-1000 g within one year, and they are produced in polyculture systems (Imtiaz-Ahmed *et al*., 2004). These constitute the commercially important fish species in India, Bangladesh, Pakistan and Nepal. Currently, *Cirrhinus mrigala* is over jerked around in the Ganga River system. The species is of economic importance owing to its aquaculture achievable and high consumer preference (Mayank, Dwivedi 2015).

P. fluorescens has been widely recorded as the causative agent of bacterial haemorrhagic septicaemia in pond - cultured fish (Mastan, 2013). Therefore, an assay has been made in the present work to assess the effect of garlic bulb (*A. sativum*) extracts on the immunological, biochemical, enzymological and growth parameters of the Indian major carp *Cirrhinus mrigala* fingerlings against pathogenic bacteria *P. fluorescens.*

MATERIALS AND METHODS

Acclimatization of the fishes

Normal fingerlings of mrigal carp having an average weight of 8.5 ±1.0g and total length of 9 ± 1.0cm, were procured from Aliyar Dam, Tamil Nadu Fisheries Corporation, India. (Located at a distance of about 65 km from Coimbatore). Initially, 300 fishes were introduced into a large cement tank and retained for 30 days. Control diet was given during this acclimatization period. Subsequently, the fishes were divided into five different groups (1-control group, 2, 3, 4 and 5-experimental groups) of 60 fishes each. The water exchange ratio was 50% of the total volume on a daily basis.

Plant material

Commercially available healthy garlic bulbs (*A. sativum Linn*.) belonging to the genus-*Allium* in the family-*Liliaceae* were procured from the local market Coimbatore and they were confirmed by comparison with the reference herbarium specimens. The plant parts were authenticated by Botanical Survey of India (Ministry of Environment, Forest and Climate Change - Government of India) Southern Regional Centre, Coimbatore. Initially, the clean and healthy bulbs were sun-dried for 10 days. Later, they were crushed with pestle and mortar reduced to a fine powder using a laboratory mixer, sieved and then filed in airtight containers until being used for the experiments.

Preparation of *A. sativum* bulb extracts

The powdered *A. sativum* bulbs were extracted alone to exhaustion using with a help of Soxhlet apparatus using various organic solvents in the increasing polarity order. Twenty grams of the powder were extracted in 450 ml of each solvent namely (petroleum ether, toluene-99.5%, ethyl acetate-99%, methanol-99%). The extracts were collected carefully and then concentrated using evaporating beakers at room temperature (27^o - $35\ ^o$ C) for 2 - 4 days until the final volume was reduced to one-fourth of the original total volume and stored at 4^o - 6^o C in airtight containers until further use. These extracts were tested against the Gram-negative bacterium *P. fluorescens.*

Preparation of the experimental feed

Five different feeds were prepared as outlined below

1- The Control normal balanced feed was composed of the following (per 100gm of the feed preparation) corn meal-10gm, fish meal-15gm, wheat meal-15gm, soy bean meal-15gm, fish oil-2gm, mineral and vitamin mix-2gm, rice bran-15gm, ground nut oil cake-20gm, starch-1gm and egg white-5gm 2- The treatment diet was prepared by mixing the normal balanced feed with the petroleum ether extract of *A. sativum* bulb in a ratio 10grams per kilogram of food (0.10 %). 3- The treatment diet was prepared by mixing the normal balanced feed with the toluene extract of *A. sativum* bulb in a ratio 10grams per kilogram of food (0.10 %). 4- The treatment diet was prepared by mixing the normal

balanced with the ethyl acetate extract of *A. sativum* bulb in a ratio 10grams per kilogram of food (0.10 %). 5- The treatment diet was prepared by mixing the normal balanced feed with the methanol extract of garlic bulb in a ratio 10grams per kilogram of food (0.10 %).

Antibacterial activity

Antibacterial effect of the *A.sativum* extract was tested by the disc diffusion method. Overnight culture of *P. fluorescens* bacterial strains grown in broth medium was adapted to an inoculum's density of 100 µl. Further, 10 µl was spread on to the surface of sterile agar plates containing 25 ml of the culture medium by using a clean spread plate rod. The top of the medium was permitted to settle for about 5 - 10 min. Hygienic filter paper discs also (5-7 mm in diameter) parturient with various test extracts (10mg) were placed on the surface top of the inoculated agar medium plates. Vancomycin (VA30/disc) was used as positive control. The agar plates were then incubated at 27°C for 48 h, after which the microbial growth was resolved by measuring the diameter area of the inhibition zone using a measuring scale. Each extract was evaluated in triplicates, and the mean values were considered.

Statistical analysis

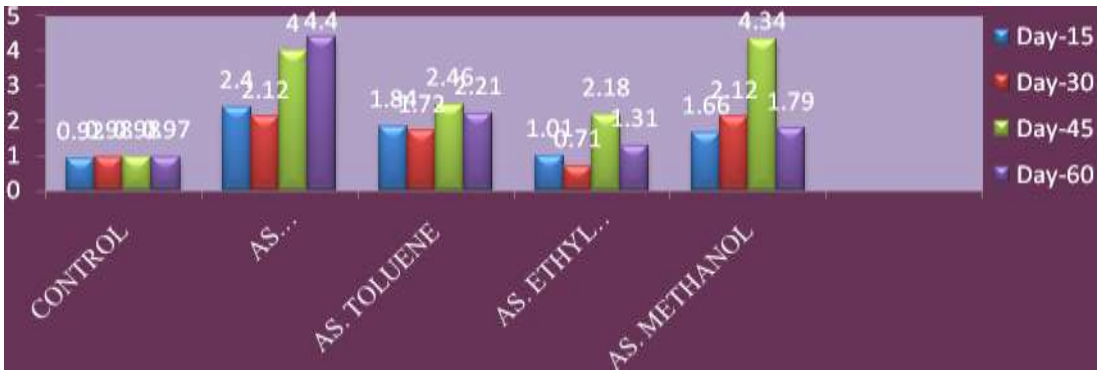
The experiment values were presented as (n = 3) arithmetic mean ± standard error (SE). The data were statistically estimated by one-way analysis of variance (ANOVA) followed by Post hoc multiple comparison tests using SPSS software (version-16). S The statistical P value 0.05 was considered as significant and P value 0.01as highly significant.

RESULTS AND DISCUSSION

The results of the present study reveal that super oxide anion production from *C. mrigala* reached the highest level on the 45th day in the *A. sativum* ethyl acetate and methanol extract treated groups (2.18 ± 0.28 and 4.34 ± 0.48 respectively). The lowest level was attained on the 60th day of the experimental study for the two extracts (1.31 ± 0.26 and 1.79 ± 0.56 respectively). Lysozyme activity was significantly increased in the ethyl acetate and methanol extract treated group (299 ± 0.14 and 468 ± 0.70) on the 45th day and it was lowest (186 ± 0.84 and 268 ± 0.70 respectively) on the 15th day of the experiment. Bactericidal activity was considerably decreased in the ethyl acetate and methanol extract treated groups. (80 ± 0.11 and 172 ± 0.34 respectively) on the 30th day and it was increased (120 ± 0.46 and 202 ± 0.05 respectively) on the 45th day. When comparisons were made between the control group and the experimental groups, the *p - value* was found to be highly significant.

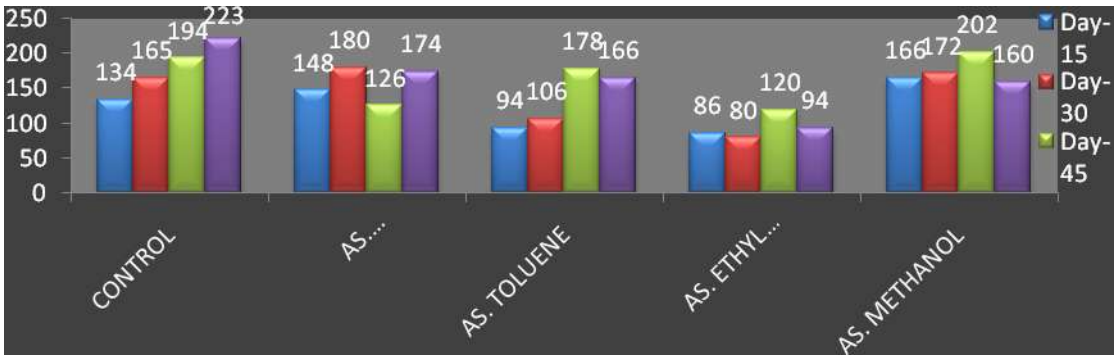
Aspartate transaminase (AST) level was significantly increased in the methanol extract treated group. (82.40 ± 0.07) on the 45th day when compared with the levels (59.80 ± 0.14) on the 60th day of the experiment. Alanine transaminase (ALT) level was significantly (P-0.017) raised in the ethyl acetate and methanol extract treated groups. (82.8 ± 0.03 and 96.4 ± 0.02 respectively) on the 45th day when compared with the levels (76.6 ± 0.03 and 82.2 ± 0.01 respectively) on the 60th day Alkaline phosphatase (ALP) level was significantly (P-0.023) augmented in the ethyl acetate and methanol extract treated group. (112.4 ± 0.04), (115.6 ± 0.06) on 45th day when compared with the levels (98.4 ± 0.05 and 91.2 ± 0.02 respectively) on the 60th day of the research.

Figure 1. Immunological parameters (A) Superoxide anion production (O. D), (B) Lysozyme activity (U / ml), (C) Bactericidal activity% (CFU) for *Cirrhinus mrigala* fed with 0.10% *Allium sativum* extracts in different solvents for varying number of days. Each value is represented as the mean ± SE of three individual observations (n = 3). Mean values within the same groups were statistically significant at P < 0.05.



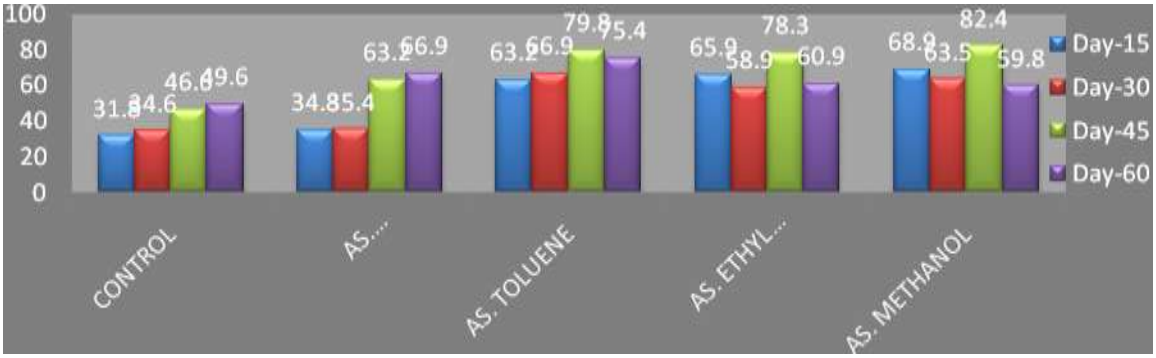
(A) Superoxide anion production (O. D)

(B) Lysozyme activity (U / ml)

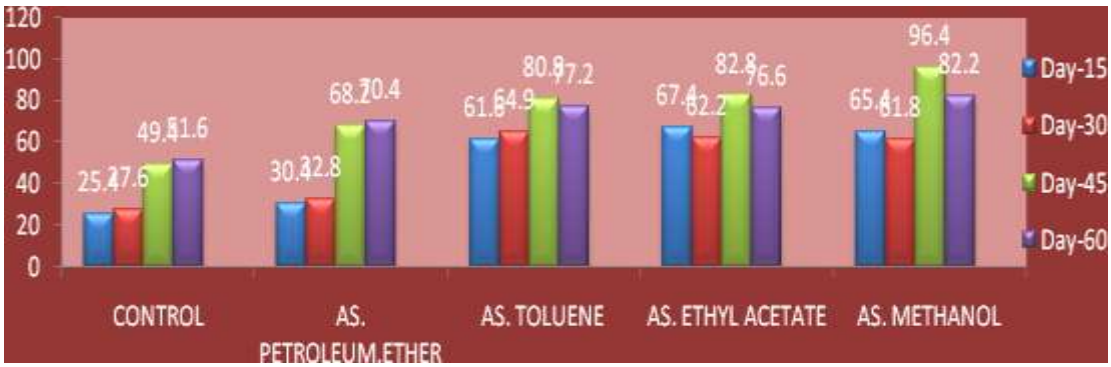


(C) Bactericidal activity% (CFU)

Figure 2. Enzymological parameters (A)Aspartate transaminase (AST) (U/ml), (B) Alanine transaminase (ALT) (U/ml), (C) Alkaline phosphatase (ALP) (U/ml) for *Cirrhinus mrigala* fed with 0.10 % *Allium sativum* various solvent extracts in different solvents for varying number of days.Each value is represented as mean ± SE of three individual observations (n = 3). The mean values within the same group were statistically significant at $P < 0.05$.



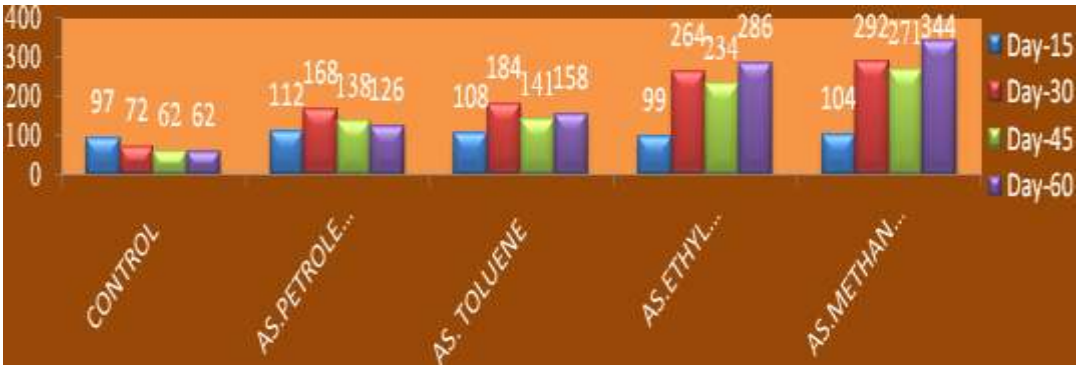
(A) Aspartate transaminase (AST) (U/ml)



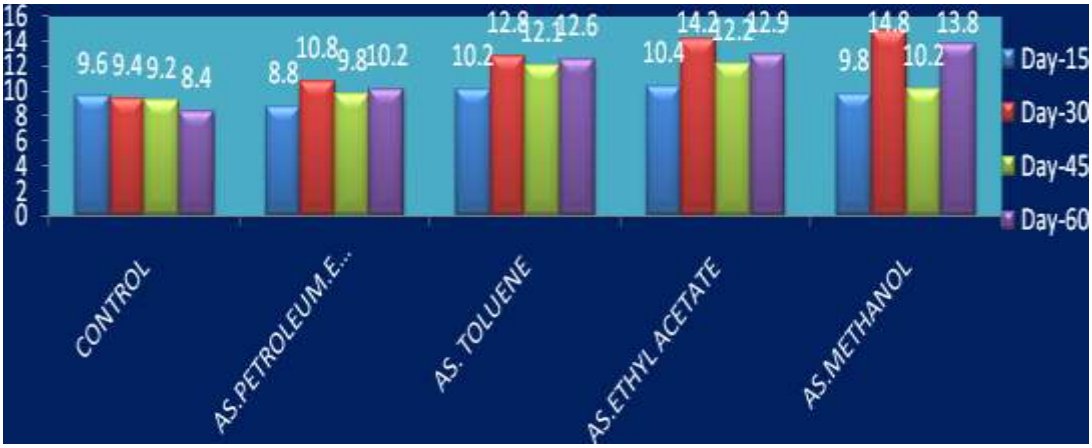
(B) Alanine transaminase (ALT) (U/ml)

(C) Alkaline phosphatase (ALP) (U/ml)

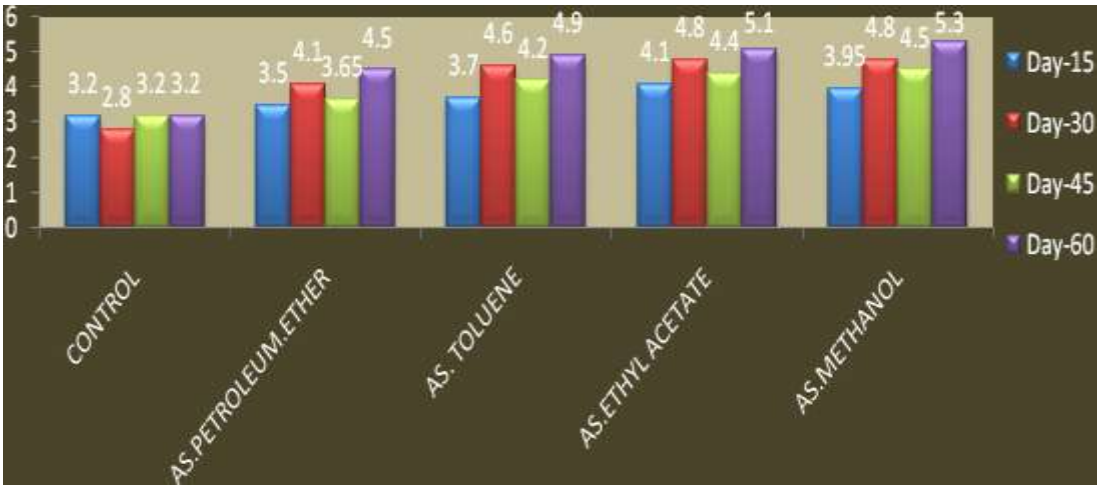
Figure 3. Biochemical parameters (A)Blood glucose (mg l^{-1}), (B) Total protein (g / dl), (C) Albumin(g / dl), (D) Globulin (g / dl), (E) Cholesterol (mg/ dl), (F) Triglycerides (mg / dl) for *Cirrhinus mrigala* fed with 0.10% *Allium sativum* extracts in different solvents for varying number of days.Each value is represented as mean ± SE of three individual observations (n = 3). The mean values within the same group were statistically significant at $P < 0.05$.



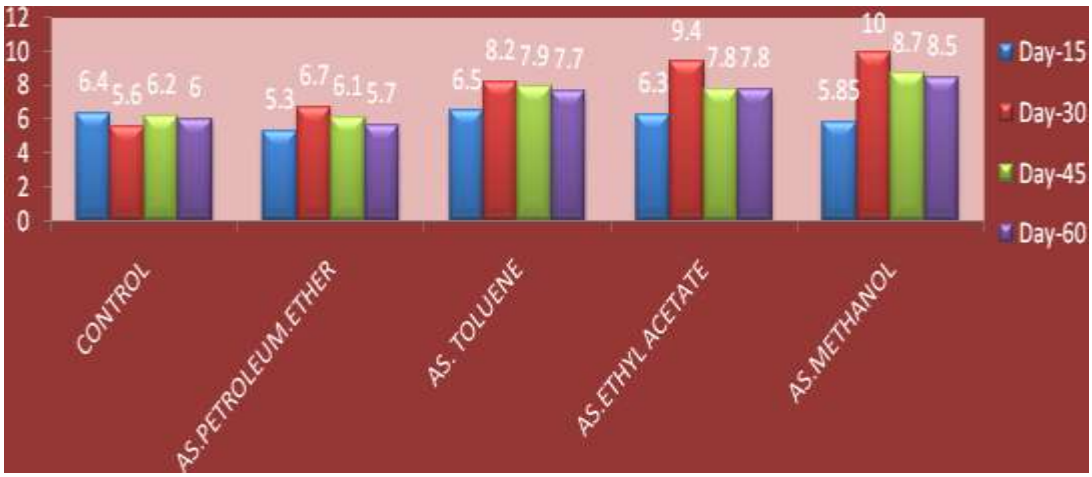
(A)Blood glucose (mg l^{-1})



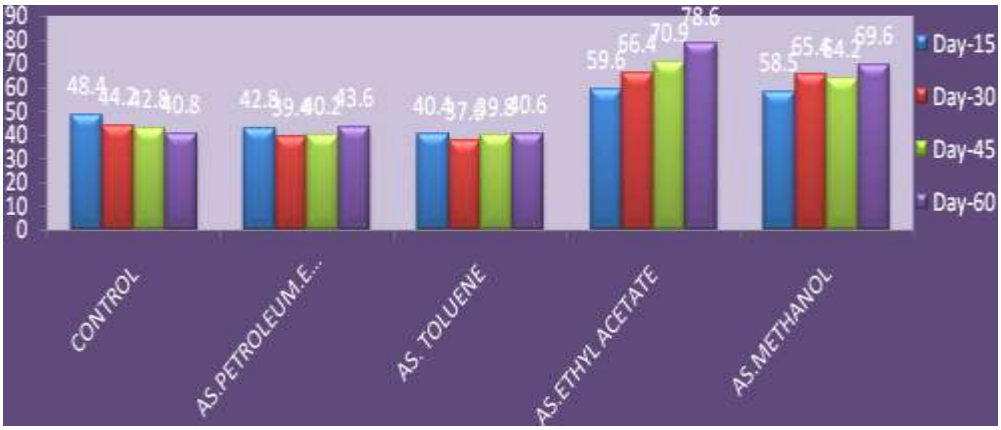
(B) Total protein (g/ dl)



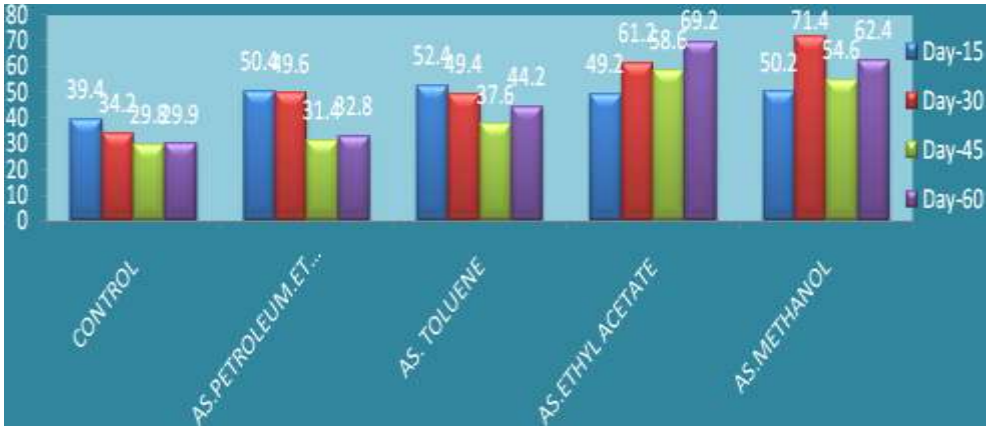
(C) Albumin(g / dl)



(D) Globulin (g / dl)

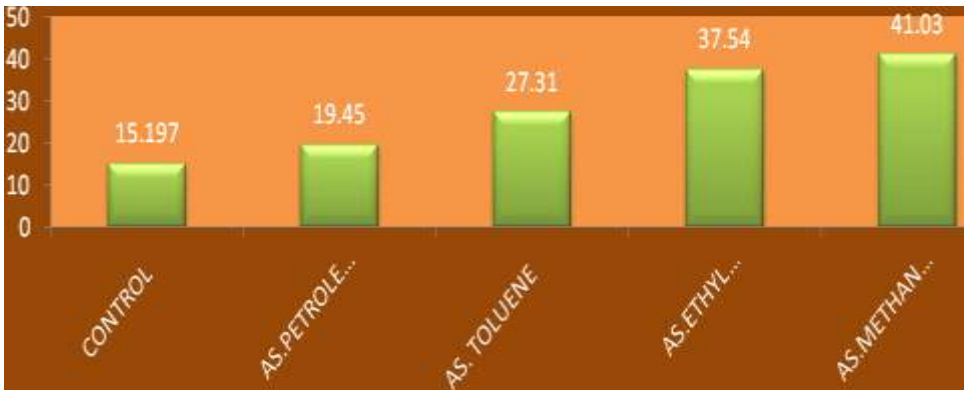


(E) Cholesterol (mg/ dl)

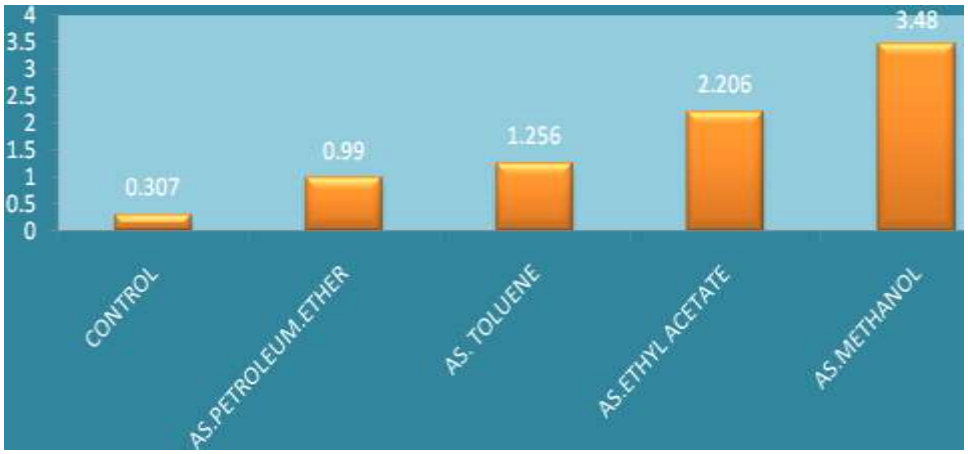


(F) Triglycerides (mg / dl)

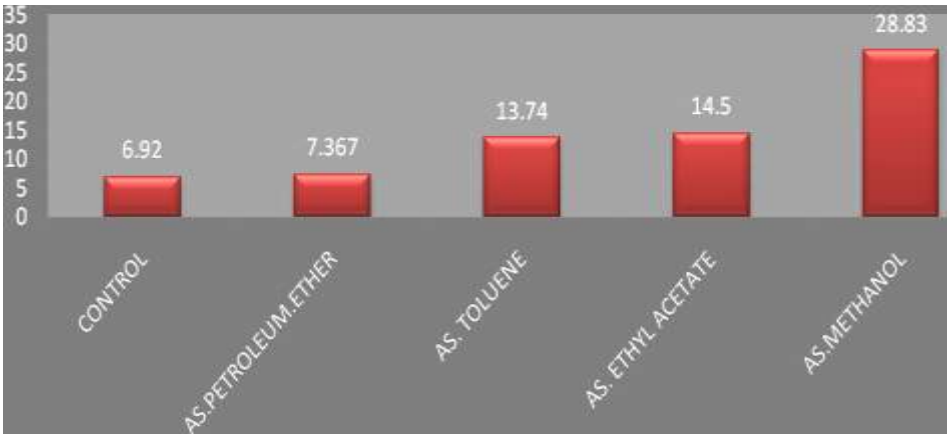
Figure 4. Growth parameters (A)Weight Gain (%), (B)Specific Growth Rate (%), (C) Feed Conversion Ratio (%) for *Cirrhinus mrigala* fingerlings fed with 0.10% *Allium sativum* extracts in different solvents. The results obtained on the 60th day of the experiment. Each value is represented as mean ± SE of three individual observations (n = 3). The mean values within the same group were statistically significant at P < 0.05**.**



(A)Weight Gain (%)



(B)Specific Growth Rate (%)

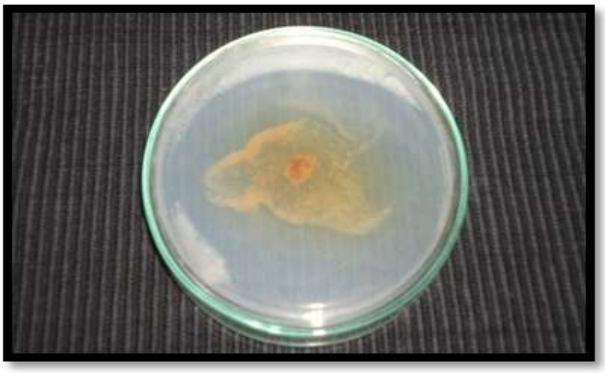


(C) Feed Conversion Ratio (%)

Figure 5. *Pseudomonas fluorescens* culture

(A)*Pseudomonas fluorescens* culture in nutrient agar plates.

(B) Control- Vancomycin (VA30/disc) (10^{-5}dilution)



(C)Antibacterial activity of *Allium sativum* methanol extract against *Pseudomonas fluorescens*. (10^{-5}dilution)

The present study indicates that the blood glucose levels were notably raised in all the groups and the levels were quite high in the ethyl acetate and methanol extract treated groups (264 ± 0.14 and 292 ± 0.00 respectively) on the 30th day. However, they were and decreased (234 ± 0.70 and 271 ± 0.56) on the 45th day of the study. The total protein level was notably (P-0.002) increased in the ethyl acetate and methanol extract treated groups. (12.90 ± 0.28 and 13.80 ± 0.56 respectively) on the 60th day when compared with the levels (12.20 ± 0.17 and 10.20 ± 0.23 respectively) on the 45th day of the investigation. The albumin level was significantly enhanced in the ethyl acetate and methanol extract treated groups. (5.10 ± 0.05 and 5.30 ± 0.11 respectively) on the 60th day when compared with the levels (4.80 ± 0.04 and 4.80 ± 0.02 respectively) on the 30th day. The globulin level was significantly (P-0.010) increased in the ethyl acetate and methanol extract treated groups. (7.80 ± 0.06 and 8.50 ± 0.03 respectively) on the 60th day when compared with the levels (9.40 ± 0.00 and10.0 ± 0.08 respectively) on the 30th day. The cholesterol level was significantly raised in the ethyl acetate and methanol extract treated groups. (78.6 ± 0.00 and 69.6 ± 0.25 respectively) on the 60th day when compared with the levels (70.9 ± 0.05 and 64.2 ± 0.05 respectively) on the 45th day. The triglycerides level was significantly enhanced in the ethyl acetate and methanol extract treated groups. (69. 2 ± 0.11 and 62.4 ± 0.91 respectively) on the 60th day when compared with the levels (58.6 ± 0.84 and 54.6 ± 0.15 respectively) on the 45th day. These results were obtained upon comparison with control group.

In this study, percentage weight gain was notably increased in the fish fed on the 60th day in the ethyl acetate and methanol extract treated groups (37.54 ± 0.29 and 41.03 ± 0.02 respectively).Specific growth rate (%) was significantly on the 60th day in the ethyl acetate and methanol extract treated groups (2.206 ± 0.39 and 3.48 ± 0.51 respectively).The feed conversion ratio (%) was significantly increased on the 60th day in the ethyl acetate and methanol extract treated groups (14.5 ± 0.14 and 28.83 ± 0.80 respectively). These results were obtained upon comparison with the control group and other groups. The application of herbal immunostimulants, in the aquaculture field, offers a notable advantage because of their safe and eco-friendly nature (Jian and Wu, 2004; Dugenci, *et al*., 2003). In the present study, various solvent extracts of the traditional herb garlic (*A. sativum Linn*.) bulb were chosen

because of the ability to enhance immunity and offer disease resistance. The extracts prepared using petroleum ether, toluene, ethyl acetate and methanol were tested against the pathogenic bacterium *P. fluorescens* that affects *C. mrigala.* It has been shown that the powdered extract of *A. sativum* exerts considerable stimulatory effect on the disease resistance and immune mechanism in *Cirrhinus mirgala* fingerlings.

Owing their overall activity immunostimulants seem to be valuable in controlling fish diseases and therefore can be helpful in aquaculture systems. The findings of the present study support the concept that immunostimulant plants can activate fish immune activities under any kind of stress factor (Ardo, *et al*., 2008) and reverse the injurious effects (Sahoo and Mukherjee, 2002, 2003). *A. sativum*, is a vital medicinal plant, that not only has a dynamic effect on the harmful agents but also exerts positive effects on the immune system development and cardiovascular systems (Harris, *et al*., 2001). As a result of garlic-mixed feed application, the red blood cell counts increased, which might suggest an immunostimulant effect (Sahu, 2004). The findings of this work support the report of Sahu, *et al*. (2007). In traditional medicine, onion and garlic have been employed for many years against fungal, viral, parasitic, and bacterial infections. Contemporary chemical characterization of their sulphur components has assessed that they are very active antimicrobial agents (Rose, *et al*., 2005). However, saponins, some proteins and phenolic components can also be responsible for this activity (Griffiths, *et al*., 2002). Due to the strong antimicrobial activity of garlic and onion both could be used as natural preservatives, to prevent microorganism growth (Pszczola, 2002). Delaha and Garagusi (1985) proved that garlic can impede the growth of acid-fast bacteria, Gram negative and Gram-positive bacteria as well as toxin production. The bacteria against which *A. sativum* is effective include strains of *Escherichia coli, Proteus, Pseudomonas, Klebsiella, Salmonella, Staphylococcus aureus, Clostridium Micrococcus, Bacillus subtilis* and *Mycobacterium.* Miron, *et al*., (2000) discerned that *A. sativum* is highly effective against actively pathogenic enterobacteria, probably because of their increased sensitivity to allicin. In addition to organosulphur components, it has been currently recorded that certain quercetin oxidation products present in onion and garlic also exert antibacterial activity against *H. pylori* and *S. aureus*(Ramos, *et al.*, 2006; Marta, *et al*., 2007).

Fazlolahzadeh, *et al*., (2011) and Nya and Austin, (2009) explained that the use of *A. sativum* enhances the immunological functions and improves the hematological parameters in aquatic fish species. Roch (1999) showed that several reactive oxygen species (ROS) are produced by the phagocytic cells of fishes during their respiratory burst activity. Once fungi or bacteria are enveloped by the leucocytes, the host's NADPH-oxidase is activated, which in turn elevates oxygen utilization and latterly produces ROS such as superoxide anion (O_2-·), hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$) and hydroxyl radical (OH). Secombes and Fletcher 1992 reported that the discharge of superoxide anion from the cell is called the respiratory burst activity, and its derivates collectively exert bactericidal effect. Since O_2- is the initial product discharged from respiratory burst activity, its measurement has been accepted as an accurate and direct way of calculating respiratory burst activity (Roch, 1999; Secombes and Olivier, 1997). Kim, *et al*., (2001) and Yang, *et al*., (1993) revealed that after challenge with *A. hydrophila* flashed out decreased nitroblue tetrazolium (NBT) activity when matched with the day-60 before challenged specimens, but the reduction was not powerful. A number of things could have happened but it is reasonable to speculate that the creation of superoxide anions by the *A. sativum* in fishes acted against *A. hydrophila*. It has been present that aqueous extract dried powder and raw garlic are capable of scavenging the superoxide anion and hydroxyl radicals (Kim *et al*., 2001). Yang, *et al*., (1993) and Kim, *et al*., (2001) established that the same types of activities might have existed occurred during their work. Sea basses fed with garlic diets at concentrations of 10 g and 15 g / kg of fish feed exhibit significant elevation in respiratory burst activity, thus, developing their immune system capacity. *A. sativum* contains beneficial therapeutic compounds, such as superoxide anions and hydroxyl radicals, which offer protection against diseases. In this work, the production of superoxide anions and hydroxyl radicals by the *A. sativum* in fishes might have provided protection against the adverse effects of the challenging pathogen. The growth enhancing the functions of many stimulant plants have been proven in aquatic animals (Sivaram, *et al*., 2004; Citarasu, *et al*., 2002). The specific growth ratio and feed conversion rate in fishes fed with garlic were insignificantly ($P > 0.05$) elevated when compared with the control group. The specific growth rate value was increased 32.14%, 36.74% and 37.88% in the treated groups respectively, when compared with control (Shahu, *et al*., 2007).

CONCLUSION

In the present study, feed supplementation with *A. sativum (Linn.)* bulb extract has facilitated valid antibacterial activity against the pathogenic bacterium *P. fluorescens.* This result appears to have been achieved by improvements in immunological, enzymological, biochemical and growth parameters. Therefore, the data were reported in this work assert that incorporating 0.10% *A. sativum* bulb extract in the feed could increase the resistance and also improve the survival rate of the fishes. Further analysis is needed for the identification and isolation of the active constituents in the extracts which could be exploited for fish diet formulation and infection prevention in the fresh water aquaculture ecosystem.

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