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Antioxidant enzyme activity of *Aeromonas* infected *Catla catla* under the exposure of *Couroupita guianensis* fruit extract

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ABSTRACT

Aquaculture has become the most important part in supplying the source of animal protein, in addition to husbandry for terrestrial animals. For instance, Rohu is a preferred carp variety in most part of the due to its highest market demand whereas *Catla* is also preferred in the carp farming community owing to its fast growing nature. Diseases are recognized as one of the major constraint to sustainable animal production which can cause significant economic loss especially in aquaculture. Diseases in fish caused by pathogenic bacteria, particularly *Aeromonas* are most widespread. Septicaemia caused by motile *Aeromonads* is a ubiquitous problem that affects fishes found in warm, cool and cold fresh water around the world. In the present investigation has aimed to study the efficacy of a *Couroupita guianensis* fruit extracts antioxidant activity such as catalase and SOD characteristics in an edible fish infected with the bacterium *Aeromonas hydrophila*. After infection the enzyme concentration will be increased. The post infective *Catla* fish show decrease the enzyme concentration by the treatment of *Couroupita guianensis* fruit extract. Conclusion of this present study is *Couroupita guianensis* fruit extract drastically decrease the *Aeromonas hydrophila* infection of *Catla catla*.

Keywords: Aquaculture, *Aeromonas hydrophila, Catla catla*, *Couroupita guianensis*, Superoxide dismutase

INTRODUCTION

Aquaculture has become the most important part in supplying the source of animal protein, in addition to husbandry for terrestrial animals. One marine species cultured with high economic value is humpback grouper (*C. altivelis*), which has been intensively applied in several places in Indonesia. However, intensive aquaculture practices were facing various obstacles related to growth rate, feed conversion and diseases, thus limiting their production. Humpback grouper nutrition has been widely researched (Shapawi et al., 2014; Williams, 2009). Aquatic animal diseases are the most significant constraints to the development and management in aquaculture (Subasinghe, 2009). India has a long coastline of 8118 km and equally large areas under estuaries, backwaters, lagoons etc., conducive for developing capture as well as culture fisheries. The inland fishery resources include 1.96 lakh kms stretch of rivers and canals, 29.07 lakh hectare reservoirs, 24.40 lakh hectare ponds and tanks, 7.98 lakh hectare of beels, derelict water bodies and 12.40 lakh hectare brackish water areas. Fish farming is profitable only when fishes are reared free from diseases. Diseases bring mass mortality of fishes and heavy economic loss. The amount of mortality is variable among ponds ranging from a few to large number of fish, but high mortality is the exception. There are three main environmental concerns with dead fish carcasses and associated bacteria may be released from ponds if outflow of water occurs decomposition of dead fish following sudden, massive mortality could impair in-pond water quality and cause effluent water quality to decline; and where ponds are near dwellings, bad odour from large fish kills could be a problem (Salinas, et al., 2004). *Aeromonas* sp. is a ubiquitous inhabitant of aquatic ecosystems such as freshwater, coastal water and sewage. These bacteria are usually microbiota as well as primary or secondary pathogens of fish and amphibians. Some motile species of *Aeromonas* such as, *Aeromonas caviae*, *A. hydrophila* and *A. veronii* are opportunistic pathogens of humans.

Among the species belonging to *Aeromonas* genus, the most important species is *A. salmonicida*, a fish pathogen which causes a common disease among salmonids, named furunculosis or ulcerative furunculosis (Seethalakshmi, et al., 2008). *Aeromonas* species are gram negative bacilli which have worldwide distribution they can cause disease in fish, reptiles and amphibians (Wen-Chien and Yin-Ching, 1995). *Aeromonas* have been found in brackish, fresh, estuarine, marine, chlorinated and dechlorinated water supplies worldwide with highest numbers obtained in the warmer months. *Aeromonas* is responsible for the disease known as motile aeromonad septicaemia and is considered as one of the most important pathogens of fresh water fish (Bullock and McLaughin, 1970). One of the major bacterial pathogens in India, *Aeromonas hydrophila* is known to cause a variety of diseases in fish, such as, haemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease and fin rot leading to heavy mortality in aquaculture forms (Karunasagar et al., 1997). *Catla catla* and *Labeo rohita* contribute a major portion to the freshwater fish production in South India. The Indian major carp *Catla catla* mainly inhabits in rivers. The application of medicinal plants as natural and innocuous compounds has potential in aquaculture as an alternative to antibiotics and immune-prophylactics. The growing interest in these plants has increased world-wide because they are easy to prepare, cheap and have few side effects on animals and the environment. A wide range of medicinal plants such as, herbs, spices, seaweeds, herbal medicines, herbal extracted compounds, traditional chinese medicines and commercial plant derived products has been studied in various aquatic animals (Hai, 2015).

Enzymes play an important role in metabolism. They are efficient and very specific in terms of nature of reaction catalyzed and the substrate utilized. Their synthesis and final concentration are under genetic control and is greatly influenced by very small molecules of substances. These cellular catalysts control the formation of biochemical intermediates essential to all physiological functions. Hence, the present investigation has aimed to study the efficacy of a *Couroupita guianensis* fruit extracts modify the composition of antioxidant enzymes in a *Catlsa* fish infected with the bacterium *Aeromonas hydrophila*.

LITERATURE REVIEW

Preparation of Fruit Extract

The fruits were collected and dried under shade at room temperature, powdered and extracted in Soxhlet apparatus. 25 grams of fruit powder were extracted in Soxhlet apparatus for 8 hours over a mantle heating at 60°C using methanol as solvent. The extract was filtered and then concentrated. After complete evaporation of the solvent, residue of extract was stored in a refrigerator and used whenever needed after re-dissolving in methanol, following the method of Peach and Tracey (1956).

Preparation of Live Bacteria

For the present study *Aeromonas hydrophila* (strain: MTCC 646) was obtained from Institute of Microbial Technology, Chandigarh, India, where it was previously isolated from diseased fish. The strain was inoculated in 50 ml of nutrient broth in a conical flash. Sub cultures were maintained on Tryptone Soya broth (Himedia).

Intraperitoneal Injection of *A. hydrophila*

A. hydrophila was harvested from the original stock culture and diluted in 0.85% sterile saline. Appropriate volume (0.1 ml per 100 g fish) of the selected serial dilition (10^5 CFU/ml) was injected intraperitoneally, using a tuberculin syringe into the fish. Ten fishes (11 ± 1 g) were inoculated with of the chosen dose of the pathogen. The volume of the medium was 3 lit water/10 g fish. Mortality was monitored continuously for 10 days and dead fish, if any, were removed with a forceps once in 2 hrs. The fish was considered dead, when there was no respiratory gill movement and no response to gentle prodding. Three replicates were maintained. The medium was not changed or aerated during this period. 0.1 ml of 10^5 CFU/ml of *A. hydrophila* was prepared and intraperitonially injected for negative control and experimental fishes.

Collection of Blood

The blood samples were collected seven days after inoculation or administration of *A. hydrophila* from the control and experimental groups. The blood samples were collected from the cardinal vein puncture using sterilized 2 ml plastic syringe. Care was taken to avoid foaming when drawing the blood, because foaming would result in haemolysis.

Antioxidant Enzyme Activity Assay

The enzymic antioxidants analyzed in the parts of *Catla catla* were super oxide dismutase, catalase. The antioxidant status in precision-cut liver exposed to oxidative stress and confirming the results in experimental animals.

Catalase Assay

Catalase (CAT) is a common enzyme found in nearly all organisms that are exposed to oxygen and its function is to catalyze the decomposition of H_2O_2 to O_2 and H_2O. It is a very important enzyme in protecting the cell from oxidative damage by Reactive Oxygen Species (ROS). Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water

and oxygen each second. Catalase activity was assayed following the method of Aebi (1974).

Preparation of Enzyme Extract

20% homogenate of the liver tissue part of *Catla catla* was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay.

Assay

H_2O_2 phosphate buffer (3.0 ml) was taken in an experimental cuvette, followed by the rapid addition of 40 µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer. The enzyme solution containing H_2O_2 free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

To do the calculations:

(Decrease in absorbance × 100/1) divided by protein amount in mg divided by time in min=units/mg protein/ min.

Superoxide Dismutase Assay

SOD was assayed according to the method of Kakkar et al.

Preparation of Enzyme Extract

A 20% homogenate of the liver tissue part of *Catla catla* (0.5 g), were ground with 3.0 ml of potassium phosphate buffer, centrifuged at 2000 rpm for 10 minutes and the supernatants were used for the assay.

Assay

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2 ml of the enzyme preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

To do the calculations:

% inhibition of NBT reduction by SOD=((Control Ab-Treatment Ab) × 100)/Control Ab

Units/ml enzyme=(Percent inhibition) (DF)/(50%)(0.10)

DF=Dilution Factor

50%=Inhibition of the rate of SOD reduction per the unit.

0.10=Volume (in milliliters) of enzyme used in each test.

RESULTS

In the present study, the cumulative percentage of catalase, super oxidase dismutase, were studied in *Catla catla* intraperitoneally injected with 0.1 ml of 10^5 CFU/ml of *A. hydrophila* and treated with different concentrations (10, 20 and 30 mg/g) of *Aegle marmelos* and *Couroupita guianensis* extracts formulated diet.

Catalase Activity

The catalase enzyme activity (U/mg protein) of *Catla catla* inoculated with 0.1 ml of 10^5 CFU/ml of *Aeromonas hydrophila* and treated with different concentrations of the fruit extract of *Aegle marmelos* was carried out and presented. The positive control that are non-inoculated with bacterium and non-treated with the fruit extract the catalase activity has been found fluctuation value from 98.67 ± 0.50 on 0 day to 98.04 ± 0.25 on 35th day of the treatment. The negative control fishes shown (inoculated with the bacterium *Aeromonas hydrophila* and non-treated fruit extract), increased from increasing days of treatment in the enzyme activity from 94.53 ± 0.30 on 0 day to 106.33 ± 0.25 on 35th day of treatment. In the experimental fishes that are inoculated with bacterium and treated with 10 mg/100 g diet showed 95.00 ± 0.50 on 0 day of treatment that has further decrease due to reduce bacterial stress upto 86.34 ± 0.82 on 35th day of treatment. Similarly the experimental fishes that are inoculated with bacterium and treated with 20 mg/100 g diet showed 95.20 ± 0.65 on 0 day of treatment that has further decrease due to reduce bacterial stress upto 81.33 ± 0.11 on 35th day of experimental period and at other doses of 30 mg/100 g diet treatment from 95.80 ± 0.55 on 0 day that has further decrease due to reduce bacterial stress upto 88.18 ± 0.34 on 35th day have been found. The results were statistically significant at $p<0.05$ (t-test).

The catalase enzyme activity (U/mg protein) of *Catla catla* inoculated with 0.1 ml of 10^5 CFU/ml of *Aeromonas hydrophila* and treated with different concentrations of the fruit extract of *Couroupita guianensis* was carried out and presented. The positive control that are non-inoculated with bacterium and non-treated with the fruit extract the catalase activity has been found fluctutation value from 98.67 ± 0.50 on 0 day to 98.04 ± 0.25 on 35th day of the treatment. The control fishes shown (inoculated with the bacterium *Aeromonas hydrophila* and non-treated fruit extract), increased from increasing days of treatment in the enzyme activity from 94.53 ± 0.30 on 0 day to 106.33 ± 0.25 on 35th day of treatment. In the experimental fishes that are inoculated with bacterium and treated with 10 mg/100 g diet showed 96.23 ± 0.43 on 0 day of treatment that has further decrease due to reduce bacterial stress upto 94.56 ± 0.57 on 35th day of

treatment. Similarly the experimental fishes that are inoculated with bacterium and treated with 20 mg/100 g diet showed 96.20 0.18 on 0 day of treatment that has further decrease due to reduce bacterial stress upto 8 9.33 ± 1.00 on 35th day of experimental period and at other doses of 30 mg/100 g diet treatment from 95.93 ± 0.42 on 0 day that has further decrease due to reduce bacterial stress upto 86.96 ± 0.67 on 35th day have been found. The results were statistically significant at $p<0.05$ (t-test).

Superoxidase Dismutase Activity

The superoxidase dismutase activity (U/mg protein) of *Catla catla* inoculated with 0.1 ml of 10^5 CFU/ml of *Aeromonas hydrophila* and treated with different concentrations of the fruit extract of *Aegle marmelos* was carried out and presented. The positive control that are non-inoculated with bacterium and non-treated with the fruit extract the superoxidase dismutase activity has been found more or less stable value from 17.67 ± 0.50 on 0 day to 17.22 ± 0.05 on 35th day of the treatment. The negative control fishes shown (inoculated with the bacterium *Aeromonas hydrophila* and non-treated fruit extract) increased from increasing days of treatment in the superoxidase dismutase activity from 14.53 ± 0.30 on 0 day to 21.36 ± 0.25 on 35th day of treatment. In the experimental fishes that are inoculated with bacterium and treated with 10 mg/100 g diet showed 26.14 ± 0.34 on 0 day of treatment that has further decrease due to reduce bacterial stress upto 19.48 ± 0.12 on 35th day of treatment. Similarly the experimental fishes that are inoculated with bacterium and treated with 20 mg/100 g diet showed 26.34 ± 0.13 on 0 day of treatment that has further decrease due to reduce bacterial stress up to 16.00 ± 0.32 on 35th day of experimental period and at other doses of 30 mg/100 g diet treatment from 26.15 ± 0.24 on 0 day that has further decrease due to reduce bacterial stress upto 20.32 ± 0.57 on 35th day have been found. *Aegle marmelos* fruit extract treated fishes showed highly protective the fishes and the enzyme activity is decreased due to reduce bacterial stress.

The superoxidase dismutase enzyme activity (U/mg protein) of *Catla catla* inoculated with 0.1 ml of 10^5 CFU/ml of *Aeromonas hydrophila* and treated with different concentrations of the fruit extract of *Couroupita guianensis* was carried out and presented. The positive control that are non-inoculated with bacterium and non-treated with the fruit extract the superoxidase dismutase activity has been found more or less stable value from 17.67 ± 0.50 on 0 day to 17.22 ± 0.05 on 35th day of the treatment. The negative control fishes shown (inoculated with the bacterium *Aeromonas hydrophila* and non-treated fruit extract), increased from increasing days of treatment in the superoxidase dismutase activity from 14.53 ± 0.30 on 0 day to 21.36 ± 0.25 on 35th day of treatment. In the experimental fishes that are inoculated with bacterium and treated with 10 mg/100 g diet showed 27.18 ± 0.45 on 0 day of treatment that has further decrease due to reduce bacterial stress up to 24.02 ± 0.09 on 35th day of treatment. Similarly the experimental fishes that are inoculated with bacterium and treated with 20 mg/100 g diet showed 27.34 ± 0.53 on 0 day of treatment that has further decrease due to reduce bacterial stress up to 18.62 ± 0.32 on 35th day of experimental period and at other doses of 30 mg/100 g diet treatment from 27.42 ± 0.21 on 0 day that has further decrease due to reduce bacterial stress up to 17.19 ± 0.13 on 35th day have been found. *Couroupita guianensis* fruit extract treated fishes showed highly protective the fishes and the enzyme activity are decreased is due to reduce bacterial stress.

DISCUSSION

In the present study, the experimental fishes treated with *Aegle marmelos* and *Couroupita guianensis* formulated diet showed antioxidant enzyme activity was decreased with increasing days of treatment in all concentrations than the control discussed below.

Catalase Assay

Catalase is a tetrameric haemin enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa. Therefore, it contains four ferriprotoporphyrin groups per molecule; its molecular mass is about 240 kDa. Catalases are found in nearly all living organisms exposed to oxygen. This enzyme protects the cellular environment against the oxidative stress by utilizing H_2O_2 which reduces the harmful effect of this reactive oxygen compound by converting it into water and oxygen. The three families of catalases are Mn catalases, bi-functional catalase-peroxidases and monofunctional or true catalases. The catalases and several peroxidases catabolise H_2O_2 in the aerobic organisms and protect the cells from H_2O_2 generated within them. Winston and Giulio, reported that the antioxidant activities can be modulated by several factors including the parasitic infections which can disturb the metabolic pathways of its host. Therefore, the increase or decrease in different antioxidant enzymes in liver of infected *C. catla* during the present study can also be linked to the modulated metabolic activity in the presence of *A. hydrophila*. According to Tabatabaie and Floyd, the enzyme activity can also be decreased by negative feedback from excess of the substrate or it damages by oxidative modification. Modulation of oxidative stress due to altered metabolic processes is found to vary between different organs in the infected fish.

The result was supported with Liu et al. They reported that the catalase activity was increased in control and the activity was decreased in Total Dissolved Gas Supersaturation (TDGS) treated fishes rock carp (*Procypris rabaudi* Tchang). SOD and CAT activity are the most widely used measures of oxidative stress. Superoxide anion production and serum bactericidal level

were enhanced in fingerling, rohu *L. rohita* fed with diet enriched with Achyranthus at different doses (Rao et al., 2006). Both fruit extract treated fishes showed highly protective the fishes and the enzyme activity is decreased is due to reduce bacterial stress. The enzyme activity decreased with increasing days of treatment when compared with control. In the present investigation, we find out the antioxidant phytochemical, such as, 9-Octadecanoic acid, propanoic acid and n-Hexadecanoic acid (palmatic acid, 9.22%) found in the *Aegle marmelos* and *Couroupita guianensis* fruit extracts. These compounds may protect as an indication of elevated oxidative stress due to *A. hydrophila* infection.

Super Oxide Dismutase (SOD)

SOD is an oxido-reductase enzyme which catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich, 1989). SOD enzymes are present in all aerobic cells and in the extracellular fluids and are essential for the survival of oxygen utilizing organisms. SOD also protects the different cells against the toxic effects of superoxide radicals (Fridovich, 1978). It can be categorized into three different classes namely (copper-zinc, manganese and iron) on the basis of metal content in them (Fridovich, 1975). In the present study, the different concentrations of fruit extract formulated diet treated fishes showed the super oxide dismutase activity was gradually decreased with increasing days of treatment. The result was suppored by Metwally. The effect of garlic (*Allium sativum*) on antioxidant system in the fish tilapia nilotica (*Oreochromis niloticus*) treatment groups had a diffrerent sources of garlic added to their diets (natural garlic 40 g/kg, garlic oil capsules 250 g/kg and garlic powder tablet 32 g/kg) at 90 days experiments. Results indicated glutathione peroxidase, superoxide dismutase, alkaline phosphatase, aspartate aminotransfrase and alanine ainotransferase decreased stress all treated groups of garlic. Ivestigator concluded that addition of garlic in any for of fish diet can promote decrease mortality rate and increase the antioxidant activity in fish. SOD catalyses the dismutation of the superoxide anion radical to H_2O and H_2O_2 which is then detoxified by catalase. The increased activity of SOD and catalase in the liver and intestine of the infected fish in the present study is an indication of elevated oxidative stress due to Aeromonas hydrophila infection. Similarly Jordanoska, et al. studied the fish were subject of activities of Superoxide Dismutase (SOD) and Catalase (CAT) in the blood of fish and marker to prooxidant effects of different pollutants present in the aquatic environment. The results indicated that decrease of superoxide dismutase activity and increase of level of catalase enzyme in several samples. Superoxide Dismutatase (SOD) and Catalase (CAT) have been detected in a wide variety of mammalian cells. These enzymes play important roles in protecting the cell against the potentially toxic effects of environmental pollutants (Kuthan et al., 1986). Superoxide dismutase catalyzes the dismutation of the superoxide ion (O_2^-) to hydrogen peroxide and oxygen molecule during oxidative energy processes. The reaction diminishes the destructive oxidative processes in cells. The level of antioxidant enzymes have been extensively used as an early warning indicator of lake pollution (Lin, et al., 2002).

According to Zikic, et al. cadmium induces the appearance of anemia and alters the metabolism of carbohydrates and proteins in goldfishes. Their results also show the decreased activity of SOD in erythrocytes of goldfishes during acute exposure to cadmium, which indicates the presence of ROS-induced peroxidation, which leads to the destruction of RBC membrane. The freshwater silver catfish infected by *Clinostomum detruncatum* has been reported to induce oxidative stress, without inducing the significant changes in the activities of SOD and catalase in the muscle (Bello et al., 2012). Increased antioxidant enzyme activities in GST, GR and catalase in liver and head kidney of infected common carp have been reported by Dautremepuits, et al. It is postulated that the parasitic infection can lead to an increase in activities of antioxidant enzymes in the hosts which is a type of strategy to reduce the host oxidative response by the parasite and this helps in their long-term survival inside the host. From this study the fruit extracts are reduced the bacterial stress due to reduce the enzyme activity in all treatment when compared with control.

CONCLUSION

Efficacy of fruit extracts (*Aegle marmelos* and *Couroupita guianensis*) supplementation diet on antioxidant enzymes (CAT and SOD) swere studied in Indian major carp *Catla catla* against *Aeromonas hydrophila*. The catalase and superoxide dismutase activity were increased with increasing days of treatment and the enzyme activity was decreased with increasing concentrations of both fruit extract treatment. Based on the results it can be concluded that the fruit extract formulated feed used have great potential for against pathogen. They can be used in the treatment infectious diseases by facilitating the function of phagocytic cells and immune system to enhance the immunity level. Further investigations are needed to fully understand the interaction between this herb and different fish species even on the molecular level.

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