



EVALUATION OF ANXIOLYTIC POTENTIAL OF ETHANOLIC EXTRACT *HYPERICUM HOOKERIANUM* IN STRESS INDUCED SWISS ALBINO MICE

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ABSTRACT

The aim of the present work is evaluate to Anxiolytic profile of *Hypericum hookerianum* in stress induced Swiss albino mice. The study was carried out using Swiss albino mice (25- 30 g). The Anxiolytic effect of aerial parts of ethanolic extract of *Hypericum hookerianum* was evaluated by using behavioral analysis like Elevated plus maze (EPM) test, Open Field Test (OFT), Hole Board Test (HBT), Light dark exploration Test (LDE) in restraint stress induced animals. Behavioral test parameters for anxiety were assessed followed by biochemical parameters (lipid per oxidation, super oxide dismutase, catalase, glutathione per oxidase, reduced glutathione, etc.) and Diazepam 1 mg/kg served as a standard Anxiolytic drug, administered intraperitoneally. The results were shown that, ethanolic extract of *H.hookerianum* (Hh 100mg/kg and Hh 200 mg/kg, p.o.) significantly increased the percentage of time spent and number of entries in open arm in EPM. In LDE, the extract produced significant increase in time spent, number of crossing and decrease in the duration of immobility in light box. In OFT, the extract showed significant increase in number of rearings, assisted rearings and number of square crossed, all of which are demonstrations of exploratory behavior. Biochemical analyses revealed an increase in lipid per oxidation, depletion of super oxide dismutase, reduced glutathione, catalase activity and glutathione per oxidase in stress induced animals as compared to unstressed animal. Six days treatment of Hh (100 mg/kg, 200 mg/kg) comparable with Diazepam, significantly attenuated restraint stress-induced behavioral and oxidative damage. The results of the present study suggest that an ethanolic extract of *H.hookerianum* may possess Anxiolytic activity in stressed animals and provide a scientific evidence for its traditional claim.

Keywords: *Hypericum hookerianum*, stress induced, Anxiety, behavior

INTRODUCTION

Mental Stress is defined as the non-specific response of the body to any demand imposed upon it¹. It is known to alter the physiological homeostasis of the organism and complex mechanisms contribute to the breakdown in adaptation processes resulting in various visceral, endocrinal, and behavioral changes². Stress plays the main role in pathogenesis of mental disorders^{3, 4}. A host of chronic psychiatric disease states like melancholic depression, anorexia nervosa, panic disorders, anxiety disorders and cognitive dysfunction have been reported to involve abnormality of stress axis⁵. Anxiety disorders in particular, affect 1/8th of total population worldwide and have become one of important research interest in psychopharmacology during this decade⁶. The hallmark of anxiety disorders is marked, persistent and excessive or unreasonable fear that is experienced to a degree that significantly interferes with everyday life⁷.

A search for novel pharmacotherapy from medicinal plants for psychiatric illnesses has progressed significantly in the past decade. This is reflected in the large number of herbal preparations for which psychotherapeutic potential has been evaluated in a variety of animal models. Restraint stress can induce a series of dysfunctions of central nervous system, such as cognitive impairment, anxiety, depression, amnesia, and insomnia. Therefore, close attention was paid to studies of protecting effects of natural potential ingredients on stress-induced injury. Several plants have been investigated, which were once used as tonics due to their adaptogenic and rejuvenating properties in traditional medicine. The drugs of plant origin are gaining increasing popularity and are being investigated for remedies of a number of disorders including anti stress adaptogenic activity⁸.

Hypericum hookerianum Wight and arnott is belongs to the family of *Hypericaceae* is a well known plant among the 20 different species of *Hypericum* found in India⁹. It is mainly

present in Asia – tropical areas, Bangladesh, Bhutan. In India *H.hookerianum* mainly in the areas of Arunachal Pradesh, Karnataka, Manipur, Meghalaya, Sikkim, Tamilnadu mainly in Nilgris. Antibacterial spectrum of *Hypericum hookerianum* has been reported¹⁰. *Hypericum hookerianum* stem parts posses potent antitumor activity against DLA induced tumor in mice¹¹. The wound healing potential of *H.hookerianum* leaf and stem extracts has been evaluated^{12, 13}. In the present the study we attempted to evaluate Anxiolytic potential of *Hypericum hookerianum* in stress induced Swiss Albino mice.

MATERIALS AND METHODS

Plant Material

The plant material in this study was collected from the Nilgris, Western Ghats of Tamil nadu, India. The plant was authenticated by Dr.S.Rajan, Field Botanist, Survey of Medicinal Plants & Collection Unit, (Central Council for Research in Homoeopathy), and Department of AYUSH. The collected aerial plant parts were subjected to shade drying for about 5 weeks. The dried plant material was further crushed to powder mechanically and sieved and stored in air tight container for further analysis.

Preparation of the Extract

The shade dried aerial parts of *Hypericum hookerianum* was extracted with pet ether, chloroform and ethanol successively by soxhlation method, water by maceration method at room temperature and concentrated over water bath and evaporated under reduced pressure. The extract obtained was filtered and solvent was evaporated at 50°C under reduced pressure, and then lyophilized.

Animals

Albino mice (20±2 gm) of either sex were obtained from the Central Animal House, KMCH College of Pharmacy. These were randomly housed in groups of six in polypropylene cages at an ambient temperature of 25± 10C and 45-55% Relative Humidity, with a 12 h light/ dark cycle. The animals

had free access to standard pellet (Hindustan Lever Ltd., India) and water *ad libitum*. Experiments were conducted between 8:00 and 14:00 h. Prior permission was obtained from Institutional animal ethics committee to carry out the experiments.

Experimental Design

Twenty groups of Swiss albino mice were used in this study. Five set of groups were individually divided for the each behavioral analysis. In all sets of behavioral analysis first group treated as control and second was of restraint stress induced (3 h/day for six consecutive days). Third group treated with standard reference drug Diazepam. Fourth and fifth groups were treated with the post-treatment of ethanolic extract of *Hypericum hookerianum* (100 mg/kg) and 200mg/kg respectively.

Stress Procedure

The animals were subjected to restraint stress in a wire mesh restrainer for 4 h/day for six consecutive days. The size of restrainers could be adjusted according to the size of the mice ensuring that every mouse was immobile. Immediately after the stress procedure, the mice were exposed to behavioral studies.

Acute toxicity test

Acute toxicity test was performed according to the Organization for Economic Cooperation Development (OECD) Guidelines no. 423 (OECD Guidelines 1996). It was based on a stepwise procedure with the use of a minimum number of animals per step. Three female nulliparous and non pregnant albino mice were used for each step (each dose level). The dose level to be used as the starting dose was selected from one of three fixed dose levels of 25, 200 and 2000 mg/kg, for body weight (OECD Guidelines 1996)¹⁴. The animals were fasted before the oral administration of the extract and observed individually, after dosing, at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h and daily thereafter, for a total of 14 days. The animals were observed for general behavior and any toxic symptoms produced by the extract.

Behavioral analysis

Elevated plus-maze

In order to test the Anxiolytic and sedative properties of ethanolic extract of *Hypericum hookerianum*, the animals were tested in an elevated maze. The maze consist of two open arms (60 x 10 cm) facing each other, and two closed arms (60 x 10 x 24 cm walls), placed perpendicularly to the open arms. This apparatus was fabricated in acrylic; the arms originated in a central platform and it was placed at 65 cm height over a dark surface¹⁵. After placing the animal in the center of the maze the following parameters were recorded, unaware of the treatment received by each animal, during a 5 min test period: number of entries into any of the arms and total time spent in either open or closed arms. Entry into an arm was considered only when the animal placed the four paws inside the arm. After each test, the maze was thoroughly cleaned with diluted ethanol.

Open Field Test

This activity was quantified for 5 min in an open field. It was consisted of a square arena 96 X 96 cm² with 60 cm high walls. The walls and the floor were painted in white. The floor was divided in to 16 squares by parallel and intersecting white lines¹⁶. Four squares defined as the center and the 12 squares along the walls as the periphery. Mice were placed in the very center of the open field and (a) latency, (b)

ambulation, and (c) rearing were observed during a 5 min exposure period for both control and treated animals.

Hole- board test

The animals were subjected to the hole-board test during 5 min. In one set of five groups (control, stress induced, stress induced+ Diazepam (1mg/kg), stress induced+ 100 mg/kg of H.h, stress induced + 200mg/kg H.h) the test was performed before the administration of the treatments and 60 min later. In the second set, the testing was done before and 120 min after administration of the drugs. This apparatus consisted of a 72 x 72 cm plastic square with 28 cm height walls. The floor had 16 evenly spaced holes (4 and 3 cm of diameter and depth, respectively). The animal was placed in the center of the square and allowed to explore the area by dipping its head into the holes¹⁷.

Light dark exploration test

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of the animals, applying mild stressors, i.e. novel environment and light. The apparatus consisted of two polyvinylchloride boxes (20 x 20 x 14 cm) covered with Plexiglas. One box was dark and covered with cardboard and the second box had a 100-watt bulb suspended 25 cm above it as the only source of light. An opaque tunnel (5 x 7 x 10 cm) between the two boxes. The apparatus was placed on a stand in the mouse room. Each mice were placed individually in the darkened box and recordings were made over a 5-minute period, counting the time spent in the lit box (TLB) and the number of transitions (TRANS) across the tunnel, light box rear no, duration of light box rears, vertical activity urination defecation grooming. Each mouse with all four paws in the destination box was said to have made a transition^{18,19}. All the values were recorded in stress induced period and after the administration of drugs.

Preparation of Tissue homogenate

After the experimental period, the animals were sacrificed, brain was isolated and tissue homogenate was prepared. The tissues of brain were weighed and 10% tissue homogenate was prepared with 0.025 M Tris- Hcl buffer, (pH 7.5). After centrifugation at 10,000 rpm for 10 minutes, the clear supernatant was used to measure thio barbutric acid substances (TBARS).

The tissues of brain were minced and homogenized with (10%w/v) in 0.1 M tris- buffer. (ph 7.4) and centrifuged at 10,000 rpm for 10 min and the resulting supernatant was used for the determination of SOD and CAT activity where as the tissue homogenate as such was used for the estimation of GPx and GSH activity.

Biochemical analysis

Estimation of lipid per oxidation

The quantitative measurement of lipid per oxidation in the whole brain was assessed as per method of Okhawa²⁰.

Assay of superoxide dismutase

SOD was assessed by the inhibition of formation of NADH-phenazine methosulphate nitroblue tetrazolium formazon²¹. The reaction was initiated by the addition of NADH after incubation for 90 s and stopped by the addition of glacial acetic acid. The color formed at the end of the reaction was extracted into the butanol layer and measured at 520 nm.

Assay of catalase

CAT was assayed colorimetrically as per the method of Sinha²². Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of H₂O₂. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to

split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of a dichromate-acetic acid mixture and the remaining H_2O_2 was determined colorimetrically as chromic acetate.

Assay of glutathione peroxidase

GPx was estimated as described by Rotruck *et al.*²³. Consider amount of homogenate was allowed to react with H_2O_2 in the presence of GSH for a specified time period, then the remaining GSH was allowed to react with DTNB and the developed yellow color was measured at 412 nm.

Estimation of reduced glutathione

GSH in brain homogenate was measured according to the method of Ellman²⁴. This method is based on the development of a yellow color when 5, 5'- dithio-bis-2-nitrobenzoic acid (DTNB) is added to compounds containing sulphydryl groups. Estimation of tissue protein

Protein was estimated by Lowry's method²⁵. The method is based on the biuret reaction, formation of protein complex and reduction of phosphomolybdo tungstate reagent (Folin Cio Calteau phenol reagent) by tyrosine and tryptophan residues of protein to form a colored product.

Statistical analysis

One way analysis of variance followed by Dunnet was employed for the analysis of behavioral variances and Biochemical parameters. $P < 0.05$ and $P < 0.01$ was considered significant.

RESULTS AND DISCUSSION

Acute toxicity test

In acute toxicity testing were analysed by OECD guidelines. As even the mice receiving the highest dose of *H.hookerianum* (2000mg/kg body weight) did not show any mortality, dose levels at $1/10^{\text{th}}$ (200 mg/kg body weight, p.o) and $1/20^{\text{th}}$ (100 mg/kg body weight, p.o.) of this highest dose were selected for the Anxiolytic activity.

Elevated plus maze

Elevated plus maze data revealed that restraint stress (3h/day for 6 consecutive days induced a significant reduction in open arm entries and time spent in open arm ($P < 0.05$). Post treatment of ethanolic extract of *H.hookerianum* (100 mg/kg and 200 mg/kg for 10 days) reversed the restraint stress-induced changes in both open arm entries and time spent in open arms and the significant is $P < 0.05$, $P < 0.01$ respectively. Standard drug diazepam also increases the open arm entries and time spent in open arm ($P < 0.01$).

Open field test

Restraint stress-induced a significant increase in the latency decrease in ambulation and rearing as compared to control ($P < 0.05$). Post-administration ethanolic extract of *H.hookerianum* (100mg/kg, 200mg/kg) attenuated the restraint stress effects on the open field behavior. Latency was decreased, ambulation was increased by. Rearing activity was also increased by treatment of *H.hookerianum* respectively, as compared to stress ($P < 0.01$ for Hh (200 mg/kg), $P < 0.05$ for Hh 100 mg/kg). Diazepam 1mg/kg significantly decrease in latency increase in ambulation and rearing in stress induced groups.

Hole board test

Restraint stress decreased the number of head dipping in stress induced group when compared to normal groups ($p < 0.05$). Post administration of Diazepam, *H.hookerianum* (200mg/kg) significantly increase the head dipping of mice when compared to stress induced group ($P < 0.01$). Post administration of *Hypericum hookerianum* (100 mg/kg) also increase the number of head dipping.

Light dark exploration test

Restraint stress induced group, the values shown that mice spent more time in dark box, Number of crossings of mice is also reduced, transfer latency is decreased when compared to control or vehicle group. Post administration of ethanolic extract of Hh (100 mg/kg) significantly increase the time spent in light box, increase the number of crossings and decrease the transfer latency ($P < 0.05$) when compared to control group. Post administration of *H.hookerianum* (200mg/kg) and diazepam (1mg/kg) significantly increase the time in light box and automatically decrease the time spent in dark box, increase the number of crossings and decrease the number of latency in treated groups ($P < 0.01$).

Neuro biochemical analysis

Lipid per oxidation

In this study, the TBARS level was significantly increased during stress condition and was found to be decreased on treatment with Diazepam and Hh (100mg/kg) and Hh (200 mg/kg); this effect was also dose dependent.

Neuro antioxidant enzymes

SOD, CAT, GPx and GSH activities were significantly decreased ($P < 0.05$) in stress-induced animals as compared to the control group. These activities were significantly restored ($P < 0.01$) in Hh (200 mg/kg) and Diazepam treatment group. Hh (200 mg/kg)-treated group significantly restored the activities as compared to Hh (100 mg/kg)-treated group ($P < 0.05$).

Tissue total protein

The tissue (brain) total protein was analyzed by Lowry *et al.*'s method. The protein value was found to be decreased in the tissue of animals belonging to stress induced group and it was more pronounced in stress induced animals. On treatment with the Diazepam (1mg/kg), Hh(200mg/kg), Hh (100mg/kg) the reversal of the concentration was identified and it was dose dependent, increase in dosage increased the protein concentration. The protein levels in animals treated with the Hh (200 mg/kg) and Diazepam alone were near normal. Significant increase was noted in stress induced animals treated with 200 mg of the drug. This decrease in the total protein content might be attributed to the degeneration of tissue protein in the brain.

Discussion

In the present study, restraint-stressed animal showed significant loss of memory and anxiety behavior, impaired memory activity indicating stress-induced neurobehavioral alterations²⁶. Both EPM and OFT have been used very effectively to assess neurobehavioral profile of animals under the influence of anxiogenic/Anxiolytic agents^{27, 28}. Our results indicated that restraint stress caused a significant reduction in the % number of entries and % time spent in open arms in EPM. Similarly in the OFT restraint stress induced behavioral alterations as evidenced by increase in latency and decrease in ambulation and rearing. In stress induced animals, post treatment with Diazepam (1mg/kg), Hh(100mg/kg), Hh(200 mg/kg) produced reversal behaviors of stress i.e., the results shown that the rodents spent more time in light arm and increased the number of entries in EPM. Similarly in OFT standard drug and plant extract treated groups produced decrease in latency and increased in ambulation and rearing. Hh (200 mg/kg) produced well pronounced anxiolytic activity compared with Hh (100 mg/kg).

The anxiolytic-like activity was also observed in the light/dark box. L and DT is an ethological-based approach-avoidance conflict test and it is sensitive to drugs that affect

anxiety. In this test, the number of transitions between the light and dark compartments as well as the time spent in the light side is recognized as anxiety indices, despite the transition parameter being highly dependent on loco motor activity²⁹. Mice treated with *Hh* (100 and 200 mg/kg) showed increase in the time spent in the light compartment and no changes in the numbers of shuttle crossings, confirming the activity upon the main anxiolytic parameter. The observed anxiolytic effect of *H.hookerianum* may be due to the agonistic effect on GABA/benzodiazepine receptor complex, or antagonize the 5-HT1B receptor or agonize the 5-HT1A receptor^{30, 31}. Hole-board test indicated that the head-dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state may be reflected by an increase in head-dipping behavior¹⁷. In our study, *Hh* ($P < 0.01$) increased the numbers and duration of head poking compared to the stress induced group.

In this study, the TBARS level was significantly increased during stress induced condition and was found to be decreased on treatment with Diazepam, *Hh*200mg/kg and *Hh* (100 mg/kg); this effect was also dose dependent. The *Hh* plant extracts and Diazepam caused a decrease in TBARS level in enzymatic assay in stress induced animals. The enzymatic NADPH-dependant Lipid Peroxide (LPO) is catalyzed by the NADPH-cytochrome P450 reductase and propagated by cytochrome P450 with generation of free radicals, i.e., $O_2^{\cdot-}$ and $ROO^{\cdot}$. The *H.hookerianum* ethanolic extract might have inhibited the activity of NADPH-dependant LPO due to its association with its free radical scavenging ability. Elevations in the levels of products of free radicals like TBARS in brain of acute and chronic stress induced group again support the low antioxidant enzyme activity that elevates the lipid per oxidation while TBARS is the product of lipid per oxidation. Another possibility for such an elevation in TBARS may be due to ischemia-reperfusion phenomenon^{32,33} or due to high rate of catecholamine secretion that generates free radicals either through auto-oxidation or through metal ion or superoxide-catalyzed oxidation³⁴.

Stress induced oxidative damage in mice brain has been established here by noting the low activities of SOD, CAT, and GST, important antioxidant enzymes, which are consistent with the observation of others³⁵. The decrease in antioxidant enzyme activities due to immobilization, might be due to their use against the free radicals destruction and/or their inhibition by free radical species³⁶. It is well established that SOD activity is inhibited by hydrogen peroxide that reduced Cu^{+2} to Cu^{+1} in SOD³⁷. The reduced Cu^{+1} can act as a promoter of hydroxyl by Haber-Weis reaction. Low antioxidant enzyme activities further facilitate the increased susceptibility to lipid per oxidation³⁸. To the reduction of hydrogen peroxide is catalyzed by CAT that protects the tissues from highly reactive hydroxyl radicals³⁹. Reduction of hydrogen peroxide and hydro peroxides to non-toxic products is catalyzed by GST and per oxidase. The key findings of this research are that the antioxidant nature of this *H.hookerianum* would probably relieve stress in individuals who remain immobilized either due to their job profile or physical conditions.

In the present study, stress-treated groups increase level of lipid per oxidation is indicated that the stress caused significant oxidative damage and depletes SOD, catalase, Gpx and GSH activity. Tsuboi *et al.* reported an increased oxidative damage and weak antioxidant defense events are

implicated in psychiatric illness⁴⁰. Dongre *et al.*⁴¹ have reported that *H.hookerianum* contains many phenolic compounds namely flavonoids and phenolic acid suggesting that could have potent antioxidant property. Super oxide dismutase, catalase, glutathione peroxidase, reduced glutathione levels were significantly restored to significant levels. It suggests the possible role of *H.hookerianum* as an antioxidant and has been reported earlier by Raghu Chandrasekhar *et al.*⁴² about the antioxidant effect of *H.hookerianum* against superoxide anion. Flavonoids, specifically quercetin and its glycoside derivatives, are a major class of compounds present in the total *Hypericum* species. Such free radical scavenging capacity was found to correlate with the content of several flavonoids including quercetin and hyperoside⁴³. In the present study Diazepam, *Hh* (200 mg/kg) and *Hh* (100 mg/kg) produced anxiolytic potential in experimental stress induced swiss albino mice. Same time *Hh* (200 mg/kg) produced more Anxiolytic activity similar with standard drug diazepam when compared with *Hh* (100mg/kg).

CONCLUSION

In the study reported that the well pronounced Anxiolytic potential of ethanolic extract of *H.hookerianum* in all stress induced behavioral analysis EPM, OFT, LDE and HBT. Neuro antioxidants and Lipid per oxidation results were shown that the values were significantly restored in stress induced animals with the post treatment of *Hypericum hookerianum* ethanolic extract. Thus, *H.hookerianum* has potential clinical applications in the management of stress induced anxiety disorders. Further investigations are warranted for elucidating the exact mechanism and bioactive compounds.

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Fig.1.Elevated Plus Maze Test

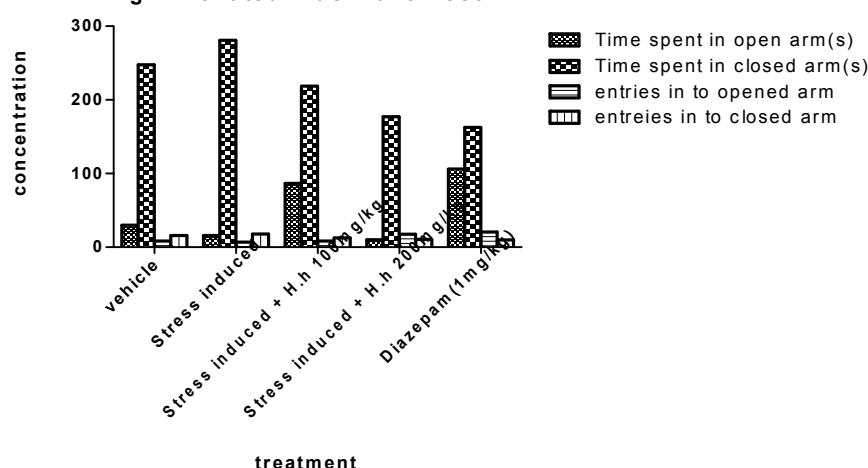


Fig 2 :Open field test

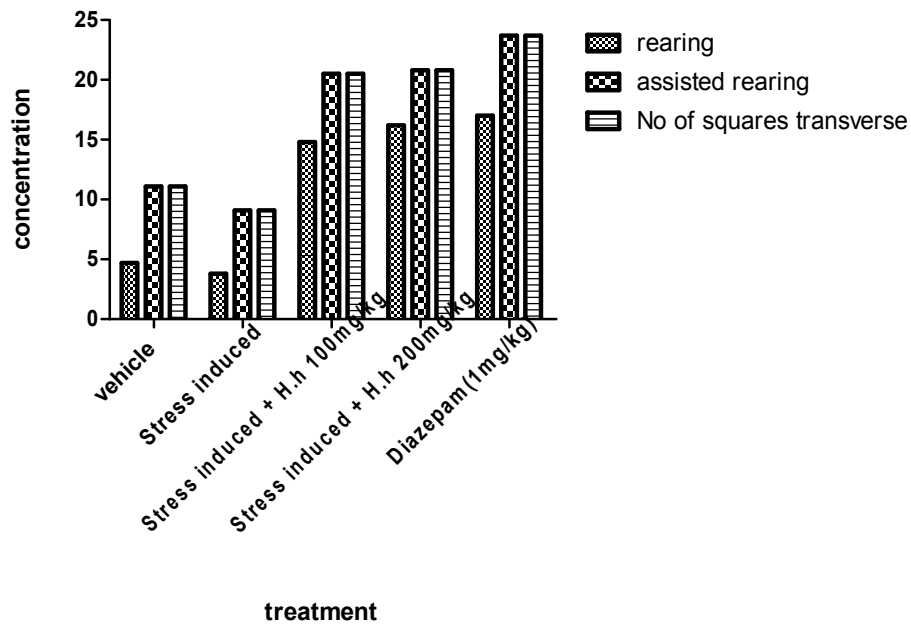


Fig 3 :Hole board test

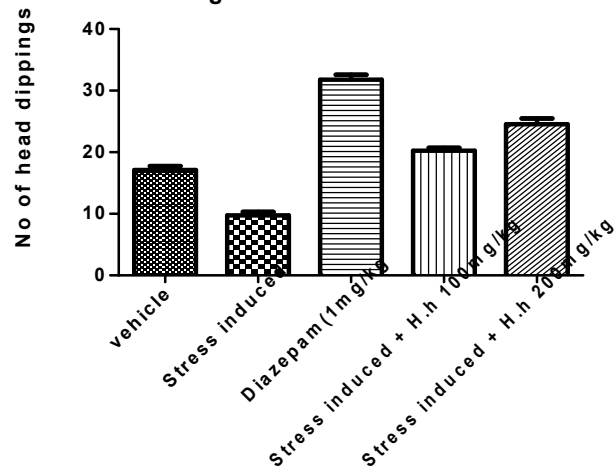
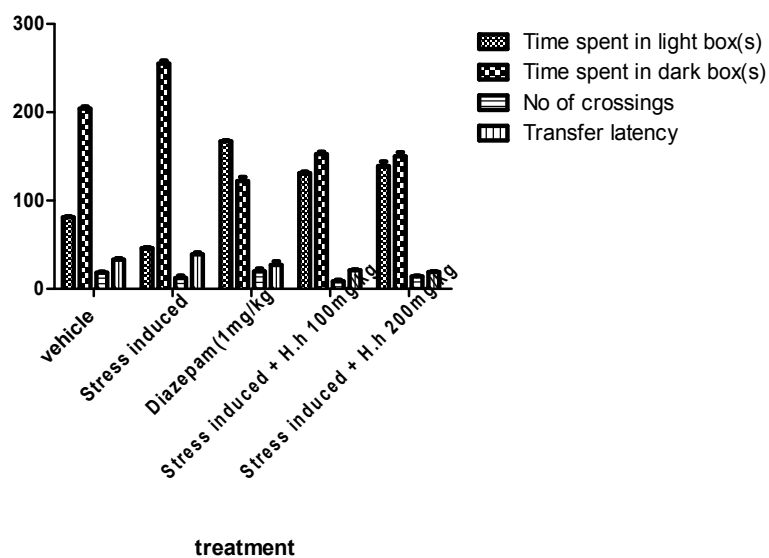
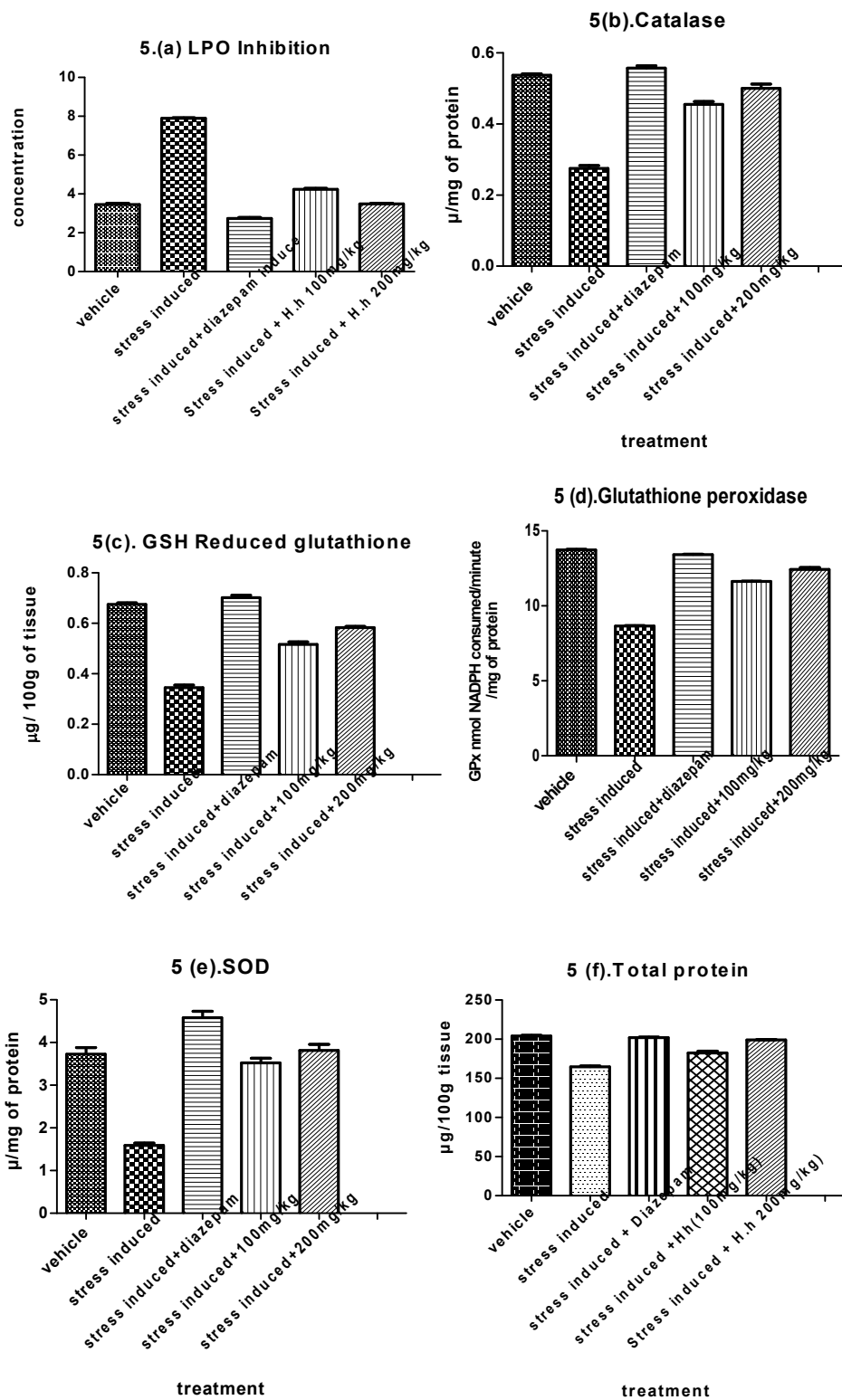


Fig 4 :Light dark exploration test





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