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EFFECT OF WATER EXTRACT OF *ACHYRANTHES ASPERA* LINN. LEAVES ON THE NEUTROPHIL ACTIVITY TO HEAT KILLED *AEROMONAS HYDROPHILA* IN *OREOCHROMIS MOSSAMBICUS* (PETERS)

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ABSTRACT

Aquaculture and fishing in India traditionally have strong export markets. But a variety of microbial agents cause diseases in aquaculture systems. *Aeromonas hydrophila* is a important pathogen and it cause a variety of diseases in fish. To overcome such problems immunostimulators can be used as protective and supportive therapy to promote host resistance. Immunostimulants are being used today both within aquaculture sector and in traditional animal husbandary to reduce mortality due to infections and to improve general performance of animals. Recently, there has been increased interest in the immune stimulating function of some herbs in aquaculture. Many studies have proved that herbal additives enhanced the growth of fishes and protected them from various diseases. For this purpose, the specific objectives of the present investigation was to screen the water extracts of *Achyranthes aspera* Linn for their neutrophil activity to heat killed *Aeromonas hydrophila* in *Oreochromis mossambicus.* Generally, the lower doses of leaf extracts administered groups significantly stimulated the neutrophil activity and the higher dose did not showed any significant difference from that of control. The results of the present study indicated, the use of leaf extracts of medicinal plants in aquaculture system has a lot of scope in maintain the health of finfish culture.

Keywords: Immunostimulants, *Achyranthes aspera*, Neutrophil activity, O*eochromis mossambicus*.

INTRODUCTION

Fisheries can be taken as a major economic activity in the country as it supports nutrition, employment and foreign exchange [1]. The farming of fish is the best option for catching fish to feed the growth masses and provides them with alternative livelihood opportunities for their socio-economic foreign exchange department [2] Aquaculture and fishing in India traditionally have strong export markets. Various kinds of marine and fresh water fish have been cultured and the worldwide production of cultured fish increases every year [3]. Fishes are regarded as a potentially a cheap source of protein, especially greater significance to developing countries like India, where problems of nutritional deficiencies persist. As a rich source of nutrient, fishes provide a good balance of protein, vitamins and minerals and relatively low caloric content. Fishes are known to provide several nutritional and therapeutic benefits for health problems like coronary heart disease, hypertension, obesity, and osteoporosis and iron deficiency [4]. Tilapia, *O.mossambicus* [Peters], is an important species for freshwater and marine aquaculture, particularly in developing countries [5]. It is considered as poor man's protein rich in food.

Word wide fish and shellfish culture are subjected to many diseases that lead to great losses and decrease in fish production [6]. Fishes have intimate contacts with their environment, which can contain very concentration of bacterial and viruses. Many of these are saprophytic, some are pathogenic and both very capable of digesting and degrading the fish tissue [7]. Diseases of fish are a great obstacle in the field of fish culture, causes of their population reduction.

Most infectious diseases of fish are opportunistic. This means that the simple presence of the pathogen in the environment of the fish is inadequate to cause a disease outbreak. Other factors usually come into play such that either the pathogen has an advantage over the host or the

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immune system of the host is compromised in some way, increasing its susceptibility to the pathogen. A variety of microbial agents cause disease in aquaculture systems. This includes viruses, fungi and bacteria [8]. One of the major pathogen in India, *Aeromonas hydrophila* is known to cause a variety of diseases in fish, such as haemorrhagic septicaemia, infectious dropsy, trophical ulcerative disease and fin rot leading to heavy mortality in aquaculture farms. *A.hydrophila* has been associated with diseases in fishes like carp, eel, milk fish, channel cat fish, tilapia and opportunist in stress related diseases in Salmonids. [9]. Intensive aquaculture practices have led to a growing interest in understanding fish diseases. So that they can be prevented or treated.

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources [10]. India is a land of rich biodiversity, the total number of lower and higher plants in India is about 45,000 species. The plants are potential source of medicines since ancient times [11]. A large population of India uses plants for its healing, preventive, curative and much therapeutic property together with immunostimulatory property [12]. These natural plant products have been reported to have various properties such as anti-stress, growth promoters, appetizers, tonic and immuno-stimulants [13].

Natural compounds from medicinal plants having antioxidant and immunostimulatory activities have potential as therapeutic agents. Plant extracts can act as an immunostimulant even at low concentrations and hence, its use could be very cost effective. It is biodegradable and environmental friendly [14]. The immunostimulants mainly facilitate the function of phagocytic cells, increase their bactericidal activities, and stimulate the natural killer cells, complement system, lysozyme activity, and antibody responses in fish and shellfish which confer enhanced protection from infectious diseases [15].

Recently, there has been increased interest in the immune stimulating function of some herbs in aquaculture. The non-specific immune functions such as bacteriolytic activity and leucocyte function were improved by some mixtures of Chinese herbs in shrimp (Penaeuschinensis) and tilapia [16]. The herbal medicines seem to have potential to be a rich source of active substances for immunostimulation. These immune stimulants are substances, which increase resistance to infectious disease, by enhancing non-specific defence mechanism and enhance the barrier of infections against pathogen [17].

Achyranthes aspera L. (family: Amaranthaceae), an erect and much branched diffuse herb is a medicinal plant, frequently found in tropical and warmer regions as weed. The plants are reported to contain following major classes of compounds: fatty acids, a number of oleonic acid, bisdesmosidic, triterpenoid based saponins, ecdysterone, n-hexacos-14- enoic, oleanolic acid, triacontanol, spinasterol, dihydroxy ketones, spathulenol, alkaloids, D-glucuronic, Betaine, Achyranthine and various amino acids. This acrid, bitter plant has been used as indigenous medicine and commonly used by traditional healers for the treatment of various diseases. Though almost all of its parts are used in traditional systems of medicines, seeds, roots and shoots are the most important parts which are used medicinally [18].

The objectives of the present investigation were to screen the water extracts of the leaves of the plant *A. aspera* Linn for their immunostimulatory properties with reference to Neutrophil activity to heat killed *A. hydrophila* in *O. mossambicus*.

MATERIALS AND METHODS

Animal maintenance

Experimental fish of both sexes weighing about 30-40g were acclimatized to the laboratory conditions one week before initiating the experiment. They were collected from kullurchanthai dam near Virudhunagar. Experiments were conducted in cement tanks with a capacity of 125liters.The cement tanks were provided with inlet and outlet facilities for exchanging the water. Water was changed on alternate days to avoid stress to ammonia accumulation. The animals were fed ad *labitum* with a balanced fish diet prepared in our laboratory. The excreta and feed were siphoned out to avoid fungal growth. Temperature of the tanks indicated only a minor fluctuation (30±1.5).

Antigen

Bacteria

The bacterial strain *A. hydrophila* was used as an antigen throughout the study. Nutrient agar and nutrient broth (Hi media private limited, Mumbai, India) were used for routine culturing of *A.hydrophila*.

Preparation of heat killed *A.hydrophila*

Single cell of colony of *A.hydrophila* from the agar plate was inoculated in the nutrient broth. The overnight culture of bacterial cells harvested from the broth was subjected to 60^{o}c for 1hr in a water bath and the sterility was checked by inoculating on nutrient agar. The heat killed bacterial culture was centrifuged at 3000rpm for 30 minutes. The packed cells were collected and required dose (10^{8}cells/fish) was prepared in Phosphate Buffered Saline (PBS).

Preparation of leaf extract

Five grams of fresh leaves of *A.aspera* was put in the freezer over night. Once the leaves were taken out, they were crushed immediately and put in 50ml of sterile distilled water and was left soaked for 48 hours. The extract was filtered in a muslin cloth and centrifuged at 100rpm for five minutes to get an extract free of debris. The extract obtained now is 10% (20g) water extract of *A.aspera* leaves [19].

Leaf extract administration and immunization

Six groups of fish(12fish/group) were individually administered with different doses of water extract of leaves viz., 20mg (10%) 2mg(1%), 0.2mg(0.1%), 0.02mg(0.01%), 0.002mg(0.001), 0.0002mgn(0.0001%). The leaf extracts were injected intraperitoneally using 1ml tuberculin syringe with 24

gauge needle. The control group (n=12) were injected only with saline. Two days after the administration of the extract, the experimental and control group were injected individually with 0.2 ml of heat killed *A.hydrophila*.

Serial bleeding

The fish were bled serially using one ml tuberculin syringe with 24 gauge needle from the common cardinal vein situated just below the gills [20] at regular intervals of five days for antibody response. Nearly 0.2 to 0.3 ml of blood was collected at a time from a fish. Leaf extracts administration ,antigen administration and serial bleeding of animals were always done between14 hours and 16 hours to avoid possible influence of circadian rhythmic variation on the immune response [21].The blood was collected in small serology tubes (dia 10mm 75mm).

Antiserum collection

The blood collected from immunized fish was kept at room temperature for 15 minutes. The clot was freed from the wall of serology tube for efficient retraction and was kept overnight at 4^{o}c.The serum was separated by spinning down the clot at 3000 rpm for 15to20 minutes and collected in sterilized storage vials. The serum was kept at47^{o}c in a water bath for 30 minutes to inactivate complement (classical pathway) and stored at 20^{0}c until further use.

Neutrophil activity

Nitroblue tetrozolium assay

The NBT assay was done by the following method [22] with slight modification [23]

Fish were bled at regular intervals of two days. From common cardinal vein 0.2ml of the blood was taken using syringe filled with 20µl of heparinised saline(dissolving 4mg of heparin in 10ml of saline).The blood was collected in silicon coated eppendrof tubes and centrifuged for 20 minutes at 200 rpm. Fifty micro liters of the buffy coat was placed in a 96 well "U" bottom Takatsi plate and incubated for one hour at 37^{0}C to facilitate the adhesion of cells. The supernatant was removed. To this cell suspension,50µl of NBT(0.03%)(dissolving 3mg of NBT in 1 ml of distilled water) was added and incubated for at 1hour at room temperature. Then NBT was removed. The cells were fixed with 100% methanol and washed thrice with 70% methanol. The plates were allowed to dry and the formazan produced inside the cells were solubilized in 60µl of 2N Potassium hydroxide(KOH) and 70µl Dimethyl Sulfoxide (DMSO).The OD was measured at 655nm using microplate reader (Model 680,Bio-rad,USA).

Statistical analysis

The data was expressed as arithmetic mean ±S.E. Analysis of varience (one way) and student's t-test were employed for statistical analysis [24].

RESULT AND DISCUSSION

Effect of water extract of *A. aspera* on the Neutrophil activity to heat killed *A. hydrophila* in *O. mossambicus*, From the result obtained (Table:1) it is evident that *For both the control and experimental groups the peak day was on 6^{th} day post immunization except the dose of 0.002mg.

*The dose of 0.002mg administered group showed enhancement on the neutrophil activity significant by at (P<010) on 6^{th} day and (P<0.001)on 8^{th} day post immunization than control.

*The dose of 0.2 mg showed enhancement on the neutrophil activity significance at(P<0.001)&the doses such as 0.02 and 0.0002 mg showed significant(P<0.005)on peak day.

*The doses such as 20 and 2mg showed no significant effect than control on the peak day.

A.aspera was found to possess antiarthritic, antimalarial, antibacterial and antiviral activity. It contains alkaloids, flavonoids, saponins, steroids and terpenoids [25].An alcohol extract of *A.aspera* showed anti-inflammatory activity on carrageenin-induced paw oedema and cotton Pellet granuloma models in albino rats(26).The aqueous solution of the base achyranthine as well as the entire plant of *A.aspera* showed antibacterial activity against *S.aureus*, *S.heamolyticus* and *B.trphosus* [27].While the alcoholic and the aqueous extract of the leaves showed antibacterial activity against *S.aureus* and *E.coli*. The ethanol extract of the root was screened for antifertility activity to proven fertile albino rats [28].

The nitroblue tetrazolium (NBT) reduction assay estimates the ability of neutrophils and macrophages to produce oxygenic radicals .The ability of macrophages to kill the pathogenic microbes is probably one of the most important mechanisms of protection against diseases in fish. Increased respiratory burst activity i.e production of oxygen radicals can be correlated with increased killing activity [29]. During the functioning of the immune system, such as in phagocytosis, reactive oxygen and nitrogen species are generated. If they are unchecked they can affect the components of the immune system by inducing oxidative damage.

Table 1. Summary of 't' test for the effect of water extract of *A.aspera* leaves on the neutrophil activity to heat killed *A. hydrophila* in *O. mossambicus* on the peak day

S.No	Dose tested (compared to control)	Degrees of freedom	't' Value	Table value	'P' Value
1	20mg	10	1.25	2.228	P>0.05
2	2mg	10	0.45	2.228	P>0.05
3	0.2mg	10	6.28	4.587	P<0.001***
4	0.02mg	10	4.5 2	3.581	P<0.005**
5	0.002mg	10	3.44	3.169	P<0.010*
6	0.0002mg	10	3.86	3.581	P<0.005**

***Significance level at 0.1% level; **Significance level at0. 5% level ; * Significance level 1% level.

This is more so in the elderly or during inflammation where there is excess generation of these reactive species than can be taken care of by the defenses in the form of antioxidants. There are some indications of possible benefits of antioxidant supplementation [30].

CONCLUSION

In the present investigation [Tab:1] neutrophil activity of water extract of *A.aspera* leaves, the dose of 0.2mg showed statistically significant enhancement (P<0.001) & the doses such as 0.02 and 0.002 mg showed enhancement (P<0.005) and the dose 0.002 mg showed enhancement (p<0.010) on 6^{th} day, the same dose persistence (P<0.001) until on 8^{th} day post immunization than control. The highest dose such as 20 and 2mg was no statistically significant effect than control on sixth day post immunization. The similar study was reported by [31] that ethanol extract of *T.cordifolia* doses of 0.8, 8 and 80mg/kg significantly enhanced the neutrophil activity on day 8 whereas the petroleum ether extract was not very effective in enhancing the neutrophil activity. However, 0.8mg, the lowest dose of petroleum ether extract tested increased the level of neutrophil activity on day 2 and 8. The results revealed enhancement the number of activated neutrophils when compared with control. The use of leaf extracts of medicinal plants in aquaculture system has a lot of scope in maintaining the health of finfish because of their easy availability and application. The use of medicinal plants as immunostimulants prevent disease outbreak in aquaculture systems and then by increasing fish production.

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