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PROTECTIVE EFFICACY OF *Tribulus terrestris* ON ATRAZINE EXPOSED FRESH WATER FISH *Oreochromis mossambicus* (W.K.H Peters, 1952)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Atrazine is considered moderately toxic to aquatic animals. In the present study protective effect of *Tribulus terrestris* on hepatotoxicity in fresh water fish *Oreochromis mossambicus*, at sub lethal concentration of atrazine (16.5mg/l) exposure fish for 120h was studied. After completing the exposure time experiment were carried out to find the level of lipid peroxidation, reduced glutathione, superoxide dismutase, catalase and glutathione peroxides (LPO, GSH, GPx, SOD, and CAT) activities in liver tissues. The results showed increases LPO and decreased level of GSH, GPx, SOD, and CAT when compared to normal. During the recovery period *Tribulus terrestris* (1.2g/l) plant exposure drastically restored to the normal level when compared to atrazine treatment. Present investigation concluded that the *Tribulus terrestris* significantly alters the metabolic activity in liver tissues of atrazine exposed fishes.

Keywords: LPO; GSH; SOD; CAT; herbicide; *Oreochromis mossembicus*; *Tribulus terestris*.

1. INTRODUCTION

Environment is that the interrelationships of water, air and land, the human being, different living organisms and other property. It includes all the physical and biological surroundings and their interactions. The environmental degradation crosses the limit so that. It becomes lethal to living organisms [1]. Aquatic ecosystems that run through agricultural and industrial areas have high probability of being contaminated by runoff and ground water leaching by a variety of chemicals [2]. Ventura et al. [3] reported that pesticides are presents in aquatic environments can affect aquatic living organisms in different ways. Agricultural pesticides are released into the atmosphere in different ways by the spray drift, post application, volatilization and wind erosion of soil [4]. In India, pesticides constitute an important

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components in agricultural development and protection of public health since the tropical climate is very conducive to pest breeding [5,1]. In the aquatic ecosystem is a serious problem and fish are more frequently exposed to these pollutants and may be taken in through the gills, skin and contaminated food [6]. The application of environment toxicology studies on aquatic living organism especially fish is rapidly expanding for the evaluation of the effects of environmental contamination by noxious compounds [7]. Fish is included as the main component of human food chain with the high nutritional value, however, they are very sensitive to the environmental changes, and are often used as bio-indicators to detect the degree of pollution in aquatic environment [8].

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is one of the most commonly usable herbicides and it's found in rural environments. It was first introduced in twentieth century and often used alone or in amalgamation with other herbicides for agricultural applications [9,10,11]. It is the second most widely consumed pesticide in the world with annual consumption of about 70,000–90,000 tons [12]. In India, about 340 tones of atrazine are consumed annually [13,14,15]. It is extensively used for corn, sorghum, sugarcane, pineapples, and other some extent on landscape vegetation [16,17]. Rated as moderately toxic elements to aquatic environment species, atrazine is mobile in to the environment and it's among the most detected pesticides in streams, rivers, ponds, reservoirs and ground waters. Recent studies have indicated that the pesticide toxicity in fish may be related to an increased production of reactive oxygen species (ROSs), leading to oxidative damage [55]. Atrazine increase chromosomal abnormalities in lymphocytes of exposed to atrazine [18]. Patalac et al. [56] suggested that many chlorinated pesticides, including atrazine, trigger breast cancer development by affecting the metabolism. Estradiol is metabolised 16-alpha-hydroxyestrone (C_{16}), which strongly activates the estrogens receptor, promoting breast cell proliferation, and 2-hydroxyestrone which weakly interacts with the estrogens receptor without triggering the growth promoting genes. High levels of C_{16} have been linked to DNA damaged and increased to risk of breast cancer [19]. Atrazine could cause damage the renal excretion of sodium, chloride and protein in the rainbow trout and carp [20].

Tribulus terrestris an annual herb of the Zygophyllaceae family used for the treatment of various diseases [21] its commonly used traditional Chinese-Mongolian medicine. Its mainly affected constituent is terrestrosin, which is primarily composed to steroid saponins [22,23,24]. Modern pharmacological research has demonstrated the effects of steroid saponins against tumours, cardiovascular and cerebrovascular diseases, senescence, and inflammation [25,26,27]. The significant anti-inflammatory effects of steroid saponins originate from the N-trans-q-caffeoyl tyramine structure [28].

In this present studies was investigated to oxidant and antioxidant activities were examined using the standard methods.

2. MATERIALS AND METHODS

The fresh water fish *Oreochromis mossambicus* were collected from surroundings of Cheyyar Town, Thiruvannamalai District. The collected fish was acclimated to laboratory condition for 15 days. They were checked thoroughly for injury and disease conditions, and only healthy fishes were used for this study. After washing with 0.01% KMnO4 solution for 15 min, they were placed in nine plastic pools (500L) containing non-chlorinated water. Prior to the start of the experiment, the fishes were acclimatized to food and laboratory conditions with 12h dark and 12h light cycles, pH range of 6.95 to 7.60 and temperature ranging from 16 to 24°C for 15 days. The fishes measuring 16-20cm in length and 80-100g n weight were selected irrespective of their sex for the experiments. Fishes were divided into four equal groups each comprising of 25 fishes. Each group was kept in separate plastic tanks. The first group was kept as negative control; the fishes were maintained in water containing normal water without any treatment. The second group of fishes was exposed to a sub-lethal concentration of (16.5mg/l) of atrazine added in the water for 24, 48, 72, 96 and 120 h respectively. And third groups of fishes were treated with *T. terrestris* (1.2g/l) followed by atrazine exposure. Fourth group of fishes were treated in only T. terrestris only. Solutions were renewed once daily after exposure period, animals were sacrificed and isolated for their liver tissues, homogenized and stored at −80°C for further biochemical analyses.

2.1 Supplement Feed

Health disease free plant of *T. terrestrisis* were collected from around the areas cheyyar, the plant was identified. The plants were washed in running tap water for 10 minutes and were dried, 1kg of *T. terrestrisis* were macerated thrice at room temperature and prepared n powdered form and equal amount of rice bran was mixed well and small

amount of water was added and small pellet were made.

2.2 Estimation of Lipid Peroxidation (TBARS)

The concentration of TBARS in the selected liver tissue was estimated by adopting the method of Nichans and Samuelsen (1968) [54]. Known amount of whole kidney tissue homogenate was prepared in Tris-HCl buffer (pH 7.5). 1ml of the tissue homogenate was taken in a clean test tube and 2.0ml of TBA-TCA-HCL reagent was added and then mixed thoroughly. The mixture was kept in a boiling water bath (60ºC) for 15 minutes. After cooling, the mixture was taken to read the absorbance of the chromophore at 535nm against the reagent blank in a UV-visible spectrophotometer (Spectronic-20, Bausch and Lamb). 1, 1', 3, 3' tetra methoxy propane was used to construct the standard graph. Values were expressed as **n** moles of MDA released / 100 mg.

2.3 Estimation Reduced Glutathione (GSH) Activity

The level of reduced glutathione in the selected liver tissue was estimated by the method of Ellaman (1959) [32]. A known weight of tissue was homogenized in phosphate buffer (0.1M. pH 7.0) and centrifuged at 2500 rpm for 5 minutes. 0.2ml of the sample (Supernatant) was taken in a clean test tube and 1.8 ml of EDTA solution was added. To this 3.0 ml of precipitating reagent was added and mixed thoroughly and kept for 5 minutes before centrifugation at 3000 rpm for 10 minutes. In a clean test tube, 2.0ml of the content mixture was taken and to this 4.0ml of 0.3M disodium hydrogen phosphate solution and 1.0ml of DTNB reagents were added. The appearance of yellow colour was read at 412nm in UV-visible spectrophotometer (Spectronic-20, Bausch and Lamb). A set of standard solution containing 20-100µg of reduced glutathione was treated similarly. Values are expressed as µg/100mg protein.

2.4 Estimation of Superoxide Dismutase (SOD) Activity

Superoxide dismutase in the selected liver tissue was assayed by adopting the method of Kakkar [29]. The kidney tissue was homogenized with 2.0ml of o.25M sucrose solution and the centrifuged the contents at 10,000 rpm for 30 minutes in a cold centrifuge. After completing the centrifugation the supernatant was taken in a clean test tube and the content was dialysed against the Tris-HCl buffer and then mixed the contents thoroughly. The contents were centrifuged again at 3000 rpm for 15 minutes. The supernatant was taken in a clean test tube and then 1.2ml of sodium pyrophosphate buffer, 0.1ml of phenazine methosulphate and 0.3ml of nitroblue tetrazolium reagents were added. The sample mixture (enzyme preparation) was kept in water bath at 30ºC for 90 seconds and appropriately diluted enzyme preparation in a total volume of 3 ml with double distilled water. The reaction was started by the addition of 0.2ml NADH. After completing the incubation period, the reaction was stopped by the addition of 1m1 glaicial acetic acid. The reaction mixture was stirred vigorously and shaken with 4ml n-butanol. The mixture was allowed to stand for 10 min and then centrifuged the contents at 3000 rpm for 5 minutes and n-butanol layer was separated the colour density of the chromogen in n-butanol was measured in an UV spectrophotometer at 510nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit. The specific activity of the enzyme was expressed as unit/min/mg of protein for tissues.

2.5 Estimation of Catalase (CAT) Activity

The activity of catalase in the selected liver tissue was determined by the method of Sinha [30]. Tissue homogenate was prepared by phosphate buffer (0.01 M. pH 7.0) in a clean test tube 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate and 0.4ml of hydrogen peroxide were added. The reaction was arrested after 15, 30, 45 and 60s by adding 2.0ml of dichromate -acetic acid mixture. The tubes were kept in a boiling water bath for 10 min. and then cooled with the help of tap running water and the colour developed was read at 620 nm in an UV spectrophotometer. Standards in the concentration range of 20-100µ mol were taken and preceded as for the test. The specific activity was expressed as µ mol of H_2O_2 consumed/min/mg of protein for tissues.

2.6 Estimation of Glutathione Peroxidase (GPx) Activity

The activity of GP_X in the selected liver tissue was measured by the method of Rotruck [31]. The known quantity of whole liver tissue was homogenized with tris buffer. After completing the homogenization the content was centrifuge at 2500rpm for 5 minutes. 0.2ml of supernatant was taken in a clean test tube and then 0.2 ml of EDTA and 0.1 ml of sodium azide reagents were added. By lateral shaking the test tube the above said reagents were mixed well. To the mixture, 0.2ml of GSH followed by 0.1ml of H_2O_2 reagents was added. The contents were mixed well thoroughly and incubated at 37ºC for 10 min and then

0.5ml of 10% TCA was added. Simultaneously reagent blank was also used which is containing all reagents except tissue homogenate. The contents were centrifuged and then supernatant was used for GSH assay by using the method of Ellman [32]. The activity was expressed as µ mol of GSH consumed /min/mg of protein tissues.

2.7 Statistical Analysis

Values are given as mean ± S.D. for 25 fishes in each group. The data for various biochemical parameters were analyzed using analysis of 't'-test and the group means was compared by Duncans [33] multiple range test (DMRT).Values were considered statistically significant when $p<0.05$ and the values sharing a common superscript did not differ significantly.

3. RESULTS AND DISCUSSION

Table 1 during the course of present investigation were observed that the both groups results showed at the end of 120h atrazine exposure antioxidant enzyme level of the hepatic tissues were slightly increasing LPO and decreased level of GSH, GPx, SOD, and CAT. In addition, a recovery of *Tribulus terrestris* plant exposure drastically restored near normal level when compared to atrazine treatment.

Table 1. The level of lipid peroxidation(LPO) (n moles of MDA released / 100 mg protein) activity of *T. terrestris* on atrazine exposed fresh water fish *O. mossambicus* liver tissue

Groups	24h	48h	72h	96h	120h
Control	12.06±1.325	12.46±0.456	12.65±0.978	12.79±0.987	12.99±0.006
Atrazine	20.16±0.265	22.26±0.657	24.65±0.567	25.21±0.987	26.29±0.545
Atrazine + *Tribulus terrestris*	16.26±0.549	18.26±0.526	17.26±0.265	19.52±0.144	20.22±0.656
Tribulus terrestris	13.26±0.550	13.26±0.564	14.65±0.015	15.26±0.055	15.26±0.665

Table 2. The level of Catalase (CAT) (µ moles of H_2O_2 utilized by min/ mg protein) activity of *T. terrestris* on atrazine exposed fresh water fish *O. mossambicus* liver tissue

Groups	24h	48h	72h	96h	120h
Control	11.55±0.265	11.46±0.266	11.56±0.798	11.65±0.895	11.33±0.645
Atrazine	8.99±0.424	9.45±0.130	9.78±1.319	9.45±0.796	10.12±0.465
Atrazine + *Tribulus terrestris*	11.54±0.966	11.78±0.589	12.06±0.415	12.36±0.215	12.56±1.146
Tribulus terrestris	14.65±0.455	14.32±0.979	14.78±0.464	15.56±0.186	17.63±0.0545

Table 3. The level of Superoxide dismutase (SOD) (units/min / 100 mg protein) activity of *T. terrestris* on atrazine exposed fresh water fish *O. mossambicus* liver tissue

Groups	24h	48h	72h	96h	120h
Control	47.56±0.421	47.65±0.109	47.64±0.321	48.50±0.315	48.97±0.121
Atrazine	53.64±0.424	55.68±0.130	61.52±0.319	63.14±0.796	63.15±0.310
Atrazine + *Tribulus terrestris*	51.35±0.416	52.21±0.145	52.98±0.164	53.04±0.063	54.21±0.146
Tribulus terrestris	48.12±0.564	48.15±0.665	48.56±0.656	49.16±0.785	49.26±0.945

Table 4. The level of Glutathione peroxidase (GPx) (µg of GSH utilized/min / 100 mg protein) activity of *T. terrestris* on atrazine exposed fresh water fish *O. mossambicus* liver tissue

Groups	24h	48h	72h	96h	120h
Control	4.303±0.053	4.336±0.042	4.353±0.045	4.355±0.028	4.378± 0.036
Atrazine	3.218±0.085	3.145±0.044	3.133±0.051	3.103±0.058	3.048±0.059
Atrazine + *Tribulus terrestris*	3.815±0.044	3.828±0.051	3.851±0.042	3.866±0.069	3.908±0.058
Tribulus terrestris	4.268±0.039	4.305±0.053	4.323±0.056	4.378±0.057	4.396±0.037

Table 5. The level of Reduced Glutathione (GSH) (µg/ 100 mg protein) activity of *T. terrestris* on atrazine exposed fresh water fish *O. mossambicus* liver tissue

Groups	24h	48h	72h	96h	120h
Control	2.523±0.051	2.563±0.078	2.591±0.072	2.625±0.068	2.606±0.048
Atrazine	1.623±0.069	1.568±0.069	1.541±0.074	1.503±0.085	1.428±0.061
Atrazine + *Tribulus terrestris*	2.323±0.066	2.376±0.063	2.408±0.063	2.465±0.065	2.495±0.072
Tribulus terrestris	2.528±0.051	2.563±0.049	2.586±0.049	2.516±0.073	2.595±0.044

Fish are often used as sentinel organisms for ecotoxicological examine because they play number of roles in trophic web, accumulate to toxic substances and respond to less concentration of mutagens therefore, the use of fish biomarkers are indices the effects of environmental pollution are of increasing importance and can permit early identification of aquatic environmental problems [34]. It is possible to detect toxic symptom of herbicides by studying cytological and serological indices in fresh water fish.

The toxic effects of atrazine involve interaction with a large number of cellular processes, including the formation of complexes with free thiols and protein thiol groups, which may lead to oxidative stress in animals. Oxidative stress due to free radical mediated lipid peroxidation has been implicated in the pathogenesis of several diseases [35]. In the present experimental study, the level of LPO content was drastically increased in liver tissues of fish when treated with sub-lethal concentration of atrazine for 120h. The formation of lipid peroxidation is an autocatalytic free radical process whereby polyunsaturated fatty acids (PUFA) in cell membranes undergo degradation by a chain reaction to yield lipid hydroperoxides which subsequently decompose to form a variety of products in clouding malondialdehyde. Increased ROS production may, thus, be associated with the metabolism of atrazine herbicide leading to the peroxidation of membrane lipids of the liver [36]. The liver is noted as site of multiple oxidative reactions and maximal free radical generation. To the observed LPO resulting from ROS generated by the atrazine may lead to cell apoptosis. ROS and oxidative stress have been demonstrated to be triggers of apoptosis [34,37]. Oxidative stress may also be due to the depletion of cellular GSH content below the critical level which prevents the conjugation of xenobiotics like atrazine to GSH and thus enables them to freely combine covalently with cell proteins. Oxidative stress may also be due to the depletion of cellular GSH content below the critical level which prevents the conjugation of xenobiotics like atrazine to GSH and thus enables them to freely combine covalently with cell proteins [36]. However, organisms are equipped with interdependent cascades of enzymes to alleviate oxidative stress and repair damaged macromolecules, produced during normal metabolism or due to exposure to xenobiotics.

Livingstone [38] has stated that the oxidative stress caused by pesticides in aquatic organisms may lead to ROS production and alterations in antioxidants enzymes. The production of ROS may attack nearby molecules resulting in damage of the molecular structure and function or dysfunction of many organs and systems [39]. The excessive production of ROS production and their damaging effects can be minimized by the cellular antioxidant systems [40,41]. Generally, organisms under stress conditions use the antioxidant enzymes to adapt to environmental stress and altered activities depend on the dose, species and route of exposure [42]. The enzymes such as SOD and catalase play a major role in eliminate the ROS produced during bioactivation of xenobiotics and the induction of SOD/CAT system may be the first defense mechanism against ROS [34,36]. Moreover, SOD and CAT are highly sensitive and respond more quickly thereby protecting organisms from oxidative stress [43,44]. Superoxide dismutase and CAT are among the most important detoxifying agents to ameliorate harmful effects of ROS in living organisms. Superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen, and, subsequently, CAT decomposes hydrogen peroxide to water and oxygen [45]. Additional key antioxidant enzymes providing cellular defense against ROS are glutathione peroxidase (GPx) [45], and the non-enzyme antioxidant GSH [46]. According to Pandey et al., [47], says to the SOD-CAT system provides an important line of defense against ROS and their activities are usually correlated. When intracellular levels of H_2O_2 are high, more CAT is required than GPx, the enzyme that reduces numerous peroxides, including H_2O_2, by GSH oxidation [48,49]. The lack of activation of GPx could be a mechanism for preserving the GSH content in the cell above a critical level favoring the conjugation of atrazine to GSH by GST [48,49].

Antioxidant enzyme activities have been used as an early warning sign of environmental pollution [50]. In

vertebrates, superoxide dismutase is one of the most important antioxidant enzymes that detoxify superoxide anion radical (O_2-) while catalase (CAT) reduces hydrogen peroxide to water (H_2O) and oxygen (O_2). Thus CAT and SOD provide the first line of defense against stress [35]. Both enzymes (CAT and SOD) are inducible and may have been produced in response to the toxicity of atrazine.

In the current study, atrazine exposure provoked a decrease in hepatic antioxidants, GSH levels, as well as the SOD and CAT activities. All these effects are involved in the cascade of events leading to the atrazine mediated liver damage resulting from the oxidative stress that arises from the excessive generation of ROS, which have been reported to attack various biological molecules. Santos and Martinez [46] suggested that atrazine interferes with the synthesis of these enzymes. Thus, the most likely cause for the inhibition of these enzymes would be a general reduction in the liver cell metabolism caused by the herbicide.

The alteration in the antioxidant status in common carp subchronically exposed to atrazine may be explained as follow: when the generated superfluous ROS exceed the scavenging abilities of SOD and CAT, the ROS inhibit the CAT activity by oxidation of the cysteine in the enzymes [51,52]. Moreover, the non-enzymatic antioxidant GSH was extraordinarily declined in the atrazine exposed group. As GSH prevents free radical damage and aids in cellular detoxification by conjugating with toxicants, the decrease in GSH likely prompts the increased susceptibility of the tissues to peroxidative damage and makes the system more susceptible to radical generation [35,53]. It could be suggested that the atrazine exposure was massive free radicals production (represented by an elevation in MDA level), which utilizes the hepatic oxidative machinery, further prompts a decrease in the activities of antioxidant enzyme catalysts by inhibiting the biosynthesis of the enzymes. The result of these studies evidence that the biochemical responses are dependent on stressor type, species and exposure time. Furthermore, the herbicide may lead to the occurrence of transformation products in water with a potential or actual similar [34,36]. The treatment of *T. terrestries* restored near normal level antioxidant and oxidant activity.

4. CONCLUSION

In conclusion, our experimental results are indicates that *T. terrestris* may play a protective role of reducing the toxic effects of atrazine exposed oxidative damage in liver, in which could be due to the antioxidant potentially by scavenging the free radicals. In this present study therefore provides biologically supporting the efficacy of *T. terrestris* against atrazine exposed toxicity in freshwater fishes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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