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LIPID PEROXIDATION AND ANTIOXIDANT DEFENCE IN LIVER TISSUE OF HIBERNATING COMMON ASIAN TOADS, *Duttaphrynus melanostictus*

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Abstract: **In this study, lipid peroxidation in terms of TBARS formed and antioxidant defence comprising both antioxidant enzymes and non-enzymatic antioxidants were assessed in the liver tissue of common Asian toads during hibernation and compared with the summer active toads. A significant (P<0.001) increase in lipid peroxidation was found in liver tissue during hibernation. Concomitantly significant (P<0.001) increase in catalase activities was also found during hibernation, with an elevated superoxide dismutase activity both during hibernation and the summer active period. Ascorbic acid, a potent non-enzymatic antioxidant, was also found to be augmented during hibernation compared to summer-active toads. However, reduced glutathione (GSH) was found at low levels during hibernation compared to the summer active period. It may be due to its low rate of biosynthesis during the hypo metabolic condition of hibernation. Increased lipid peroxidation, an indication of oxidative stress and augmented antioxidant defence that were found in this investigation, are not only an adaptive response during hibernation but also a preparation for the oxidative burst expected during arousal from hibernation.**

Keywords: **Hibernation, oxidative stress, Lipid peroxidation, antioxidant enzymes, and non-enzymatic antioxidants.**

1. INTRODUCTION

Animals adapt to the changed environmental conditions and follow different life-saving strategies during cold winters with scarcity of food materials and dry weather. Some homeothermic mammals go heterothermic by reducing their body temperature to as low as 2°-4°C and enter into a state of dormancy or torpor for some days during extreme cold climatic conditions of the winter season (Geiser,2013; Lyman et al,1982). They became active for some period after few days of torpor during which their body temperature rises to 37°C, metabolic rate rises to normal level, they move here and there in search of food material and again go into torpor for some days, This cycle of torpor and arousal during winter season to avoid scarcity of food material and loss of heat energy from their body (due to homeothermy) is known as hibernation (Geiser,2013; Allison et al., 2023). Hibernation has been reported largely as a life-saving adaptive response in small-sized endothermic mammals (Geiser, 2004; Humphries et al, 2003). However, many ectothermic animals, including amphibians, have also been reported to show hibernation during the winter season (Bagnyukova et al, 2003; Yonggang et al, 2018) (Afifi and Alkaladi,2014; Holenweg and Reyer,2000). Though ectothermic animals are slow in their metabolism and show changes in their body temperature as per Q_{10} relationship (Withers,1992; Withers and Cooper,2010), some ectothermic animals exhibit additional metabolic depression, reduced body temperature and dormancy during cold winter, which is also known as hibernation(Withers and Cooper, 2010).

Although hibernation physiology has been studied well in endothermic animals, reports on ectothermic animals are limited to some Antarctic fishes, *Notothenoids* (Campbell et al,2008).Amphibians like *Rana ridibunda* (Bagnyukova et al,2003), *Rana esculanta* (Holenweg and Reyer,2000), *Nano rana parkeri* (Yonggang et al,2018) and some reptiles like Garter snakes, *Thamnophis sirtalis parietalis* (Joy and Crew,1987), American box turtles (Dannis et al,1991).Moreover, studies on hibernation in anuran amphibians are limited to temperate regions, and studies on hibernation in Tropical amphibians are rare (Pratihar and Kundu,2009,2010; Pratihar et al., 2010) and limited to the common Asian toad, *Duttaphrynus melanostictus.*

Hibernation has been largely reported with metabolic depression in both endothermic animals(Storey K.B et al., 2010; Carey H.V. et al., 2003; Chazarin et al., 2019) and ectothermic animals (Seymour,1973; Withers and Thompson, 2000; Rossi et al,2020). In our previous study, metabolic depression with reduced activities of some key aerobic oxidative metabolic enzymes has been reported in the liver tissue of hibernating common Asian toads (Sahoo and Acharya,2025). Metabolic depression, evidenced by low oxygen consumption, low body temperature and low thyroid activities, has also been reported earlier (Pratihar and Kundu,2009) in hibernating common Asian toads. Low metabolic activities with low oxygen consumption is supposed to produce low reactive Oxygen species (ROS), leading to a low rate of oxidation of Lipid molecules, resultingin lipid peroxidation and other oxidative stress markers like protein carbonylation and GSSG/GSHratio. However, both an increase in lipid peroxidation (Yonggang et al.,2018; Carey et al.,2000; Chauhan et al.,2002) and a decrease in lipid peroxidation (Wei et al.,2018; Orr et al.,2009) have been reported during hibernation of ectothermic as well as endothermic animals. This instigated us to investigate the status of lipid peroxidation in the liver of hibernating common Asian toads.

Aerobic organisms have developed different strategies to deal with lipid peroxidation and other oxidative stress-related oxidised products of proteins, carbohydrates and even DNA molecules. One such strategy is to minimise the level of oxygen uptake to reduce the production of reactive oxygen species (ROS) (Halliwell and Gutteridge, 2006) and thus result in low lipid peroxidation. Another strategy is to evolve an antioxidant defence system to counteract the oxidative stress conditions. This antioxidant defence system comprises antioxidant enzymes for catalytic removal of ROS (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidants (ascorbic acid, glutathione, α-tocopherol, uric acid) for scavenging ROS (Halliwell and Gutteridge, 2006; Chainy et al., 2016; Sohal et al., 1995). Like lipid peroxidation, the status of antioxidants during hibernation has been reported with conflicting views. While upregulated antioxidant defence has been reported during hibernation of endothermic mammals (Okamoto et al., 2006; Yin et al., 2016; Wei et al., 2018). Both steady and down-regulated antioxidant defence have been reported in some ectothermic hibernators (Tang et al., 2021; Yonggang et al., 2018). Moreover, studies relating oxidative stress and antioxidant defence during hibernation of ectotherms in general and amphibians in particular are very scarce and need investigations involving diverse groups of ectothermic animals.

So in this study, levels of lipid peroxidation and status of antioxidant enzymes, like superoxide dismutase (SOD), catalase and non-enzymatic antioxidants like reduced glutathione, ascorbic acid were investigated in the liver tissue of hibernating common Asian toads, collecting them from their natural hibernaculum.

2. MATERIALS AND METHODS

Ethics statement: - Directives of the Institutional Animal Ethics Committee, Berhampur University, India, Registration no.-2020/Go/Re/S/18/CPCSFA, and a resolution number - 1have been followed for this study.

Animal collection:

Middle-aged (2-4 years old, with 8.0–8.3 cm snout-vent length) male common Asian toads (*Duttaphrynus melanostictus*) found in nature were selected for this study. Males were identified by observing a brick red or orange-red hue on their throat region, along with black nuptial pads on the inner side of the first two fingers of the fore limb. Their age was determined by skeletochronology (Sahoo and Kara, 2017), i.e., by counting the number of lines of arrested growth (LAG) in the bone matrix of transverse sections of long bones and phalanges.

Fourteen (14) middle-aged toads comprising seven (07) summer active toads collected during June to August of 2023 and seven (07) hibernating toads collected from their hibernaculum in moist soil of a protected area located in Paralakhemundi (10° 45’ N, 84° 6’ E), India during December- 2023 and January - 2024 were used in this study.

Experimental condition:-

For comparison of lipid peroxidation and antioxidant status in liver tissue of summer active toads and hibernating toads, they were collected during nighttime from their natural habitat. They were sacrificed then and there to collect liver tissue in an ice bucket for transferring them into the laboratory immediately for future processing. While summer active toads collected during nighttime of June to August 2023 with 37° atmospheric temperature were considered as control one, hibernating toads collected from their hibernaculum during December 2023 and January 2024 with 9° C atmospheric temperature were experimental specimens. At the time of collection, control specimens were of29±1° C body temperature and 43-45 times per minute heartbeat rate against the experimental specimen's body temperature of 9±1° C and 13 to 15 times per minute heartbeat rate.

Tissue Preparation:-

Whole liver collected from summer active or control toads (n=7) and hibernating or experimental toads (n=7)were stored in ice-cold (2° C) amphibian Ringer's solution. Adherent tissues were removed, the required amount of liver tissue was weighed and immediately processed for estimations of lipid peroxidation and different antioxidant status.

Lipid peroxidation Assay:-

Lipid Peroxidation (LPO) level in terms of TBARS (Thio Barbituric Acid Reactive Substance) formed in the 2.5% (W/V) tissue homogenate (50 mg of tissue in 2 ml. cold 50 mM potassium phosphate buffer (pH = 7.0) having 0.5 mM EDTA, 2 – 3 crystals of phenyl methyl sulphonyl fluoride) was estimated by the TBA test following Sestini*et al.*, (1991) and modification suggested by Jena *et al.*, (1991). For this measurement, the experimental test tube contained 0.5ml. of 2.5% (W/V) tissue homogenate, 1.5ml. of orthophosphoric acid (1%, pH= 2.0) and 0.5 ml of TBA (0.6%) solution, whereas the control test tube contained an equal amount of distilled water instead of tissue homogenate. All the tubes were heated inside a water bath at 90°C for about 45 minutes. Then the test tubes were allowed to cool down to room temperature, followed by the addition of 3 ml chloroform, 1ml of glacial acetic acid to each test tube and centrifuging the contents at 1000 X g for 10 minutes. Supernatant from the upper phase containing TBARS was taken for measurement of extinction at 535 nm (Fletcher *et al.*, 1973) against the control. Calculation of TBARS content in the tissue homogenate was made using the molar extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$ for MDA (malonaldehyde) following Sinhuber *et al.* (1958). Tissue TBARS content was expressed as µ mol/g. tissue wet weight.

Assay of superoxide dismutase (SOD) activity:-

Cytoplasmic Cu and Zn forms of SOD (E.C.1.15.1.1) activity was estimated following Das *et al.* (2000) in liver tissues. Briefly, 2ml of 2.5% (W/V) tissue homogenate (50mg tissue homogenised in chilled 50 mM phosphate buffer (pH=7) with 1mM EDTA and a little amount of phenyl methyl sulphonyl fluoride) was centrifuged at 10000 x g for 20 minutes at 4°C using a refrigerated centrifuge. A cocktail (1.4ml) was prepared by adding 1.11ml. of 50 mM phosphate buffer (pH=7.0), 0.075ml. of 20 mM L-methionine, 0.04ml. of 1% (v/v) Triton X-100, 0.075ml. of 10 mM HAC (hydroxylamine hydrochloride) and 0.1 ml of 78.125mM EDTA. To this 1.4ml. of cocktail inside the experimental tube, 0.1ml. of the tissue extract and 0.1ml. of 40µM riboflavin were added. A control was prepared without tissue extract, and a blank was prepared without riboflavin. All the above tubes were exposed to fluorescent light for 10 minutes. After this, 1ml of Griss as a reagent was added to each tube. The extinction of both experimental and control tubes was measured at 543 nm against the blank. The SOD activity was expressed as units/mg. protein where one unit of SOD activity is equal to $(V_o/V) - 1$, where 'V_o' is the extinction of the control and 'V' is the extinction of the experimental tube.

Assay of catalase activity:-

Catalase (CAT) (E.C. 1.11.1.6) activity of the tissue homogenate was estimated following Aebi (1974). In this method decrease in absorbance of H_2O_2 at 240 nm due to the action of catalase in the tissue homogenate indicates catalase activity. Briefly, 0.1 ml. of tissue extract obtained by centrifugation (10000 X G for 20 minutes at 4°C) of 2.5% tissue homogenate in 50 mM cold phosphate buffer (p^H=7.4) was added to 2.9ml of phosphate buffer having 12mM H_2O_2 to initiate enzymatic reaction and decrease in OD at 240 nm was recorded at 1 minute interval vide the blank having only phosphate buffer. Decrease in the H_2O_2 concentration was calculated from its extinction coefficient of 43.6 X M^{-1} $cm^{-1,}$ and catalase activity was expressed as µMH_2O_2 consumed/ min/mg protein.

Estimation of ascorbic acid:-

Ascorbic acid content in the deproteinised tissue homogenate was measured following Roe (1954) and modifications suggested by Tewari and Pandey (1964). Briefly, 50 mg of tissue was homogenised with 2 ml of 6% ice-cold trichloroacetic acid (TCA) in a pre-cooled all-glass homogeniser. The homogenate was centrifuged in a laboratory centrifuge (Remi R 8 C, Bombay) at 1000 X G for 10 minutes. The supernatant was collected and designated as supernatant-I. To the residue, 2ml of 6% TCA was again added, stirred with a glass rod and the content was centrifuged at 1000 X g for 10 minutes. The obtained supernatant-II was mixed with supernatant-I, and the total volume of the pooled supernatant was measured. 2 ml of deproteinized tissue extract was taken in an experimental tube, and a blank was allowed to run simultaneously, taking 2.0 ml of 6% TCA only. Similarly, four different standards of ascorbic acid of known concentrations were also taken in four test tubes, and they were also processed along with the blank and experimental tubes (containing unknown concentrations of the ascorbic acid). To each of the above tubes, two drops of bromine water were added to oxidise the ascorbic acid. Then the tubes were shaken thoroughly to remove excess bromine. After that, 0.5 ml of 2,4-dinitrophenyl hydrazine-thiourea reagent was added to each tube, and then they were incubated at 57°C for 45 minutes in a water bath. Then to each sample (blank, unknown and standards) 5ml of 85% H_2SO_4 was added drop wise. After waiting for 30 minutes, the extinction of the coloured product was measured at 530 nm against the blank. The concentration of ascorbic acid in the unknown samples containing tissue extracts was determined from a standard linear curve plotted with known ascorbic acid concentration and their respective extinction value. The tissue ascorbic acid content was extrapolated from it and expressed in µg./g. tissue wet-weight.

Estimation of total glutathione:-

Glutathione equivalents (GSH_{eq}) comprising reduced glutathione (GSH)and oxidised glutathione (GSSG)were estimated following Griffith (1980). Briefly, tissue homogenate (1:5w/v) was made protein-free by centrifuging it with chilled (2°C) sulfosalicylic acid at 10000 X g for 15 minutes. Then it was divided equally into two parts. To one part, 0.2 mM NADPH, 5mM EDTA,0.6 mM DTNB and 125 mM sodium phosphate buffer (pH=7.5) was added to make a cocktail of total volume 1 ml. To this cocktail, glutathione reductase (0.5 U) was added to start the enzymatic reaction. This enzymatic reaction is proportional to glutathione equivalent (GSH_{eq}) concentration. This was compared with a reduced glutathione (GSH) standard curve (0-6 µM) by recording the rate of reduction of 5, 5–dithio–bis (2-nitrobenzoic acid) (DTNB) at 412 nm. To another part of the tissue extract, 170 mM 2 – 2-vinyl pyridine was added and allowed to derivatise reduced glutathione (GSH) for 1 hour. The remaining oxidiser glutathione (GSSG) was estimated, and total glutathione was calculated using the formula GSHeqGSH+2GSSG. It was expressed in terms of µM/g tissue weight. Reduced glutathione (GSH) content was also calculated using the formula GSH = GSH_{eq}- 2GSSG.

Statistical analysis:-

Data were expressed as Mean ± SEM (n=7). One-way ANOVA (DUNCAN multiple range tests) was made using IBMSPSS-25-0. Moreover, Student'st-test was used to compare the mean value of two different groups at a time with a $P<0.05$ significance level.

3. RESULTS

Lipid Peroxidation:-

Lipid peroxidation in terms of TBARS formed was found significantly higher ($P<0.001$) in liver tissue of hibernating common Asian toads than their control counterpart, that is, summer active toads (n=7) (Fig. 1, Table 1).

Antioxidant enzymes:-

Superoxide dismutase (SOD) enzyme activity was found to increase, but not significantly, in the liver tissue of hibernating common Asian toads than their control counterparts.(Fig. 2, Table 1).

However, activities of the catalase enzyme were found significantly ($P<0.001$) higher in the liver tissue of hibernating toads thanin their control summer active toads.(Fig-3,Table-1)

Non-enzymatic antioxidant:-

Ascorbic acid, a potent non-enzymatic antioxidant, was found significantly higher ($P<0.001$) in the liver Tissue of hibernating common Asian toads than their summer active toad counterparts (Fig. 4, Table 1).

On the contrary, reduced glutathione (GSH), another non-enzymatic, tripeptide antioxidant, was found significantly at lower levels ($P<0.001$) inside the liver tissue of hibernating toad compared to their summer active counterparts (Fig. 5, Table 1).

4. DISCUSSION

The status of Lipid peroxidation and antioxidant defence, comprising both enzymatic antioxidants and non-enzymatic antioxidants, was investigated in the liver tissue of hibernating common Asian toads. In our previous study, metabolic depression has been reported with decreased activities of some key metabolic enzymes in the liver tissue of hibernating common Asian toads (Sahoo and Acharya,2025). Hibernation in general is characterised by low metabolic activity, low oxygen consumption and low body temperature (Carey et al.,2000; Carey et al.,2003) and so has a significant effect on reactive Oxygen Species (ROS) generation and oxidative stress (Emre et al.,2014; Halliwel and Gutteridge, 2015; Yonggang et al., 2018). In this investigation significant increase in lipid peroxidation was found in the liver tissue of hibernating common Asian toads compared to their active toad counterpart. Metabolic depression, low body temperature and low Oxygen consumption are likely to produce low levels of ROS and so low levels of lipid peroxidation and oxidative stress (Adelman et al., 1988). Hernansanz-Agustin et al.,(2014) have reported that reduced oxygen consumption maintains the redox state of the mitochondrial electron transport system towards a reduced state, favouring the production of superoxide radicals and thus increased cellular oxidant production(Smith et al., 2017). Hibernating toads with metabolic depression and low oxygen consumption might have produced a considerable amount of superoxide radicals that have caused increased lipid peroxidation in liver tissue. Besides this, He J et al. (2015) have reported that the cold exposure maintains fluidity of the membrane by increasing polyunsaturated fatty acids (PUFA) in the membranes. Low body temperature in hibernating toads could have resulted in increased PUFA content in the cell membranes, making them susceptible to lipid peroxidation. Moreover, the increased lipid peroxidation level in the liver tissue of hibernating toads points to their reduced lipid peroxidation scavenging mechanisms and continuous accumulation of damaged products in lipid reserves, which are not replenished by poor or no dietary intake and very low or no biosynthesis of lipids during hypometabolic hibernation. Our findings about increased lipid peroxidation during hibernation corroborate with the results in different ectothermic hibernators or estivators (Grundy and Storey, 1998; Yonggang et al., 2018)as well as endothermic hibernators (Carey et al.,2000; Carey et al.,2003; Emre et al., 2014).

Along with increased lipid peroxidation, augmented antioxidant defence was also found in the liver tissue of hibernating common Asian toads. An elevated level of superoxide dismutase activities both during the summer active period and the hibernation period in common Asian toad, shows its important role in catalytic conversion of super oxide radicals to hydrogen peroxide(Halliwell and Cross,1994) which again converts into water and oxygen by another antioxidant enzyme, catalase (Halliwell and Cross,1994; Chainy et al.,2016). Augmented catalase activities that were found in this investigation corroborate the findings of earlier workers on both ectothermic and endothermic hibernators (Hermes Lima and Storey,1995; Ohta et al.,2006; Okamoto et al.,2006; Yin et al.,2016; Wei et al.,2018). Both superoxide dismutase(SOD) and catalase enzymes act hand in hand for the catalytic conversion of ROS into harmless products during hibernation.

Like antioxidant enzymes, non-enzymatic antioxidant Ascorbic acid was found to increase during hibernation in liver tissue compared to its control counterpart. Ascorbic acid has been reported as a Potent antioxidant(Chakrabarty et al., 1992; Ames et al.,1993)and acts as a free radical trap Sandness K, (1991) Drew et al., (1999) and Toien et al., (2001) have reported ascorbic acid as a protective antioxidant during hibernation as well as recovery from hibernation due to its capacity to scavenge free radicals produced during hibernation and oxidative burst during rewarming from hibernation. Our result of augmented ascorbic acid content in liver tissue during hibernation, corroborates with previous findings. Increased ascorbic acid content in liver tissue during hibernation could be a preparatory mechanism to minimise the expected oxidative injury due to increased formation of ROS during hibernation and recovery from hibernation (Hermes-Lima et al., 2001; Hermes-Lima, 2004; Moreira et al., 2017; Giraud-Billoud et al., 2019). Reduced glutathione(GSH) is another water-soluble, endogenous antioxidant tripeptide capable of neutralising free radicals and maintaining other non-enzymatic antioxidants, like ascorbic acid, tocophenol, in their reduced state (Dringen, 2000). In this investigation, a significant decrease in reduced glutathione was found in the liver tissue of hibernating common Asian toads compared to their control counterpart. This may have been due to its decreased rate of biosynthesis during a hypometabolic condition and its regeneration from oxidised glutathione (GSSG) during hibernation. It has been reported earlier about the biosynthesis of GSH as an energy-consuming process (Hermes-Lima 2004) and decreased synthesis during hibernation (Orr et al., 2009), which corroborates our findings.

5. CONCLUSION

Hibernation is an adaptive response shown by the common Asian toad against cold environments and scarcity of food. They pass the cold climatic conditions and food scarcity by lowering their metabolic rate and body temperature so as to manage the vital processes with a minimum energy budget. Even under hypometabolism, an increased lipid peroxidation, indicating oxidative stress during hibernation, was found in this study. Concomitantly, an augmented antioxidant defence was also found in the hibernating common Asian toad. Reduced glutathione (GSH), being a non-enzymatic antioxidant, was found at low levels due to its low rate of biosynthesis to conserve energy during hibernation. Oxidative stress and augmented antioxidant defence are not only an adaptive response during hibernation, but also a preparatory strategy to face the oxidative burst expected during arousal from hibernation.

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APPENDICES

Figure And Table

Fig: - 1 Effect of hibernation on the level of Lipid Peroxidation (LPO) of liver tissue of Male common Asian Toad, *Duttaphrynus melanostictus*. Data are expressed as the mean ±SEM,(n = 7) significant differential calculation (t-test) from animals during the active period are designated as ***(P<0.001)

Fig – 2: Effect of hibernation on the Super Oxide Dismutase (SOD) content of liver tissue of male common Asian Toad, *Duttaphrynus melanostictus*. Data are expressed as the mean ± SEM, (n = 7) significant differential calculation (t-test) from animals during the active period are designated as (NS) Non Significant.

Fig: - 3 Effect of hibernation on Catalase Activity (CAT) of liver tissue of male common Asian Toad, *Duttaphrynus melanostictus*. Data are expressed as the mean ±SEM, (n = 7) significant differential calculation (t-test) from animals during the active period are designated as ***(P<0.001)

Fig – 4: Effect of hibernation on the Ascorbic Acid (ASA) content of liver tissue of male common Asian Toad, *Duttaphrynus melanostictus*. Data are expressed as the mean ± SEM, (n = 7) significant differential calculation (t-test) from animals during the active period are designated as ***(P<0.001)

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Fig – 5: Effect of hibernation on the Reduced Glutathione (GSH) content of liver tissue of male common Asian Toad, *Duttaphrynus melanostictus*. Data are expressed as the mean± SEM, (n = 7) significant differential calculation (t-test) from animals during the active period are designated as ***(P<0.001)

Table 1. Effect of hibernation on the level of lipid peroxidation and antioxidant defence status of liver tissue of male common Asian toad, *Duttaphrynus melanostict*

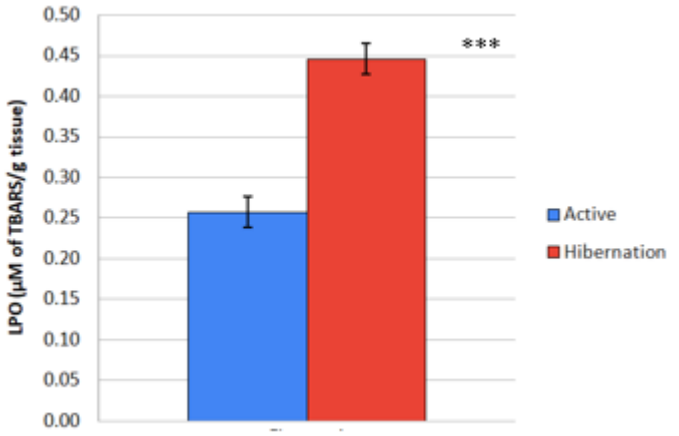

Fig: - 1

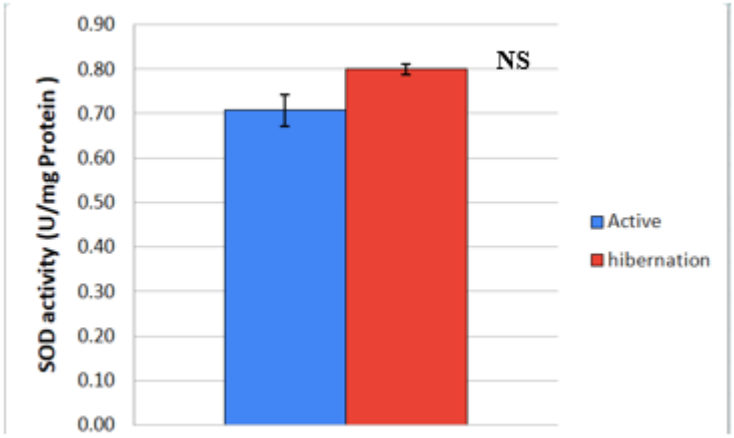

Fig – 2

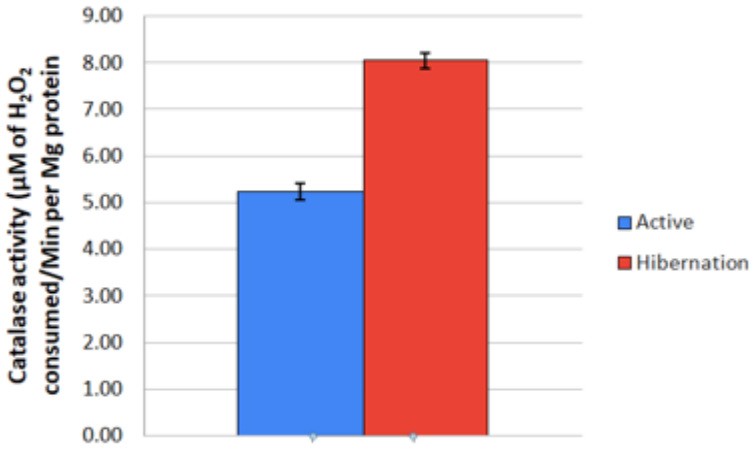

Fig -3

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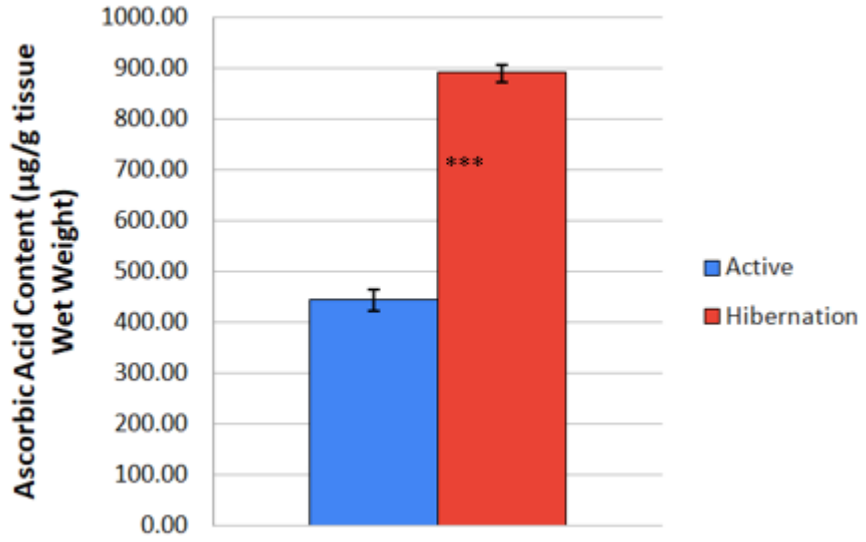

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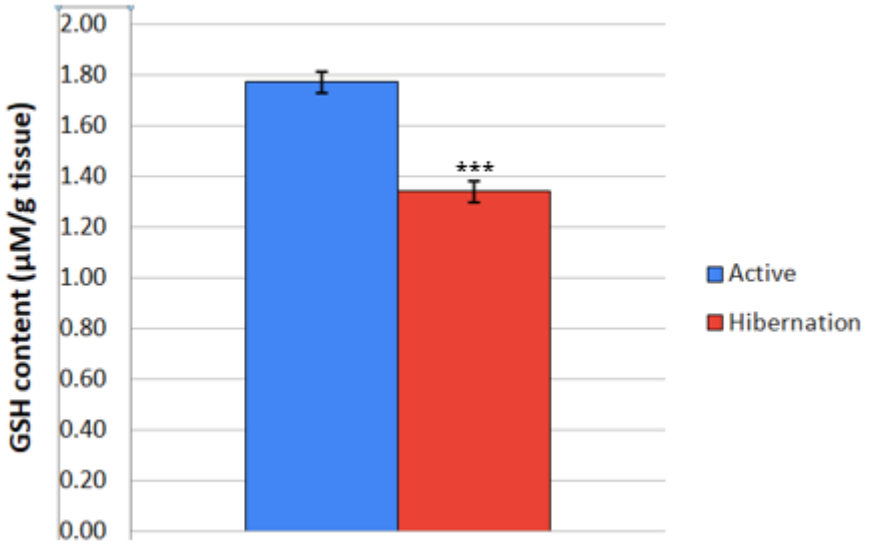

Fig – 5:

Table - 1

LIVER	CONDITION	LPO (Lipid peroxidation)	SOD (Super oxide dismutase)	CAT(Catalase)	ASA(Ascorbic acid)	GSH(Reduced Glutathione)
	Active period	0.26 ± 0.02	0.7 ± 0.01	5.24 ± 0.17	443.71 ± 3.76	1.77 ± 0.04
	Hibernation period	*** 0.446 ± 0.15	NS 0.8± 0.01	*** 8.04 ± 0.11	*** 890.86 ± 3.32	*** 1.34 ± 0.038

Data are expressed as the Mean ±SEM, (n=7). Significant differences calculated (t-test) from animals during the active period are designated as ***(P<0.001),NS(Non significant)