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Modulation of oxidant: Antioxidant imbalance by *Hygrophila Auriculata* (K. Schum) heine in mercuric chloride induced oxidative stress in rat liver

[1] Dr. Sridhar MP, [2] Dr. Geetha R, [3] Anitha D, [4] Muthukumar R, [5] Dr. Ramesh V

[1] Department of pharmacology, Tagore Dental College, Chennai, Tamil Nadu, India
[2, 3, 5] Department of Biochemistry, Sri Lakshmi Narayana Institute of Medical Sciences, Puducherry, India
[4] Department of Biotechnology, SRM University, Chennai, Tamil Nadu, India

Abstract
The deleterious effect of mercury has been attributed to mercury induced oxidative stress with the consequence of lipid peroxidation. The present study was designed to investigate the effect of ethanolic extract of *Hygrophila auriculata* on mercury induced oxidative damage in rat liver. Mercuric chloride ($HgCl_2$) (1mg/kg b.w, i.p) was administered three times in a week for two weeks to induce mercury toxicity. Mercury damage to the liver was evident from increase in the activity of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and acid phosphatase (ACP) in serum. The level of lipid peroxidation (LPO) was increased in liver tissue along with decrease in both the enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamin C and vitamin E. Post treatment with ethanolic extract of *Hygrophila auriculata* (100 mg/kg b.w, p.o) for 10 days after mercury induction completely ameliorated the mercury induced oxidative damage.

Keywords: *Hygrophila auriculata,* Mercuric chloride, Antioxidant, Lipid peroxidation

1. Introduction
It is a well known fact that the heavy metals such as aluminum, cadmium, lead, zinc and mercury affect many tissues like liver, kidney and brain etc. [1]. Due to industrialization and changes in the environment during the twentieth century, humans and animals are exposed to numerous chemical form of mercury. All forms of mercury cause toxic effects in a number of tissues and organs depending upon the level of exposure, the duration of exposure and the route of exposure [3]. Since mercury is being widely used in different fields such as medical, agricultural and industrial fields, its exposure cannot be avoided by humans [4].

Heavy metals are known to produce oxidative damage in the liver tissues by enhancing peroxidation of membrane lipids [5], a deleterious process solely carried out by free radicals [6]. Many studies have investigated possible relationship between lipid peroxidation (LPO) and cellular damage in hepatic tissues under various pathological conditions [7]. Lewis and Willis [8] have suggested that peroxide formation may lead to oxidative destruction of thiol groups of amino acids and proteins.

Antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, glutathione -S-transferase and glutathione reductase scavenge free radicals and lipid peroxides and deoxicify them [9]. Previous reports by Dwivedi *et al*., 1984 [9]; Tan *et al*., 1984 [10], have shown that LPO is enhanced by disturbances such as depletion of cellular antioxidants.

Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers [11-12]. Recently, interest has increased considerably in finding naturally occurring antioxidants in feed or medicinal flora to replace synthetic antioxidants for their adverse side effects, such as carcinogenicity, hepatotoxicity and nephrotoxicity [13]. Natural antioxidants can protect the human body from free radicals and related the progress of many chronic diseases as well as retard lipid oxidative rancidity in food, cosmetics and pharmaceutical materials [14-15]. In search of plant as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the past few decades [16-17].

Hygrophila auriculata (K. Schum.) Heine (Syn). *Asteracantha longifolia* Nees. (Acanthaceae), is a wild herb commonly found in moist places on the banks of tanks, ditches and paddy fields throughout India and is one of the source of Ayurvedic drug 'kokilaksha'. The plant has been shown to possess hypoglycemic activity in human subjects [22], hepatoprotective activity against paracetamol and thioacetamide intoxification in rats [23] and CCl_4-induced liver dysfunctions [24], antitumour [25], anabolic and adrogenic activities [26]. *H. auriculata* seeds have been reported to ameliorate the activities of antioxidant enzymes glutathione peroxidase (GPx) and catalase (CAT) in hepatocarcinoma [27]. However, there is paucity of information regarding antioxidant investigations in experimental animals have been reported in the whole part of this plant. Therefore, the present study was undertaken to investigate the effect of ethanolic extract of whole plant *H. auriculata* on the level of enzymatic, non-enzymatic antioxidants along with TBARS and hydroperoxides in mercuric chloride ($HgCl_2$)-induced rats and validate the ethnobotanical and clinical claims of the plant.

2. Materials and methods

2.1 Animals

Healthy male Wistar albino rats weighing 120±30g purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, were used for this study. Animals were housed in polypropylene cages and were provided certified rodent pellet diet and water *ad libitum*. They were maintained at 27 °C to 37 °C with 12 h light and dark cycle. All animal experiments were performed in accordance with the strict guidelines prescribed by the Institutional Animal Ethical Committee (IAEC) after getting necessary approval.

2.2 Chemical

Mercuric chloride was purchased from Sigma Chemical Company, USA. 5-5′dithiobis-2-nitrobenzoic acid (DTNB) and 1-chloro 2, 4-dinitrobenzene (CDNB) were purchased from SISCO Research Laboratories, Chennai, India. All the other chemicals used were of analytical grade and were purchased locally.

2.3 Collection of plant material

The plant is widely distributed throughout India, Srilanka, Burma, Malaysia and Nepal. The plants were collected from Red Hills, Tamil Nadu in the month of August. The plant specimen was authenticated by Dr. S. Jayaraman, Plant Anatomy Research Center, Chennai, Tamilnadu, India. A voucher specimen has been deposited at the herbarium unit of the Department of Pharmacology and Environmental Toxicology, University of Madras, Taramani, Chennai.

2.4 Preparation of plant extract

The whole plant was shade dried and coarsely powdered. The powder was then extracted with ethanol (60-70 ˚C) using Soxhlet extractor. The extract was dried under reduced pressure using rotary flash evaporator. The percentage yield of alcoholic extract was 12% w/w. The extract was stored in refrigerator for further studies.

2.5 Determination of free radical quenching capacity

2.5.1 DPPH assay

The free radical quenching capacity of ethanolic extract of *Hygrophila auriculata* was determined by a method involving the bleaching of stable DPPH [28]. A reaction mixture containing methanol, DPPH (10 mM,30 µl) and 100 µl of various concentrations of ethanolic extract of *Hygrophila auriculata* (0.01, 0.1,or 1.0 mM in dimethyl sulfoxide, DMSO) was allowed to stand at room temperature for 30 min before mixing with redistilled water (1 ml) and toluene (3 ml). The solution was then centrifuged, and the absorbance of the upper phase was read at 517 nm against a blank without ethanolic extract of *Hygrophila auriculata*, prepared and processed as described above. Vitamin C served as standard and compared with the extract.

$$\text{Radical scavenging (\%)} = \{\frac{A\text{CONTROL} - A\text{SAMPLE}}{A\text{CONTROL}}\} \times 100$$

2.5.2 NO- Scavenging effect

According to the method of Sreejayan and Rao [29], sodiumnitropruside (SNP, 5mM) in phosphate buffered saline was mixed with different concentrations (10, 20,30,40 & 50 µg) of ethanolic extract of *Hygrophila auriculata* dissolved in 50mM phosphate buffer, before being incubated at 25 °C for 150 min. the amount of NO produced by sodium nitropruside was measured by Griess reaction (1%) sulphanilamide in 5% phosphoric acid and 0.1% N- 15-napthyl ethylenediamine dihydrochloride in water). 0.5 ml of the extract (dissolved in 50mM phosphate buffer) was mixed with same volume of Griess reagent. The absorbance of the mixture was read at 550 nm. Vitamin C served as standard and compared with the extract.

2.6 Experimental design

Rats were divided into 4 groups with 6 animals in each group. The experimental design was as follows: Group I rats served as controls and were treated with normal saline (0.9% NaCl). Group II rats were administered with $HgCl_2$ (1 mg/kg b.w., i.p.) three doses per week for two weeks. Group III rats were administered with $HgCl_2$ dosage as in group II and then extract of *H. auriculata* (100 mg/kg b.w, p.o) for ten days. Group IV rats were treated with *H. auriculata* extract alone.

At the end of experimental period, animals were subjected to mild ether anesthesia, blood was collected from retro orbital plexus and the serum was separated by centrifugation at 3000 rpm for 15 min at 4 °C. Animals were sacrificed by cervical decapitation and the liver was excised, washed in ice cold saline and blotted to dryness. A homogenate of the liver tissue was prepared in Tris–Hcl buffer (0.1 M; pH 7.4), centrifuged at 1000 rpm for 10 min at 4 °C to remove the cell debris. The clear supernatant used for further biochemical assays. Aspartate and alanine transaminases (AST and ALT) were assayed according to the method of [30], acid and alkaline phosphatase enzymes in serum by the method of [31] as described by [32]. Lipid peroxidation (LPO) was determined in the liver tissue as described by [33], superoxide dismutase (SOD) according to [34] and catalase (CAT) according to the method of [35]. The activity of glutathione peroxidase (GPx) was estimated by the method of [36]. The hepatic reduced glutathione (GSH) content was estimated by the method of [37]. Vitamin C and Vitamin E were estimated by the method of [38] and [39] respectively.

2.7 Statistical analysis

The data obtained was subjected to one way ANOVA and Tukey's multiple comparison tests was performed using SPSS statistical package (Version 7.5). Values are expressed as mean ± S.E.M. Value $p < 0.05$ was considered significant.

3. Results

The present study elaborates the total free radical scavenging potential of ethanolic extract of *Hygrophila auriculata* with reference to DPPH and nitric oxide (NO) inhibition activity

DPPH is generally used as a substrate to evaluate anti oxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form, DPPH – H by the reaction. The ethanolic extract of *Hygrophila auriculata* showed a concentration dependant antioxidant activity by reducing stable radical DPPH to the yellow coloured diphenyl picryl hydrazine derivative. Positive DPPH test suggest that the plant extract is a free radical scavenger. The ethanolic extract showed strong

antioxidant activity compared with the standard Vitamin C (fig.1). Thus by depleting the DPPH it proves that the extract might be a potent free radical inhibitor.

The role of free radicals in inflammation especially with respect to nitric oxide (NO) is well documented [40]. While nitric oxide is an important second messenger in a number of physiological pathways, recent studies suggest that in the presence of oxidative stress, nitric oxide can be converted into reactive nitrogen species that contribute to cellular injury and death. For example, in the presence of superoxide anion, nitric oxide can be transformed into peroxynitrite ($ONOO^-$), a strong oxidant and nitrating species. Proteins, lipids and DNA are all targets of peroxynitrite. Over production of nitric oxide is known to be an important mediator of inflammatory state [40]. The plant extract suppressed the nitric oxide formation comparable to the standard (Fig.2). Since the plant has been used in the treatment of inflammatory diseases, the nitric oxide (free radicals) scavenging activity of this plant assumes importance.

Table 1 shows the status of serum AST, ALT, ACP and ALP in control and experimental animals. Due to mercuric chloride induced hepatotoxicity the AST and ALT showed 27.8% and 39.5% increased activity in serum. The ACP and ALP were also showed 24.5% and 28.5% increase in their activity in group II animals when compared with group I control animals. The increases in the activities of AST, ALT, ACP and ALP in serum due to mercuric chloride toxicity were significantly decreased on post treatment with *H. auriculata* (group III).

The changes in the LPO level of liver tissue of control and experimental rats were illustrated in figure -3. LPO was significantly increased (around 2 fold) in $HgCl_2$ treated rats (group II) when compared with control rats (group I) whereas the treatment with *H. auriculata* (group II) showed significant fall in the LPO level.

The changes in the level of enzymatic antioxidants such as SOD, CAT and GPx in the liver of control and experimental rats were given in figure 4, 5 and 6 respectively. A significant decrease (2 fold) in the activity of these radical scavengers was noticed after the administration of $HgCl_2$ in group II rats, when compared with group I rats. On post treatment with *H. auriculata* the activities of these enzymatic antioxidants were significantly reversed to normal.

The activities of non- enzymatic antioxidants namely GSH, Vitamin C and Vitamin E were significantly decreased (2 fold) in group II rats treated with mercuric chloride, when compared with group I rats, which were on post treatment with *H. auriculata* significantly reverted to normal level in group III (Fig 7,8 and 9).

The levels of glutathione metabolizing enzymes such as GR, G6PD and GST were depicted in table 2. The levels of all the three enzymes were significantly ($p < 0.05$) decreased in group II rats, which were reverted to normal level on treatment with *H. auriculata* when compared with control rats.

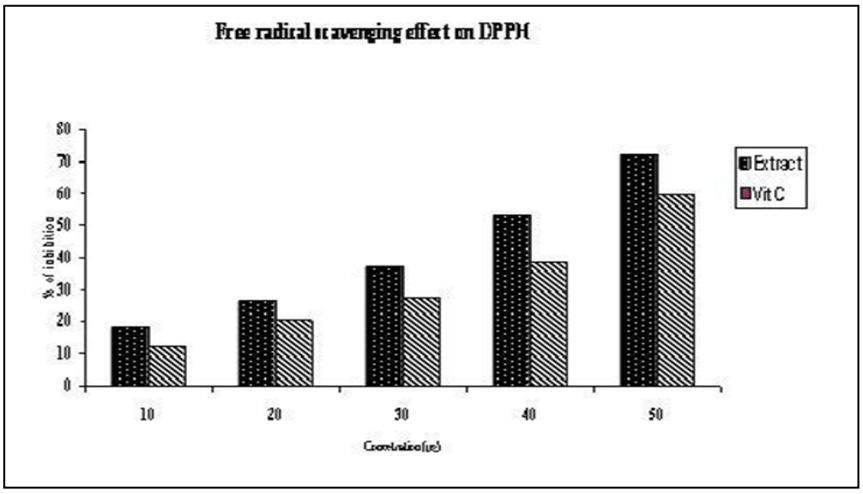

Fig 1: Free radical scavenging potential of *H. auriculata*

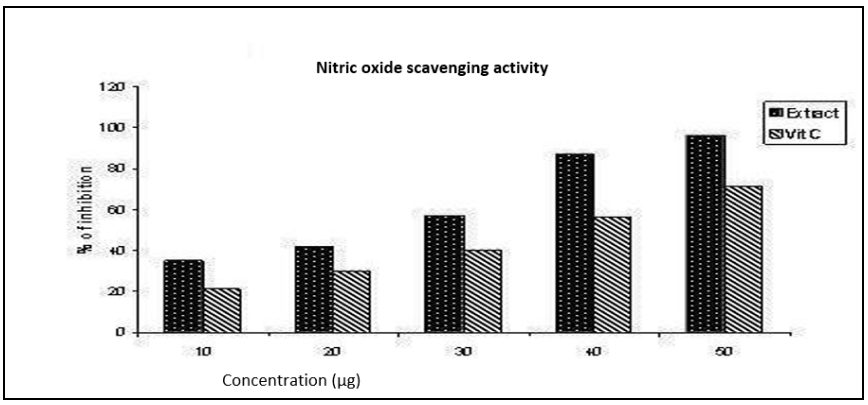

Fig 2: Nitric oxide scavenging activity of *H. auriculata*

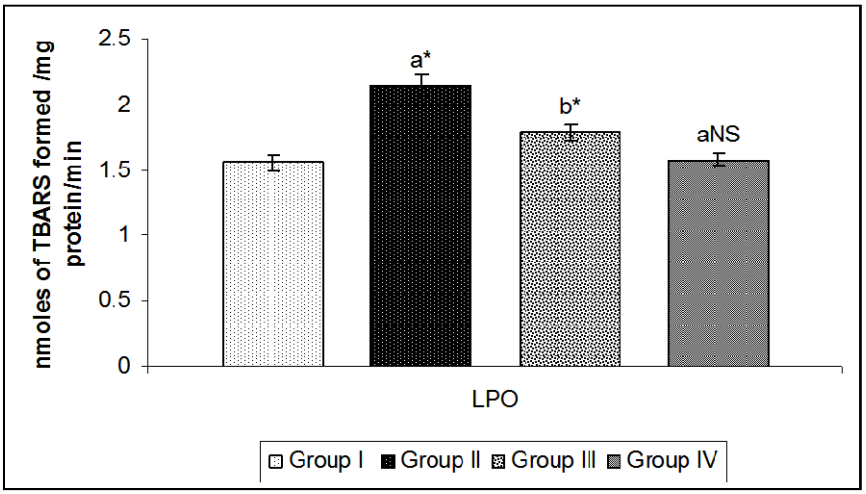

Fig 3: Effect of *H. auriculata* on lipid peroxidation in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats. * $p< 0.05$, NS – Not significant.

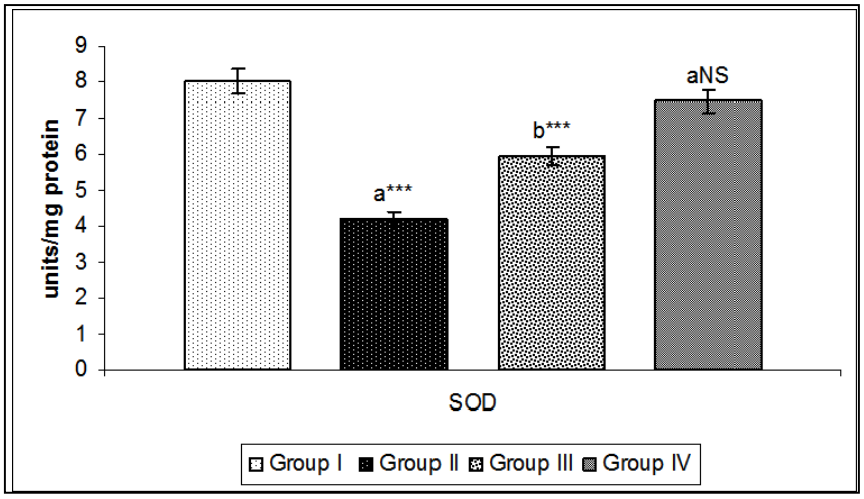

Fig 4: Effect of *H. auriculata* on the activity of superoxide dismutase in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity. Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats. *** $p< 0.001$, NS – Not significant. *** $p< 0.001$, NS – Not significant.

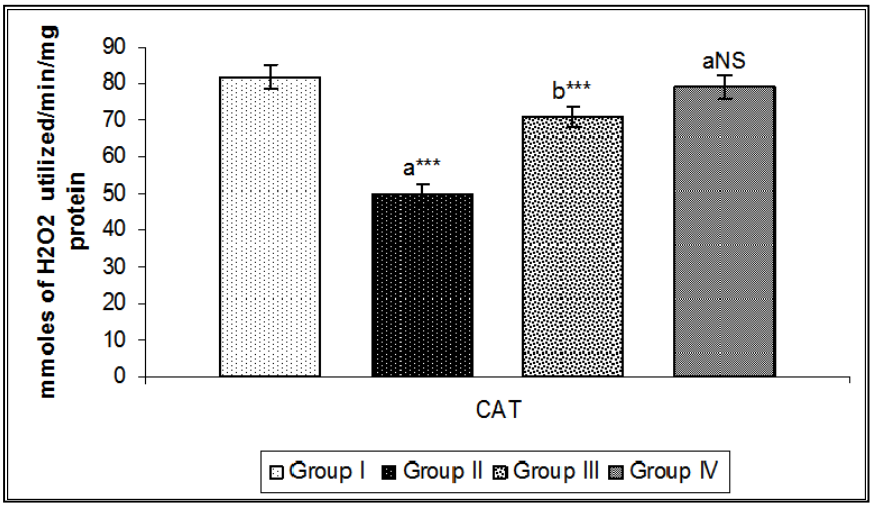

Fig 5: Effect of *H. auriculata* on the activity of catalase in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity. Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats. *** $p< 0.001$, NS – Not significant.

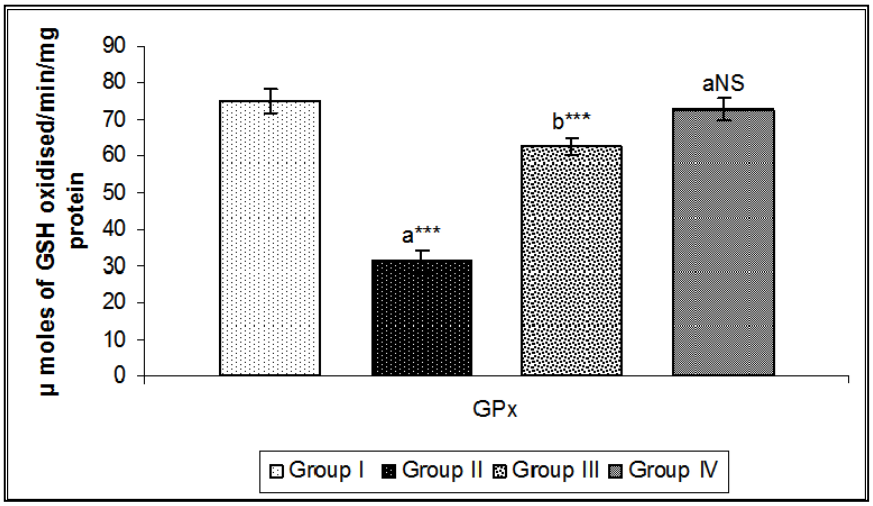

Fig 6: Effect of *H. auriculata* on the activity of glutathione peroxidase in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity. Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats. *** $p < 0.001$, NS – Not significant.

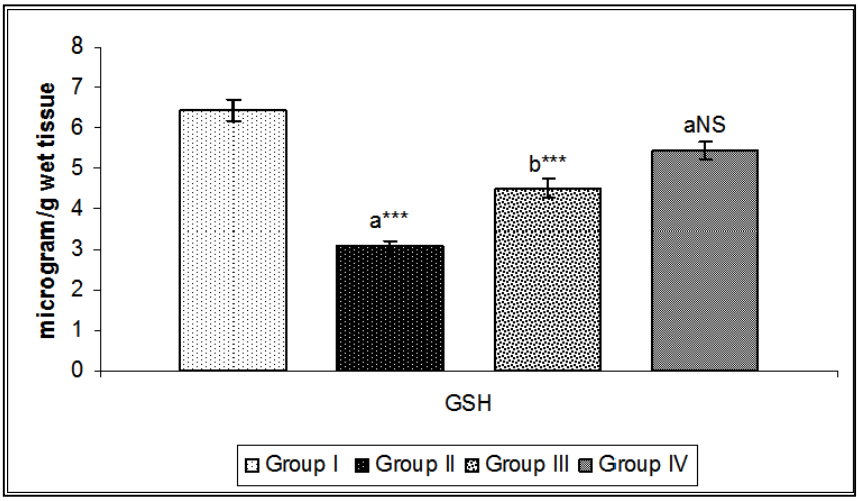

Fig 7: Effect of *H. auriculata* on the levels of GSH in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats.

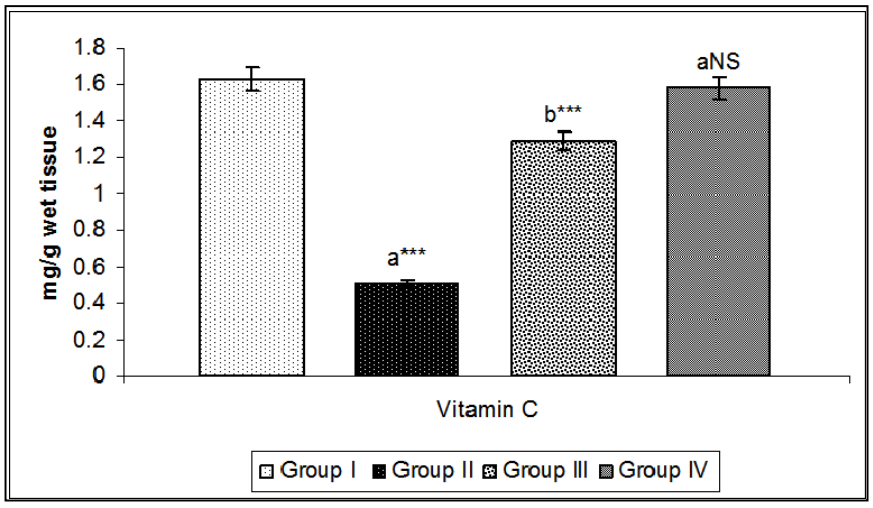

Fig 8: Effect of *H. auriculata* on the levels of vitamin - C in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats. *** $p < 0.001$, NS – Not significant.

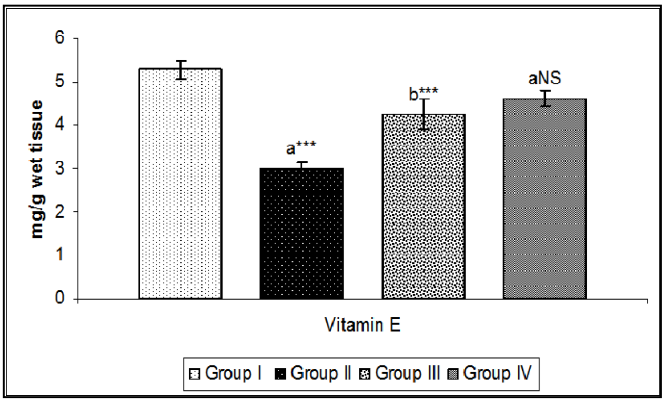

Fig 9: Effect of *H. auriculata* on the levels of vitamin - E in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity. Results are given as mean ± S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats. *** $p< 0.001$, NS – Not significant.

Table 1: Levels of serum AST, ALT, ACP and ALP in experimental animals

Parameters	AST	ALT	ACP	ALP
Group I	12.8±0.8	32.4±2.1	121.4±10.7	101.3±7.8
Group II	32.5±1.8[a]	74.1±4.8 [a]	243.7±18.7 [a]	252.6±21.5 [a]
Group III	18.6±1.2[b]	44.6±3.4 [b]	110.6±9.7 [b]	134.7±11.6 [b]
Group IV	13.4±0.7 [aNS]	33.7±2.3 [aNS]	124.5±10.9 [aNS]	104.3±6.9 [aNS]

Results are given as mean±S.E.M for six rats. Comparisons are made between: a – control rats (group I); b - $HgCl_2$ treated rats.
*** $p< 0.001$, NS – Not significant

4. Discussion

Redox disturbances are known to negatively impact body system through generation of ROS, which modify proteins, lipids and DNA [6]. Liver being one of the target organs for mercury accumulation has witnessed the toxic insult of mercury by way of alterations in the activities of transaminases and phosphatases. Transaminases (ALT and AST) being an important class of enzymes linking carbohydrate and amino acid metabolism, have established a relationship between the intermediates of the citric acid cycle. These enzymes are regarded as markers of liver injury since; liver is the major site of metabolism [41]. Phosphatases (ALP and ACP) are membrane bound and their alterations are likely to affect the membrane permeability and produce derangement in transport of metabolites. Moreover, [42] have reported that these phosphatase enzymes act as an indicator of cholestatic changes.

In this study, $HgCl_2$ administration to rats lead to marked elevation in the level of serum transaminases and phosphatases which is indicative of hepatocellular damage. This might be due to the possible release of these enzymes from the cytoplasm, into the blood circulation rapidly after rupture of the plasma membrane and cellular damage. Several researchers have reported increased serum phosphatases and transaminases during mercury poisoning [43, 44] which corroborate our study. Treatment with the ethanolic extract of *H. auriculata* reduced the level of above mentioned markers in serum of $HgCl_2$ induced rats. This indicates that *H. auriculata* tends to prevent liver damage by maintaining in the integrity of plasma membrane, thereby suppressing the leakage of enzymes through membranes, exhibiting hepatoprotective activity. This might be the reason for the restoration in the activities of marker enzymes during *H. auriculata.*

Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species (O_2, H_2O_2, and OH) generated exceeds the antioxidant capability of the cell [45]. All products of LPO inactivate cell constituents by oxidation or cause reaction oxidative stress by undergoing radical chain reaction ultimately leading to loss of membrane integrity [46]. The stimulation of LPO observed as result of Hg (II) administration could be due to the formation of free radicals thought an exhaustion of antioxidants leading to oxidative stress [47]. It has been reported that Hg (II) can inactivate a number of enzymes by blocking the functional sites through binding to sulphydryl groups, which are part of catalytic or binding domains [48]. Thus it was suggested that in addition to depletion of intracellular thiol pools, the oxidant pathway may be a primary mechanism of induction of the response for Hg to induce oxygen free radicals or promote formation of lipid peroxides. Free radical scavenging enzymes like SOD and CAT protect the biological systems from oxidative stress. The SOD dismutates superoxide radicals (O_2-) into hydrogen peroxide (H_2O_2) and O_2 [49, 50]. CAT further detoxifies H_2O_2 into H_2O and O_2 [49, 50]. GPx also functions in detoxifying H_2O_2 similar to catalase. Thus, SOD, CAT and GPx act mutually and constitute the enzymatic antioxidative defense mechanism against reactive oxygen species [51]. The decrease in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the metabolism of $HgCl_2$. This is further substantiated by an elevation in the levels of lipid peroxidation. Similar reports have shown an elevation in the status of LPO in liver during $HgCl_2$ exposure [1, 52] and our results are in accordance with these reports.

Restoration in the levels of lipid peroxidation after administration of *H. auriculata* could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane lipids. Our results are in line with previous studies by [53] who have shown that *H. auriculata* exhibits excellent antioxidant property. Therefore this property of *H. auriculata* might have resulted in the recoupment in the activities of the above antioxidant enzymes to normalcy.

Excessive liver damage and oxidative stress caused by $HgCl_2$ depleted the levels of non-enzymatic antioxidants like GSH, vitamin-C and vitamin-E in our study. Non-enzymatic antioxidants like vitamin-C and E act synergistically to scavenge the free radicals formed in the biological system. GSH acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation [54]. Vitamin-C also scavenges and detoxifies free radicals in combination with vitamin-E and glutathione [55]. It plays a vital role by regenerating the reduced form of vitamin-E and preventing the formation of excessive free radicals [55]. The decreased levels of these antioxidant vitamins and GSH observed during $HgCl_2$ administration might be due to the excessive utilization of these vitamins in scavenging the free radicals formed during the metabolism of $HgCl_2$. *H. auriculata* treatment effectively restored the depleted levels of these non-enzymatic antioxidants caused by $HgCl_2$. *H. auriculata* has been reported to maintain the GSH homeostasis in the system [56] and this might be the reason for elevated glutathione levels observed during *H. auriculata* treatment. Increase in GSH levels in turn contributes to the recycling of other antioxidants such as vitamin-E and vitamin-C [57]. This

shows that *H. auriculata* maintains the levels of antioxidant vitamins by maintaining GSH homeostasis, thereby protecting the cells from further oxidative stress. A significant decrease in the activities of GSH dependent enzymes, G6PD, GST and GR was observed in $HgCl_2$ treated rats, which may be due to the decreased expression of these antioxidants during hepatic damage. Further, the decreased levels of cellular GSH might have also caused a reduction in their activities as GSH is a vital co-factor for these enzymes. Our observations are in accordance with the reports of [58] who demonstrated that $HgCl_2$ induced hepatic injury was escorted by a substantial fall in hepatic GSH level, GPx and GST activity, which improved on administration of antioxidants. It is likely that post treatment of *H. auriculata* maintaining GSH levels by inhibiting lipid peroxidation. This is indicative of the potent antioxidant activity possessed by *H. auriculata.*

5. Conclusion

From the present study, it may be concluded that $HgCl_2$ induced toxicity involves oxidative stress in its pathophysiology. The ethanolic extract of *H. auriculata* possesses a potent free radical scavenging and antioxidant activities. The extract is capable of increasing/maintaining the levels of antioxidant molecules and antioxidant enzymes in the liver and also of protecting against oxidative damage to liver tissues. The preliminary chemical examination of ethanolic extract of *H. auriculata* extract has shown the presence of polyphenols, flavonoids, triterpenes and sterols which may be responsible for the antioxidant activity. Further studies on the isolation of more of antioxidants are in progress.

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7. References

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