CHAPTER – 4

ANXIOLYTIC LIKE EFFECTS OF ETHANOLIC EXTRACT OF
H. hookerianum (EEHh) AND ITS GLYCOSIDIC FLAVONOID ENRICHED
EXTRACT (GFHh) IN INDUCED STRESS SWISS ALBINO MICE

4.1. Objective

To analyze the anxiolytic like effects of ethanolic extract of H. hookerianum (EEHh) and its glycosidic flavonoid enriched extract (GFHh) in induced stress Swiss Albino mice by behavioral and biochemical methods.

4.2. Introduction

Homeostasis of the body is mainly imbalanced by stress and further the body copes with stress via an adaptation identified as “fight or flight” response (Johnson et al., 1992). The stress also affects the physiological and mental regulations of the body and the conditions may vary depending on the severity of stress, type and time of its exposure (Kaouane et al., 2012). Some of the reports have confirmed that stress leads to neurological disorders, such as anxiety (excessive fear), depression (mental tiredness) and insomnia (sleeping disturbance) (Gold and Chrousos, 2002; Goldstein, 2010).

Anxiety is caused by stress which refers to the physical and mental status that are induced by marked, continual and excessive or irrational reaction to fear (Cryan and Holmes, 2005). The rate of prevalence of anxiety is high (18.1%) among teenagers and their lifetime prevalence is about 28.8% (Kessler et al., 2005). Anxiety related disorders such as generalized anxiety (GAD), panic, obsessive-compulsion (OCD), phobias or post traumatic stress disorders (PTSD) are widely seen in general population (Ernst, 2004) and one eighth of the people are affected by anxiety worldwide. Anxiety is also an obvious thing of other psychiatric illnesses and medical conditions (Lavie et al., 2004). Anxiety is characterized by panic or phobic conditions that are connected with significant disabilities (including educational and occupational) which has a negative impact on the quality of life.
(Kasper et al., 1998). Therefore, the present research area finds importance in psychopharmacology (Rabbani et al., 2003).

So far preclinical studies have been conducted to explore safety, more specific, and perhaps less expenditure therapies for stress induced anxiety disorders. Natural anti-stress agents (i.e., herbal medicine) have an advantage in research because they have been used in Siddha, Ayurveda, Unani and Chinese medicines to treat different diseases, including neuropsychiatric disorders, with less side effects (Carlini, 2003). The plant extracts of *Schizandra sinensis, Scutel laria baicalensis Cordyceps sinensis* have been shown to alleviate stress-induced anxiety disorders in experimental rodents (Koh et al., 2003). Moreover, the roots and stem barks of *Acanthopanax koreanum* Nakai (Araliaceae), a well-known herbal medicine in Jeju Island, Korea, have been used as a tonic to treat stress-related disorders (Bae, 2000). Likewise, the ethanolic extracts of *Ocimum sanctum* and *Camellia sinensis* have been reported to posses anxiolytic activity (Tabassum et al., 2010). *Hypericum perforatum* (St.John’s wort) is known to posses anxiolytic properties in stress induced mice (Kumar et al., 2010).

*H. hookerianum* is commonly known as hooker’s St. John wort and golden lotus for its lotus shaped yellow flowers. Though there are large numbers of bioactive molecules, there is paucity of scientific reports on the biological activity of *H. hookerianum*. Therefore, the present study focuses on the anxiolytic like effects of ethanolic extract of *H. hookerianum* (EEHh) and its glycosidic flavonoid enriched extract (GFHh) in stress induced Swiss Albino mice by various behavioral and biochemical methods.

**4.3. Materials and Methods**

**4.3.1. (a) Preparation of ethanolic extract of *H.hookerianum* (EEHh)**

The procedure is previously described in chapter: 3.3.6.
4.3.1. (b) Separation of Glycosidic flavonoid enriched extract (GFHh) by acid hydrolysis method

The procedure is previously described in chapter: 3.3.8.

4.3.2. Experimental animal Study

Swiss Albino mice, weighing 25-30 gm were obtained from Small Animal Breeding Station, Kerala Veterinary and Animal Science, Mannuthy, Thrissur, South India. Animals had ad libitum up accessed food and water till the experimental period. Prior to experiment, the mice were housed in polypropylene cages in a group of six groups under natural light-dark cycle under standard laboratory conditions. Behavioral evaluations were made at room temperature in a noiseless diffusely illuminated room between 9.00 to 17.00 h. Experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) (Reg. No. KMCRET/Ph.D./11/2011). Acute toxicity study with minimum number of animals and gross behavioral analysis of mice was assessed by standard method (Irwin, 1962).

4.3.3. Experimental groups

For this purpose, Swiss Albino mice were divided into six groups (n=6), first group served as Control, second group Stress Induced (SI), while the third and fourth groups are stress induced and treated with EEHh (200 and 400 mg/kg) respectively. Fifth group stress induced and treated with GFHh (100 mg/kg). Finally, the sixth group was treated with reference drug diazepam (GABA receptor; 1 mg/kg) which was administered intraperitoneally (i.p) to the stress induced groups.

Exposure of animals to stress

The mice were subjected to restraint stress in a wire mesh restrainer and stress induced for 6 h/day. The size of restrainer could be adjusted according to the size
of the mice to induce the stress (Fig.4.1). After this induction, the mice were exposed to following behavioral studies (Yadav et al., 2008).

4.3.3.1. Elevated Plus Maze test (EPM)

The EPM test has been widely used to measure the anxiolytic activity of a new molecule, including herbal drugs (Zhang, 2004; Carobrez and Bertoglio, 2005) in experimental animals (File and Pellow, 1985; Lister, 1987). The apparatus was made of wood (painted black in color) which consisted of two open arms (30 cm length×5 cm width) and two closed arms (30 cm length×5 cm width) with 25-cm walls (Fig.4.2). The arms extended from a central platform (5×5 cm²) and the maze was elevated 38.5 cm above the floor. Each mouse was placed individually on the central platform, facing one of the closed arms. The number of open arm entries, time spent in closed and open arms and rearings of mice were recorded during total time of 5 min.

4.3.3.2. Open Field Test (OFT)

The OFT was used to evaluate the exploratory activity of the experimental animals (Archer, 1973). Ambulatory (movement from one part to another part) behavior was assessed in an OFT. The apparatus consisted of transparent acrylic walls and a box with a black floor measuring 30 cm × 30 cm × 15 cm in height. The floor of the box was divided into nine equal squares. At the beginning of the session, mouse was placed in the center of the square and allowed to explore the area freely. The number of squares crossed (with the four
paws) and number of rearings during total time of 5 min was recorded (Fig. 4.3.)

4.3.3.3. Hole Board Test (HBT)

The hole board apparatus used in this study consisted of a wooden box (40×40×25 cm) with 16 holes (each diameter 3 cm) evenly distributed on the floor. The apparatus was elevated to the height of 25 cm. After 7 days of treatment, mice were placed on the centre of the hole board apparatus and allowed to roam freely in the apparatus (Fig. 4.4). The number of head dips as well as the head dip latency in seconds was recorded in a 5 min time period (Perez et al., 2008).

4.3.3.4. Light-Dark Exploration Test (LDE)

The test apparatus consisted of a rectangular box (40×27×30 cm), divided into one small (8×27 cm) and one large (27×27 cm) area, with an open door (97.5×7.5 cm) located in the center of the partition at the floor level. The small compartment painted in black and illuminated with bulb with dull light, whereas the other large compartment wood was painted with white and brightly illuminated with 60 Watts cold light sources. Mice were placed individually in the center of the light compartment facing away from the entrance of the apparatus and allowed to explore freely. The behavior recorded during the test included (i) the number of entries to the light chamber (ii) the number of entries to the dark chamber (iii) time spent in both the light and dark chamber (Ambavade et al., 2006).
4.3.3.5. Social Interaction Test (SIT)

The apparatus used for the test was a box (60×60×35 cm) with a solid floor and was placed in a dimly lit room. The mice were placed individually in the box and allowed for 7.5 min adaptation sessions at 2 hr intervals (Fig.4.6). On day 7, mice were paired on weight and sex basis and placed in the box for 5 min. Interaction values were recorded before and after the treatment. During this schedule, total time spent by the mice pair in “social interaction”, including grooming, following, kicking, sniffing, boxing, biting and crawling was recorded by a neutral ‘blind’ observer (File et al., 1978).

4.3.3.6. Marble buried test (MBT)

Marble-buried behavior of mice was performed as previously described by Ichimaru et al (1995). In brief, each mouse was individually placed in plastic base (21 × 38 × 14 cm) containing 5cm thick sawdust beddings. Twelve small glass marbles (diameter 10–12 mm) were arranged on the bedding, evenly spaced in four rows (Fig.4.7). After the stress exposure, the number of marbles buried by mice was counted. A marble covered with at least 2/3 of its size by saw dust was considered as “buried”.

4.3.3.7. Novelty Induced Feeding Latency (NIFL)

The test apparatus is a wooden box (60×60×35 cm) with a solid floor placed in a dimly lit room. The floor of the box was covered with a 2 cm layer of wooden chips and laboratory chow pellet placed on the floor evenly. A similar arrangement was made in the polypropylene home cages of the mice. Food was removed from
the home cage at 48 h prior to testing, but water was provided *ad libitum*. Inexperienced mice were placed separately in the test chamber with novel changes and the latency to start eating (defined as chewing of the pellet and not merely sniffing or playing with it), was recorded. If the mice had not eaten within 5 min, the test was terminated and a latency score of 300 sec was allotted and the observation was made by a neutral blind observer (Bodnoff et al., 1988).

4.3.4. Antioxidant assays

4.3.4.1. Estimation of enzymic antioxidants

The activities of enzymic antioxidants Superoxide Dismutase-SOD (Kakkar et al., 1984), Catalase-CAT (Sinha, 1972), Glutathione Peroxidase –GPx (Rotruck, 1973), Glutathione-S-Transferase – GST (Habig et al., 1974) were studied using Standard methods. The protein concentrations present in the tissues were determined by Lowry's method initially.

4.3.4.2. Estimation of lipid peroxidation

Lipid peroxidation (LPO) of brain tissue homogenate was estimated spectrophotometrically by measuring thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979).

4.3.4.3. Estimation of non-enzymic antioxidants

Reduced glutathione content-GSH (Ellman, 1959), levels of vitamin C (Omaye et al., 1994) and vitamin E (Desai, 1984) were studied using standard methods.

4.3.5. Estimation of brain neurotransmitters

Gamma Amino Butyric Acid- GABA (Nishizawa et al., 1959), serotonin and dopamine (Margret et al., 1974) were studied using standard methods.
4.4. Statistical Analysis

All the data were expressed as mean ± S.D. The results were analyzed with the help of analysis of variance (ANOVA) followed by Bonferroni’s test (multiple comparisons). For all the experiments, first the comparisons were made between the control and stress induced group and also between stress induced and treated groups. Statistical difference were considered significant when the ‘p’ value was <0.005.

4.5. Results

4.5.1. Gross behavior analysis of Ethanolic extract of *H. hookerianum* (EEHh) in Swiss Albino mice

The gross behavioral analysis of Swiss Albino mice treated with the different concentrations (200, 400 and 2000 mg/kg) of ethanolic extract of *H. hookerianum* (EEHh) are described in table 4.1. After the oral administration of EEHh, the animals were observed continuously for 48 hours to monitor their gross behavior (awareness, mood, motor activity, secretory signs, general signs, central excitation, motor in-coordination and muscle tone). During this study, no visible adverse reactions were noted. Even at the highest dose of 2000 mg/kg, only decrease in alertness was observed, but did not show any sign of mortality and toxic reactions. So that dose levels selected from 2000 mg/kg were calculated to 1/10th (200 mg/kg b.wt, p.o) and 1/5th (400 mg/kg b.wt, p.o) for EEHh. But in case of GFHh, 100 mg/kg were selected for anxiolytic activity.
Table 4.1. Gross behavior analysis of experimental mice

<table>
<thead>
<tr>
<th>Gross behavior</th>
<th>EEHh (200 mg/kg)</th>
<th>EEHh (400 mg/kg)</th>
<th>EEHh (2000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Awareness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alertness</td>
<td>#</td>
<td>#</td>
<td>↓</td>
</tr>
<tr>
<td>Passivity</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stereotype</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Mood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grooming</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Vocalization</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Restlessness</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Irritability</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Motor activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous activity</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Touch response</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Pain response</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td><strong>Central excitation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Startle response</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Straub tail</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tremors</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Convulsions</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Motor in-coordination</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting reflex</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Staggering gait</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Abnormal gait</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Muscle tone</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grip strength</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Limb tone</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Abdominal tone</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td><strong>Secretery signs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urination</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Salivation</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Lacrimation</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td><strong>General signs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Writhing</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piloerection</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Respiration Rate</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
</tbody>
</table>

Note: ‘−’ absent, ‘#’ Normal, ↓ Decreased.
4.5.2. Effect of EEHh and GFHh on behavioral analysis

4.5.2.1. Effect of EEHh and GFHh on Elevated plus maze test (EPM) in induced stress mice

Fig 4.8. illustrates the effect of EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg) on induced stress mice in EPM. In this test, time spent in closed arms and open arms and also the number of rearings of mice was recorded in all the groups. Stress induced group spent more time in closed arms, but spared less time in open arms and also observed less number of rearings (p<0.001) when compared with the control group. Mice treated with EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg) spent more time in open arms and also significantly increased the number of rearings when compared with stress induced group (p<0.001).

![Graph](image)

Fig 4.8. Effect of EEHh and GFHh on EPM (A) Time spent in open arms (B) Time spent in closed arms and (C) Number of rearings in induced- stress mice

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group ; # p<0.001 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
4.5.2.2. Effect of EEHh and GFHh on Open Field Test (OFT) in induced-stress mice

![A] Number of squares transversed

![B] Number of rearings

Fig 4.9. Effect of EEHh and GFHh on OFT (A) Number of squares transverse (B) Number of rearings in induced-stress mice

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group; # p<0.001 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
This test is used to assess the locomotor activity of mice in an open field. Here, number of squares crossed and rearings were analyzed in all groups. Stress induced group showed less number of squares transversed and rearings when compared with control group (p<0.001). The mice treated with EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg) showed high number of squares transversed (p<0.001) and rearings (p<0.001) which indicates the anxiolytic activity of EEHh and GFHh when compared with the stress induced group (Fig.4.9).

4.5.2.3. Effect of EEHh and GFHh on Hole Board Test (HBT) in induced-stress mice

The effect of EEHh and GFHh on stress induced mice in HBT is depicted in figure 4.10. (A) and (B). The stress induced mice showed increased head dip latency and reduced number of head dippings when compared with control group (p<0.001). The plant extracts, EEHh (200 and 400 mg/kg) and GFHh (100 mg/kg) caused significant reduction in head dip latency and also increased the number of head dippings when compared with stress induced group (p<0.001) which is similar to standard drug diazepam.
Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group ; # p<0.001 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.

4.5.2.4. Effect of EEHh and GFHh on Light Dark Exploration test (LDE) in induced- stress mice

As shown in Fig 4.11, the anxiolytic effect of diazepam (1mg/kg), EEHh (200 and 400 mg/kg) and GFHh (100 mg/kg) was apparent in the light dark exploration test. Stress induced mice spent less time in the light zone and the
number of shuttle crossings were also less when compared with control group (p<0.001). In comparison with stress induced group, significant increase (p<0.001) in both time spent in the light zone and shuttle crossings were observed in mice treated with EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg).

![Graph A](image1.png)

![Graph B](image2.png)

**Fig 4.11. Effect of EEHh and GFHh on Light Dark Exploration Test (A) Time spent in light (B) Shuttle crossings in induced- stress mice**

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group ; # p<0.001 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
4.5.2.5. Effect of EEHh and GFHh on Social Interaction test (SIT) in induced stress mice

![Graph showing the effect of EEH and GFH on SIT in induced stress mice](image)

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group ; # p<0.001 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.

In the social interaction test, stress induced mice interacted less with the partner when compared to control group (p<0.001). On the other hand, mice administered with EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg) significantly increased their interaction time with the partner when compared to stress induced group (p<0.001) (Fig.4.12).
4.5.2.6. Effect of EEHh and GFHh on Marble Buried Test (MBT) in induced stress mice

The animal’s behavior in burying reflects the defensive action which is evaluated by marble burying test (Fig.4.13). This kind of behavior is reduced by the use of drugs having anxiolytic properties. In this test, stress induced group buried more marbles when compared with control group (p<0.001). Whereas in mice treated with EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg), a significant reduction in the number of marbles buried were observed when compared to stress induced group (p<0.001).

4.5.2.7. Effect of EEHh and GFHh on Novelty Induced Feeding Latency in induced stress mice

In this test, feeding latency was increased in stress induced group when compared with control group (p<0.001). But the other treated groups, EEHh (200 and 400 mg/kg), GFHh (100 mg/kg and DZP (1 mg/kg) showed significant (p<
0.001) attenuation of novelty induced feeding latency in mice compared to stress induced group (p<0.001) (Fig.4.14.).

![Graph showing time in seconds for different groups](image)

**Fig 4.14. Effect of EEHh and GFHh on NIFL in induced- stress mice**

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group ; # p<0.001 when compared with stress induced and treatment groups using one- way ANOVA followed by Bonferroni’s test as a post-ANOVA test.

### 4.5.3. Evaluation of *in vivo* antioxidant potential of EEHh and GFHh in induced- stress mice hippocampus

#### 4.5.3.1. Effect of EEHh and GFHh on enzymic antioxidants in induced- stress mice hippocampus

The effect of EEHh, GFHh, and DZP on enzymic antioxidants (SOD, CAT, GPx and GST) activities of mice hippocampus is shown in Fig 4.15. (A, B, C and D) respectively. Biochemical analysis showed significant decrease in SOD, CAT, GPx and GST activities in stress induced group when compared with control group (p<0.001).

When the animals treated with 200 mg/kg of EEHh exhibited increased activities of all enzymes SOD, CAT, GPX (p<0.001) and GST (p<0.005) when compared with stress induced group. The other treated groups, EEHh (400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg) showed significant increase in enzyme
activities (p<0.001) when compared with stress induced group. Interestingly, GFHh (100 mg/kg) treated group showed improved SOD, CAT, GPx and GST activities than EEHh (400 mg/kg) treated group. The GFHh treatment remarkably improved antioxidant defense in stress induced mice which is similar to standard drug diazepam.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**Fig 4.15.** Effect of EEHh and GFHh on enzymic antioxidants (A) Superoxide Dismutase (B) Catalase (C) Glutathione peroxidase and (D) Glutathione -S- Transferase in induced stress mice hippocampus

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group and # p<0.001, @ p<0.05 and $ p<0.01, when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test
4.5.3.2. Effect of EEHh and GFHh on LPO in brain of induced stress mice

The treatment effect of EEH (200 and 400 mg/kg), GFH (100 mg/kg) and DZP (1 mg/kg) on LPO level of mice are depicted in Fig.4.16. (A). Biochemical analysis of LPO indicated that the level of MDA was significantly increased (p < 0.001) in stress induced group due to increased free radical generation, as compared with the control group. While administration of EEH (200 and 400 mg/kg), GFH (100 mg/kg) and DZP (1 mg/kg) significantly (p < 0.001) brought down the level of MDA which indicated the decreased LPO level, when compared with the stress induced group.

Fig 4.16. Effect of EEHh and GFHh on non-enzymic antioxidants (A) Lipid peroxidation (B) Reduced Glutathione (C) Vitamin C and (D) Vitamin E in brain of induced stress mice

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group ; # p<0.001, @ p<0.05 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
4.5.3.3. Effect of EEHh and GFHh on non-enzymic antioxidants in brain

Reduced glutathione (GSH) and vitamins (C and E) have implications in antioxidant defense mechanism. The effect of EEHh, GFHh and DZP (1 mg/kg) on non-enzymic antioxidants is depicted in Fig 4. 16. (B, C and D). Biochemical analysis indicated that decreased GSH content and vitamin C and E levels in stress induced group when compared with control group (p<0.001).

When the animals treated with 200 mg/kg of EEHh exhibited increase in GSH content, vitamin C and E levels (p<0.005) when compared with stress induced group. On the other hand, EEHh (400 mg/kg), GFHh (100 mg/kg) and DZP (1 mg/kg) treated groups showed significant increase in all non-enzymic antioxidants. Among the extracts, GFHh treatment remarkably improved the non-enzymic antioxidants in stress induced mice which is similar to standard drug diazepam.

4.5.4. Effect of EEHh and GFHh on brain neurotransmitters

4.5.4.1. Effect of EEHh and GFHh on brain GABA of induced-stress mice

In neurotransmitter analysis, decreased GABA content was observed in stress induced group when compared with control group (p<0.001). On the other hand, GABA content was remarkably increased in EEHh (200 and 400 mg/kg), GFHh (100 mg/kg), and DZP (1 mg/kg) administered groups when compared with stress induced group (p<0.001) (Fig. 4.17. (A))

4.5.4.2. Effect of EEHh and GFHh on brain serotonin and dopamine of induced- stress mice

A significant decrease in serotonin and dopamine levels were observed (p<0.001) in stress induced group when compared with control group. Upon treatment, significant increase in dopamine and serotonin levels were observed for 200 mg/kg dose of EEHh, 400 mg/kg of EEHh, 100 mg/kg of GFHh and 1 mg/kg of DZP (p<0.001) when compared with stress induced group (Fig 4.17. (B) and (C)).
Fig. 4. Effect of EEHh and GFHh on brain neurotransmitters (A) GABA (B) Serotonin and (C) Dopamine in induced-stress mice

Data are expressed as mean ± SD. a p < 0.001 compared with control and stress induced group; # p < 0.001 when compared with stress induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
4.6. Discussion

According to WHO, anxiety disorders are the most prevalent psychiatric disorders worldwide. The treatment of anxiety is principally based on 1,4-benzodiazepines (BDZs, diazepam and related drugs) or 5-HT1A receptor agonists and selective 5-HT reuptake inhibitors (SSRIs) (David Healy, 2008). Though the benzodiazepine compounds remain the drug of choice for treating different type of anxiety disorders, they also possessed adverse side effects like sedation, myorelaxation, ataxia, amnesia, and pharmacological dependence-addiction (Lader and Morton, 1991). The antidepressant drugs which are used to treat anxiety disorders also leads to many undesirable side effects like insomnia, sexual dysfunction and gastrointestinal disturbances (Andreatini et al., 2001; Carlini, 2003; Mitte et al., 2005). Therefore, the novel therapeutic compounds obtained from medicinal plants may possess anxiolytic activity with less/nil adverse effects.

The present study was intended to assess the anxiolytic like effects of ethanolic extract of *H. hookerianum* (EEHh) and its glycosidic flavonoid enriched extract of *H. hookerianum* (GFHh) in stress induced Swiss Albino mice by behavioral and biochemical analysis of brain antioxidants and neurotransmitters. The anxiolytic like effect of extracts was compared with the standard drug diazepam.

EPM is the major behavioral anxiety model which is commonly employed to study the psychomotor performance and emotional implications of experimental rodents (Lister et al., 1990; Carobrez and bertoglio, 2005). Hence, EPM is suitable for testing GABA related compound, such as benzodiazepines or direct GABA<sub>A</sub> agonists. The majority of anxiolytic-like agents amplify open arms exploration, reflected by an increase in the percentage of entries into and time spent in the open arms, at doses that do not affect its spontaneous locomotor activity (Lister, 1987).
In EPM model, EEHh, GFHh and diazepam increased the % of open arm entries and time spent in the open arms among the stress induced mice which indicated the anxiolytic effects of *H. hookerianum* (Lister et al., 1990). The anxiolytic effect exhibited by these extracts is due to the presence of flavonoids and hypericin in *Hypericum* species. It is assumed that, these phytoconstituents modulate the GABAergic system to produce the necessary therapeutic effect (Salgueiro et al., 1997; Marder and Paladini, 2002). Moreover, the study confirmed the presence of flavonoidal constituents of quercetin and rutin in both the extracts by HPTLC studies. The results obtained in EPM are similar to the anxiolytic like effects exhibited by *Nymphaea alba* (Thippeswamy et al., 2011), *Ocimum sanctum* and *Camellia sinesis* on stress induced anxiety in rodents (Tabassum et al., 2011). *Tilia americana* exerted anxiolytic like effects in experimental rodents in EPM is due to its enriched flavonoid quercetin (Perez-Ortega et al., 2008).

The hole-board test offers simple evaluation for measuring the response of mice to an unfamiliar environment. Decrease in head-dipping behavior is due to the emotional state of experimental rodents and on the other hand increase in head-dipping behavior suggests the anxiolytic state of animals (Takeda et al., 1998). In the present study, it was observed that there was increase in the head dippings and reduction in the head dip latency in EEHh and GFHh treated groups. These results were contrary with the findings of Amosa et al (2005) and Aiyelero et al (2012). Thus results of this test confirmed the stimulatory effects of EEHh, GFHh and diazepam.

Open field test mainly depends on the tactile sensory factors. Stress induced mice showed thigmotaxic behavior as they lose tactile contact with the walls (Lamprea et al., 2008). Oral administration of EEHh, GFHh enhanced both the locomotor activity (i.e., transverse squares was increased) and the number of rearings in mice which suggested the antithigmotaxic behavior of mice. The results of the current experiment are similar to Vandenbogarede (2000) and Trofimiuk et al (2005).
The light and dark exploration tests are based on the natural aversion of mice to bright light areas. Anxiolytic agents reduce the natural aversion to light and increase the time spent in the light compartment (Yadav et al., 2008). According to Young and Johnson (1991), simply counting the time spent in light area is the most reliable parameter for evaluating the anxiolytic like effect. In this paradigm, EEHh, GFHh and diazepam treated groups spent more time in light compartment. The extracts of *Galphimia glauca* (Herrera et al., 2006), *Rauwolfia vomitoria* (Bisong et al., 2010) and *Cardiospermum halicacabum* (Kumar et al., 2011) exhibited similar pattern of results in experimental animals.

Social interaction test (SIT) model is a useful tool for evaluating anxiolytic effects that are prescribed for treating social phobia and emotional immaturity (Nakamural et al., 2001). In the present study, EEHh, GFHh and diazepam administered groups increased social interaction time with partner indicated alleviating social phobia-type of disorder in these substances. The extracts of *Camellia sinensis* (Mangal et al., 2010) and *Marsilea minuta* (Bhattamisra et al., 2007) also exhibited similar kind of results in SIT.

Marble-buried test in mice is used to analyze the anti-compulsive activity of drugs due to their high predictive and good face validity (Joel, 2006). In both natural and laboratory conditions, rodents spontaneously use the available bedding material to bury unpleasant sources of discomfort present in their home environment (Archer et al., 1987). This behavioral characteristic of burying activity reflects the anxiety state of animals (Wilkie et al., 1987; Treit et al., 1990; Londei et al., 1998). However, the defensive nature of marble-burying behavior is still actively debated (Prajapati et al., 2011). In the present study, stress induced animal buried more number of marbles whereas the treated mice (EEHh, GFHh and diazepam) buried less marbles thereby indicating the anxiolytic activity of *H. hookerianum*. Also the animals treated with the extracts of *Cymbopogon citrates* (Costa et al., 2011) and *Lagenaria sicerarias* (Prajapathi et al., 2011) buried less number of marbles which is consistent with the current results.
Novelty induced feeding latency test is based on the anxiety evoked by unfamiliar environment that suppressed the normal feeding behavior of mice when the food was deprived for 48 hours (Bhattamisra et al., 2007). Feeding latency was significantly increased in stress induced animal when compared with control group, but in the present study, decreased feeding latency in EEHh, GFHh and diazepam treated animals were observed. Similar results were observed in Marsilea minuta (Bhattamisra et al., 2007) and Withania somnifera (Bhattacharya and Muruganandam, 2003) extracts.

Anxiety is mainly caused by stress (Jacobson and Sapolsky, 1991) that is associated with oxidative damage (Liu et al., 2003; Sherki et al., 2001,). Acute restraint stress (RS) stimulates main cellular cascade that leads to increased ROS production (Goyal and Anil, 2007).

The main source for generation of free radicals is due to the increased influx of pyruvate and oxygen in mitochondria and the excess ROS production during oxidative process in Electron Transport Chain (ETC). The antioxidants (enzymic and non-enzymic) play key role in defense mechanism against oxidative damage. In enzymic antioxidants, superoxide dismutase (SOD), catalase (CAT), glutathione dependent enzymes of glutathione peroxidase (GPx) and glutathione -S - transferase (GST) are mainly involved. There are also non- enzymic antioxidants which are classified into water soluble (eg: vitamin C- ascorbic acid) and lipid soluble (vitamin E- tocopherol) (Dallaqua and Damasceno, 2011). The reduction of lipid peroxidation via the antioxidant defense mechanism is also concomitant to oxidative stress suppression. The present study showed that the administration of EEHh, GFHh were able to significantly increase the enzymic antioxidants (SOD, CAT, GPx and GST) and non- enzymic antioxidants (GSH, vitamin C and E) and also reduced the LPO; demonstrating the neuroprotective defense mechanism of brain against oxidative stress.

Both physical and emotional stress leads to oxidative stress thereby decreasing the activities of SOD, CAT, GPx and GST. Therefore, oxidative stress
caused imbalance in antioxidant defense mechanism (Lekha et al., 2010). Decrease in antioxidant activities are due to their higher usage in fighting against oxidative stress (Venkateswaran and Pari, 2013) and inhibition of SOD converts cuprous ion to cupric ion that are involved in product ion OH⁻, which are the consequences of Haber-Weiss reaction (Debnath et al., 2000). Lower antioxidant enzymatic conditions further leads to increased peroxidation of lipids in cell membrane which results in cellular damage (Marklund et al., 1974).

Glutathione peroxidase is an enzyme that is involved in the oxidation of reduced glutathione (GSH) to detoxify the peroxides. Imbalances in the glutathione oxygen scavenging system in brain have been reported in different conditions associated with oxidative challenge and/or cellular damage leading to alteration in the GPx levels (Kish et al., 1986). Glutathione reductase and glutathione peroxidase, are important constituents of GSH - redox cycle which provide major protection to oxidative injury by participating in the cellular system of defense against oxidative damage. Also, they play a crucial role in limiting the propagation of free radical reactions, which would otherwise result in extensive lipid peroxidation (Toklu et al., 2009). In this experiment, we observed reduced activity of GPx and GST in stress induced mice but their activity was restored with the treatment of EEHh, GFHh and diazepam.

The decreased content of GSH in mice brain may also result in enhanced LPO and this plays a major role in defensive mechanism against oxidative stress (Paya et al., 1992). Moreover, reduced GSH might be due to the decreased activities of SOD, GST and CAT. The GST enzymatic machinery also possess peroxidase activity which can directly attack the peroxides generated via oxidative reduction recycling (Prohaska, 1980). The reduced GST activity observed in this study might have further contributed to enhanced lipid peroxidation in stress induced mice. Both the GST and LPO activities were restored in mice treated with EEHh, GFHh and diazepam.
The enzymatic NADPH-dependant lipid peroxidation (LPO) is catalyzed by the NADPH-cytochrome P450 reductase and propagated by cytochrome P450 with the generation of free radicals, i.e, O$_2^{-}$ and ROO$^{-}$. The EEHh and GFHh might have inhibited the activity of NADPH-dependant LPO due to its free radical scavenging ability. Elevations in the levels of TBARS and LPO in stress induced group again supported the low antioxidant enzyme activities (SOD, CAT, GPx GST). Another possibility for such an elevation in TBARS may be due to high rate of catecholamine secretion that generates free radicals either through auto-oxidation or through metal ion or oxidation of superoxide-catalyzed reaction (Jewett et al., 1989).

Vitamin C is a permissive antioxidant molecule in the brain. However, it also has a number of other important functions, playing as a co-factor in several enzyme reactions including catecholamine synthesis, collagen production and regulation of HIF-1$\alpha$ in brain. Normally, higher concentration of vitamin C is found in the brain and neuroendocrine tissues such as adrenal. Ascorbate is proposed as a neuromodulator of glutamatergic, dopaminergic, cholinergic and GABAergic transmission and related behaviors (Fiona and James, 2009). During the anxiogenesis condition, imbalance of other antioxidants and catecholamine synthesis affected the vitamin C, so the stress induced animals showed reduced levels of vitamin C. In the present study, treatment with EEHh, GFHh and diazepam restored the vitamin C levels in stress induced mice.

Anxiety and cognitive defects in both human and animal models is mainly associated with oxidative stress (OS). Desrumaux et al (2005) reported that vitamin E deficiency in brain leads to ROS generation, which results in anxiety like behavior in mice. A study also suggested that a palatable diet leads to oxidative stress and anxiety like behavior (Souza et al., 2007). Liu et al (2003) have shown that oxidative stress is associated with age related cognitive effects, which are reversed by treatment with ROS scavengers. In the present study, stress induced group showed reduced values of vitamin E in the brain due to OS caused by
physiological stress. But treatment with EEHh, GFHh and diazepam treated groups restored the levels of vitamin E.

The present study clearly revealed that in vitro antioxidant effect of EEHh and GFHh against free radicals. Both the in vitro and in vivo studies demonstrated the antioxidant properties of EEHh and GFHh. Among the extracts tested, GFHh which contains higher concentration of flavonoids showed higher quenching and antioxidant activity than EEHh. There is a general conclusion that flavonoid via antioxidant activity is representative for anxiolytic activity of many plant extracts and this statement is well supported by previous reports. The plant extracts of Cytisus scoparius (Jayabalan et al., 2008), Ocimum sanctum (Samson et al., 2007,) H.perforatum (Benedi et al., 2004; Jayaprakash et al., 2010), demonstrated the anxiolytic activity by their antioxidant effects which is consistent with the current results.

Preclinical investigations revealed that low levels of GABA or serotonin contribute to the progression of anxiety disorder, where it is closely associated with low levels of serotonin, nor-adrenaline and dopamine in the synaptic cleft (Lydiard, 2003; Nutt, 2008). In this experiment, the brain neurotransmitters levels (GABA, serotonin and dopamine) were decreased in stress induced group with anxiogenic response. But in the treated groups, EEHh, GFHh and diazepam increased the values of GABA, serotonin, dopamine as well as the anxiolytic activity. Many anxiolytic agents target the GABA, by facilitating the opening of GABA-activated Cl channels. GABA$_A$ receptors is mainly involved in anxiety and their direct and indirect activation of other neurotransmitters reworked in anxiolytic like effect (Vogel et al., 2002). The mechanism of action of C. sativum in exhibiting anxiolytic activity may be similar to benzodiazepine-diazepam which acts via the GABA$_A$ receptor, as flavonoids and diazepam having structural similarity. Effect of flavonoids as anxiolytics have been observed in many plant species used in folk medicine, such as Tilia tomentosa (Viola et al., 1994) and Passiflora coerulea (Mahendra et al., 2011).
It is a well-known fact that low level of GABA in the brain is mainly associated with anxiety disorders (Griebel, 1997; Griebel, 1998). Serotonin (5-Hydroxy Tryptamine) also played an important role in the progress of anxiety disorders. Previous studies have reported that patients with anxiety ailments have genetic polymorphisms in the 5-HT transporter (Salim, 2011). Apart from GABA and 5-HT, dopamine and nor-adrenaline also have a major role in the progress of anxiety disorders. Several animal based studies, suggested that reduced dopamine activity is associated with augmented anxiety. Pre-clinical studies showed that there is an increased synthesis release and turnover of nor-adrenaline in anxiety and stress, thereby suggesting its role in anxiety. Anxiety disorders are also due to free radical-induced damage affecting the neurotransmitter system (Hovatta et al., 2010; Salim, 2011). In this study, GABA, dopamine and serotonin values found to be decreased in stress induced group. But treated groups increased or restored the levels of GABA, dopamine and serotonin. Nonetheless, it was proposed that the bioactive compounds particularly flavonoids present in EEHh and GFHh extracts could also interact with neurotransmitter system of serotonergic, GABAergic or dopaminergic together contributing to the anxiolytic like effects.

Thus in conclusion, EEHh and GFHh attenuated the behavioral and biochemical changes in mice that were induced by stress. In addition, GFHh possessed remarkable anxiolytic activity than EEHh in all the behavioral and biochemical changes while HPTLC assay revealed a higher concentration of flavonoids in GFHh. Thus, GFHh was proved to be a potential candidate in treating stress induced anxiety.
Graphical summary - Anxiolytic like activity of EEHh and GFHh induced in stress induced Swiss Albino mice

Swiss Albino mice

Stress induction

Treatment with EEHh and GFHh

Oxidative stress

Neuro protection by EEHh and GFHh

EPM
- Increased time spent in open arm
- Reduced number of rearings

HBT
- Increased head dip latency
- Decreased head poking

OFT
- Decreased in square traversed
- Decreased in rearings

MBT
- Marble buries increased

LDET
- Increased time in dark arm

NIFL
- Increased feeding latency

Decreased antioxidants
- SOD, CAT, GPx, GST
- Vitamin C, E and GSH
- Increased LPO
- Decreased brain GABA, serotonin, dopamine

Increased antioxidants
- SOD, CAT, GPx, GST
- Vitamin C, E and GSH
- Decreased LPO
- Increased brain GABA, serotonin, dopamine

- Decreased time spent in open arm
- Increased number of rearings

- Decreased head dip latency
- Increased head poking

- Increased in square traversed
- Increased in rearings

- Marble buries decreased

- Increased time in open arm
- Decreased feeding latency