CHAPTER 7

ANTIPARKINSON LIKE EFFECTS OF ETHANOLIC EXTRACT OF 
Hypericum hookerianum (EEHh) AND ITS GLYCOSIDIC FLAVONOID 
ENRICHED EXTRACT (GFHh) IN HALOPERIDOL INDUCED 
PARKINSONIAN SYMPTOMS IN SWISS ALBINO MICE

7.1. Objective

To analyse the antiparkinson like effects of ethanolic extract of Hypericum hookerianum (EEHh) and its glycosidic flavonoid enriched extract (GFHh) in haloperidol induced parkinsonian symptoms in Swiss Albino mice by behavioral and biochemical methods.

7.2. Introduction

Parkinson’s disease (PD) is one of the progressive neurodegenerative disorders with an unknown etiology. The neuropathology of PD mainly includes degeneration of dopaminergic neurons in the Substantia Nigra (SN), a region of the brain which controls motor co-ordination of the body (Meissner et al., 2011). Jankovic (2008) reported that the initial symptoms of PD include muscular rigidity, tremor at rest, bradykinesia and postural deformity and instability.

PD is associated with variety of cognitive symptoms which include thinking problems, isolated memory to severe dementia. Experimental evidences have shown that over 50% of people with PD experience some form of cognitive impairment and about 20% have more substantial nigra cognitive impairment. However, memory problems in PD are typically milder than Alzheimer’s disease (AD). In PD, the person may have symptoms like difficulty in concentration, learning new information and recalling names (Hritcu et al., 2008). Although the etiology of PD remains unknown, recent studies have confirmed that Oxidative Stress (OS) also leads to generation of more free radicals which plays an important role in its pathology (Valko et al., 2007; Hritcu et al., 2008; Shim et al., 2009; Samoylenko
et al., 2010). OS contributes to the damage of biomolecules like lipids, proteins and nucleic acids and the cascade of events leads to the dopamine cell degeneration in PD (Hritcu et al., 2011).

PD is mainly caused by state of oxidative imbalance of combined factors among which are aging of brain, predisposition of genes, dysfunction of mitochondria, generation of free radicals and environmental toxins (Fukae et al., 2007; Henchcliffe and Beal, 2008; Zhou et al., 2008; Moreira et al., 2010). Previous reports have suggested that some neurodegeneration in PD is associated with dietary habits, but deficiency in antioxidants such as folic acid (Zhu, 2004), vitamins (A, C, E and niacin) and minerals like selenium in body has shown to increase the risk of PD (Paraskevas et al., 2003; Chaturvedi et al., 2006). Imbalance in these components lead to increased production of Reactive Oxygen Species (ROS) and it has been speculated that OS possibly plays a role not only in onset of PD but also in progression of the disease (Zhu, 2004; Hritcu et al., 2008).

The imbalance in the functions of acetylcholine and dopaminergic neurons in the striatum leads to PD. PD patients show symptoms like tremor, myotonia, dyskinesia and motor dysfunction (Liu et al., 2012). Recent developments that have led to evolution in the medical management of PD are to (i) improve the dopaminergic therapies (ii) identification of non-dopaminergic drugs for symptomatic improvement and (iii) discovery of novel compounds to positively alter the course of PD. The pre-clinical study models of PD (biochemical, cellular and animal) have contributed much to the understanding of PD in humans (Schapira et al., 2006).

There have been noteworthy advances in PD therapy in preclinical investigations, together with surgical and pharmacological interventions. Even though L-DOPA is considered as the preeminent standard drug for treatment of PD, several complications such as motor fluctuations, hallucinations, and psychosis arise from long-term therapy (Simpkins and Jankovic, 2003). This led to the usage of alternative substances like dopamine receptor agonists, anti-cholinergic drugs,
MAO inhibitors, catechol-O-methyl transferase inhibitors and also, the investigation of novel therapeutic molecules that prevent neurodegeneration. It has been clearly established with evidence that oxidative stress is among the main causative factors in the induction of many chronic and neurodegenerative diseases like PD (Smith and Cass, 2007).

Haloperidol (HP) is one of the traditional antipsychotic drugs that is used worldwide with different brand names (See and Ellison, 1990; Li et al., 2011) and prolonged period of its usage is associated with parkinsonian like syndromes (Sagara, 1999). This is characterized by repetitive involuntary movements, involving face, limb and trunk muscles. The syndrome is common in older patients prevailing in those using typical antipsychotic agents (Amanpreet Singh et al., 2003; Tan et al., 2005). Even this syndrome is often irreversible in case of drug withdrawal (Andreasen and Jorgensen, 2000). The pathophysiology of haloperidol induced neurotoxicity remains enigmatic and it may be due to the reduction in striatal dopamine or increased OS and imbalance between free radical metabolism and the antioxidant defense system (Elkashef and Wyatt, 1999). Bishnoi et al (2007) also reported about the behavioural and neurobiochemical alterations of haloperidol induced orofacial dyskinesia in animal models.

Therapeutic strategies that slow or stop the neurodegenerative processes of PD are expected to have a major impact on the treatment of PD (Meissner et al., 2004). The current hypothesis about the mechanisms by which neurons come into necrotic or apoptotic processes has led to belief that the therapeutic use of antioxidants may be beneficial in aging and neurodegenerative disorders (Di Matteo and Esposito, 2003; Zhou et al., 2008). Following this line of evidence, number of studies with natural compounds or polyphenols like flavonoids (Ramassamy, 2006; Mandel et al., 2008) has increased considerably during last decade.
7.3. Materials and Methods

7.3.1. (a). Preparation of plant extract

The procedure was previously described in chapter: 3.3.6.

7.3.1. (b). Separation of Glycosidic Flavonoid enriched extract of *H. hookerianum* (GFHh) by acid Hydrolysis method

The procedure was previously described in chapter: 3.3.8.

7.3.2. Experimental animals

The procedure was previously described in chapter: 4.3.2.

7.3.3. Experimental groups

For this purpose, Swiss Albino mice were divided into six groups (n=6), first group served as Control, second group is Haloperidol Induced (HI), while the third and fourth groups are haloperidol induced and treated with EEHh (200 and 400 mg/kg) respectively. Fifth group haloperidol induced and treated with GFHh (100 mg/kg). Finally the sixth group was treated with reference drug L-Dopa (30 mg/kg) which was administered intraperitoneally (i.p) to the haloperidol induced groups.

7.3.3.1. Vacuum Chewing Movement, Orofacial Burst (OB) and Tongue protrusions (Cousins et al., 1997)

After the injection of haloperidol, mice were placed in a Plexiglass beaker for a 10 min habituation period (Fig.7.1). All mice were observed for 5 min period. A blind observer recorded the number of vacuum chewing movements (VCM), tongue protrusions (TP) and orofacial burst (OB).
7.3.3.2. Catalepsy measurement by block method (Chopde et al., 1997)

Block method was carried out in 3 phases (Fig.7.2).

**Phase I:** The mice was taken from the dwelling cage and placed in a slanting wooden board. If the mouse does not show any movement when touched or pushed smoothly on the backside and the score assigned was 0.5.

**Phase II:** In next 15 sec the mice would botch to correct the position, the front paws of the mice were placed alternatively on a 3 cm far above the ground block. For each paw score is individually calculated as 0.5.

**Phase III:** The front paws of the mice were sited alternately on a 9 cm high block. If the mice is failing to correct the position within 15 sec a achievement of 1 for each foot was further added to the scores recorded in step I and II. Finally, the score was calculated and the highest score for the animal was 3.5 (i.e. cut off scores for catalepsy) and that clearly indicates the total catalepsy (immobile posture).

7.3.3. 3. Catalepsy by metal bar test method (Kulkarni et al., 2006)

Cataleptic behavior was measured by the high metal bar test method above the table top. Catalepsy score was measured for 4 hours at one hour intervals after haloperidol administration by gently placing both forepaws of the mice over a metal bar (DIA meter of the metal bar = 2-5 mm suspended 6 table tops). Catalepsy intensity was assigned by counting the time in seconds until the mice brought both forepaws down to the table top, with a maximum cut off time of 3 min. Finally, scores at interval time period 0, 60, 120, 180 and 240 min after haloperidol induction) were added and compared the cumulative catalepsy score (Fig.7.3).
7.3.3. 4. Ptosis (Bourin et al., 1983)

In brief, mice were injected intraperitoneally with haloperidol at 1 h later and treated with respective groups except control. The degree of ptosis was determined after 1 h of haloperidol treatment. Mice were placed on a shelf (20 cm above the table top) and the degree of ptosis was recorded by using determining scale: eye open = 0; one-quarter closed = 1; half closed = 2; three-quarters closed = 3; completely closed = 4 (Fig.7.4).

7.3.3. 5. Staircase test (Vogal et al., 2005)

The staircase is composed of five identical steps with uniform dimension of 2.5 cm height and 10 cm deep. Total number of steps climbed and total number of rearings of mice were recorded over a period of 5 min. A step climbed by mice is measured only if the animal has placed all four paws on the step of the staircase (Fig.7.5).

7.3.3. 6. Beam walk test (Stanley et al., 2005)

The mice were trained to walk from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above the bench by metal bar support to an objective box. For each mouse, to ensure that the mice learnt appropriately, three trials were conducted. After 30 min of haloperidol induction, each mouse was placed on the beam made of wood (8 mm in diameter and 60 cm long elevated 30 cm above the bench by metal supports) and allowed to walk to
the objective box and the time was recorded. The number of foot slips was recorded with the aid of a tally counter (Fig. 7.6)

7.3.3. 7. Gait analysis – Forepaws stride length in walking (Tillerson et al., 2002)

To measure the gait, animals were trained to walk through a narrow alley leading into their home cage. Once trained, paper was placed along the alley floor and each animal forepaws were brushed with non toxic blue colored paint. Animals were kept at the beginning of the alley. As they walked through the alley into their home cage, they leave their paw prints on the paper. Thus method measured the distance between paw prints stride length (Fig.7.7).

7.3.3. 8. Wire Hang test (Caston et al., 1999)

Muscular traction of experimental mice was performed by placing the forepaws of the animals in a small twisted wire rigidly supported above a bench top (Fig.7.8). Normally, an experimental mouse grasps the wire with the forepaws, and place at least one hind foot on the wire within 5 sec when allowed to hang freely. Inability to put up at least one hind foot is considered as a failure in the traction test (Caston et al., 1999).
7.3.4. Antioxidant assays

7.3.4.1 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 (1951).

7.3.4.2 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).

7.3.4.3 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.

7.3.5 Estimation of brain neurotransmitters

Brain glutamate (Bernt et al., 1965), dopamine (Schlumf et al., 1974) was estimated using standard methods.

7.4 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 haloperidol induced group and also between haloperidol induced and treated groups. Statistical difference were considered significant when the ‘p’ value was <0.005.
7.5. Results

7.5.1. Effect of EEHh and GFHh on Behavioral analysis

7.5.1.1. Effect of EEHh and GFHh on VCM, OB, TP in haloperidol-treated mice

The effect of EEHh, GFHh and L-Dopa on VCM, OB and TP of haloperidol induced animal are depicted in Fig.7.9. (A) and (B)

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group; # p<0.001 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
The frequency of VCM, OB and TP were significantly increased after the haloperidol (1 mg/kg, ip) induction when compared to the control group (p<0.001). EEHh (200 mg/kg) treated group showed decreased frequency in VCM and OB when compared to haloperidol induced group (p<0.001) but there was no change in TP (non significant). Additionally, EEHh (400 mg/kg), GFHh (100 mg/kg) and L-Dopa (30 mg/kg) treated groups significantly reduced the frequencies (p<0.001) with respect to involuntary movements in vacuum chewing movement, tongue protrusions and orofacial burst when compared with haloperidol induced group (p<0.001).

7.5.1.2. Effect of EEHh and GFHh on major parkinsonian symptoms in haloperidol- treated mice

The major parkinsonian symptoms (postural deformity, resting tremor, muscular rigidity, bradykinesia, akensia) were observed after haloperidol induction on 6\textsuperscript{th} day. When these animals were treated with EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and L-dopa (30 mg/kg), significant reduction in the parkinsonian symptoms was observed (Fig.7.10).
Fig 7.10. Effect of EEHh and GFHh on major parkinsonian symptoms in haloperidol- treated mice

Data are expressed as mean ± SD. * p < 0.001 compared with control and haloperidol induced group; # p<0.001 when compared with haloperidol induced and treatment groups using one- way ANOVA followed by Bonferroni’s test as a post- ANOVA test.
7.5.1.3. Effect of EEHh and GFHh on catalepsy model by metal bar method in haloperidol-treated mice

Fig.7.11. depicts the catalepsy scores of haloperidol induced and treated groups by metal bar method.

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group; # p<0.001, $ p<0.01 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment group using one- way ANOVA followed by Bonferroni’s test as a post-ANOVA test.

The cataleptic score was increased after 60 min of haloperidol induction and even it was higher at 120 min which are statistically significant when compared with control group (p<0.001). But in treated groups (EEHh 400 mg/kg, GFHh (100 mg/kg) and L-dopa), significant reduction in catalepsy scores were observed when compared with haloperidol induced group (p<0.001). Moreover, maximum
A reduction in cataleptic score was seen in GFHh (100 mg/kg) when compared with EEHh treated group (Fig. 7.11).

### 7.5.1.4. Effect of EEHh and GFHh on catalepsy model by block method in haloperidol treated mice

In this study, the animals in control group were in motion, therefore the scores are not applicable for them. When the cataleptic scores were recorded at different time intervals (30-180 min) for haloperidol induced and treated groups, it was found that haloperidol induced group showed maximum cataleptic scores at 120 min, but there was a gradual decrease in cataleptic activity at 150 and 180 min respectively. But on the other hand, the treated groups (EEHh (200 and 400 mg/kg); GFHh (100 mg/kg) and L-dopa (30 mg/kg)) significantly reduced the catalepsy scores when compared with the haloperidol induced group (p<0.001). As expected, maximum reduction in cataleptic activity was seen in GFHh (100 mg/kg) and L-dopa (30 mg/kg) treated groups. Among the extracts, GFHh (100 mg/kg) showed better activity than EEHh.

![Fig 7.12. Effect of EEHh and GFHh on catalepsy model (block) of haloperidol induced mice at different time intervals](image)

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group and # p<0.001 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment group using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test.
7.5.1.5. Effect of EEHh and GFHh on ptosis test in haloperidol- treated mice

The haloperidol induced significant ptosis in mice (Fig.7.13). In addition, antagonized ptosis activity was observed for 200 mg/kg dose of EEHh (p<0.005) when compared with haloperidol induced group, while the 400 mg/kg of EEHh, 100 mg/kg of GFHh and 30 mg/kg of L-dopa caused a significantly antagonized effect when compared with the haloperidol induced group (p<0.001)

![Graph showing the effect of EEHh and GFHh on haloperidol-induced ptosis in mice](image)

Data are expressed as mean ± SD. @ p<0.05, # p<0.001 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test
7.5.1.6. Effect of EEHh and GFHh on staircase test in haloperidol-treated mice

In haloperidol induced group, less number of steps were climbed by animal and also there were decrease in the number of rearings when compared with control group (p<0.001). At 200 mg/kg of EEHh, we observed significant increase in number of steps climbed but there was no significant change in rearings. While 400 mg/kg of EEHh, 100 mg/kg of GFHh and 30 mg/kg of L-dopa caused a significant increase in both number of steps climbed (p<0.001) and rearings (p<0.001) when compared with haloperidol induced group (Fig.7.14)

![Fig 7.14. Effect of EEHh and GFHh on staircase test in haloperidol-treated mice](image)

Data are expressed as mean ± SD. a p<0.001 when compared with control and haloperidol induced group; @ p<0.05, # p<0.001 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment groups using one- way ANOVA followed by Bonferroni’s test as a post- ANOVA test
7.5.1.7. Effect of EEHh and GFHh on Gait Analysis (GA) in haloperidol-treated mice

Further to prove whether EEHh and GFHh treatment reduced the motor dysfunction in animal, gait analysis was performed by measuring the forepaw stride length during walking (Fig.7.15). A significant decrease in forepaw stride length was observed in haloperidol induced group when compared with control group (p<0.001). But the treated groups (EEHh (200 and 400 mg/kg), GFHh (100mg/kg) and L- Dopa (30 mg/kg)) significantly increased the forepaw stride length when compared with haloperidol induced group (p<0.001). Upon comparing all other extracts, GFHh (100 mg/kg) exhibited greater effect than EEHh.

![Fig 7.15. Effect of EEHh and GFHh on forepaw stride length in haloperidol-treated mice](image)

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group ; # p<0.001 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment groups group using one- way ANOVA followed by Bonferroni’s test as a post- ANOVA test.
7.5.1. 8. Effect of EEHh and GFHh on Beam Walk Test in haloperidol –treated mice

This study dealt with the time taken for mice to walk on the beam and the absolute number of falls by the animal (Fig.7.16). Haloperidol induced group took more time to complete the beam walk and also the number of errors made were more when compared with control group (p<0.001). But treated groups (EEHh (400 mg/kg), GFHh (100 mg/kg) and L-dopa (30 mg/kg) took less time to complete the beam walk and the number of errors were also less when compared with haloperidol induced group (p<0.001). Among the extracts high promising results were observed in GFHh (100 mg/kg) and the values were similar to standard drug L-dopa (30 mg/kg)

Fig 7.16. Effect of EEHh and GFHh on Beam walk test in haloperidol- treated mice

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group ; # p<0.001 when compared with haloperidol induced and treatment groups; ns- non significant when compared with haloperidol induced and treatment group using one- way ANOVA followed by Bonferroni’s test as a post- ANOVA test
7.5.1.9. Effect of EEHh and GFHh on Wire Hang Test (WHT) in haloperidol-treated mice

In WHT, hanging strength was calculated within 5 sec for all groups. The control group showed full grip strength, whereas haloperidol induced animal exhibited very less grip strength (p<0.001). On the other hand, EEHh (200 and 400 mg/kg), GFHh (100 mg/kg) and L- dopa treated groups showed significant grip strength when compared with haloperidol induced group (p<0.001) (Fig.7.17).

![Graph showing effect of EEHh and GFHh on Wire hang test in haloperidol-treated mice](image)

**Fig 7.17.** Effect of EEHh and GFHh on Wire hang test in haloperidol-treated mice

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group ; # p<0.001 when compared with haloperidol induced and treatment groups using one-way ANOVA followed by Bonferroni’s test as a post-ANOVA test
7.5.2. Evaluation of *in vivo* antioxidant potential of EEHh and GFHh

7.5.2.1. Effect of EEHh and GFHh on enzymic antioxidants in brain of haloperidol- treated mice

The effect of EEHh and GFHh on enzymic antioxidant levels in haloperidol induced mice are presented in Fig. 7.18. (A, B, C and D).

![Graphs showing enzymic antioxidant levels](image)

**Fig 7.18. Effect of EEHh and GFHh on enzymic antioxidants- (A) Superoxide dismutase (B) Catalase (C) Glutathione peroxidase and (D) Glutathione-S- transferase in brain of haloperidol- treated mice**

Data are expressed as mean ± SD. a p < 0.001 compared with control and haloperidol induced group and # p<0.001, @ p<0.05, ns- non significant when compared with haloperidol induced and treatment groups using one- way ANOVA followed by Bonferroni’s test as a post- ANOVA test
SOD, CAT, GPx and GST play key role in antioxidant defense system and their analysis during oxidative stress evaluation is important. In the present study, haloperidol induced group exhibited decreased SOD, CAT, GPx, GST activities (p<0.001) when compared with control group.

When the animals were treated with 200 mg/kg of EEHh, there was no significant increase in SOD, CAT, GST except GPx (p<0.005). The other treated groups EEHh (400 mg/kg), GFHh (100 mg/kg) and L-dopa (30 mg/kg) showed significant increase in the enzyme activities (p<0.001) when compared with haloperidol induced group. Interestingly, GFHh (100 mg/kg) treated group showed improved SOD, CAT, GPx and GST activities than EEHh (400 mg/kg) treated group. GFHh treatment remarkably improved the antioxidant defense in haloperidol induced mice which is similar to L-Dopa.

7.5.2.2. Effect of EEHh and GFHh non-enzymic antioxidants in brain of haloperidol- treated mice

LPO level was found to be increased in haloperidol induced group as compared to control group. In the first treated group (EEHh 200 mg/kg), no significant effect in LPO level was observed when compared with haloperidol induced group. But on the other hand, EEHh (400 mg/kg), GFHh (100 mg/kg) and L-dopa (30 mg/kg) significantly decreased the LPO levels in comparison to haloperidol induced animal. GFHh (100 mg/kg) exhibited better protection against peroxidative damage than EEHh (Fig.7.19.A)
Fig 7.19. Effect of EEHh and GFHh on enzymic antioxidants (A) Lipid peroxidation  (B) Reduced Glutathione  (C) Vitamin C and (D) Vitamin E in brain of haloperidol- treated mice

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

The administration of haloperidol significantly reduced the total GSH content when compared with control group (p<0.001). Also there was no significant
change in GSH content at 200 mg/kg of EEHh. Whereas, the treated groups (EEHh (400 mg/kg), GFHh (100 mg/kg) and L-dopa (30 mg/kg) significantly increased the GSH content when compared with haloperidol induced group (p<0.001).

Furthermore to study the antioxidant protective mechanism of EEHh and GFHh, the study analyzed the vitamin C and E in haloperidol induced and treated groups. As expected both vitamin C and E levels were found to be reduced in haloperidol induced group when compared to control group (p<0.001). But the treated groups (EEHh (400 mg/kg), GFHh (100mg/kg) and L-Dopa (30mg/kg)) increased the vitamin C and E levels when compared with haloperidol induced group (p<0.001). Surprisingly, GFHh (100 mg/kg) remarkably improved the antioxidant defense than EEHh (400 mg/kg).

7.5.3. Effect of EEHh and GFHh on brain neurotransmitters in haloperidol-treated mice

Fig.7.20. depicts the levels of dopamine and L-glutamate in both haloperidol induced and treated groups. Administration of haloperidol significantly reduced the level of dopamine in brain tissue when compared with control group (p<0.001). Treatment with EEHh (400 mg/kg), GFHh (100mg/kg) and L–Dopa (30 mg/kg) significantly increased the dopamine levels (p<0.001) but there was no significant increase in dopamine at 200 mg/kg of EEHh group when compared with control group. Among the treated groups, GFHh (100mg/kg) restored the high level of dopamine than EEHh.
Fig 7.20. Effect of EEHh and GFHh on Brain Neurotransmitters (A) Dopamine (B) Glutamate in haloperidol-treated mice

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

In haloperidol induced group, significant increase in L-glutamate was observed when compared with control group (p<0.001). On the other hand, treated groups (EEHh (400 mg/kg), GFHh (100mg/kg) and L-Dopa) significantly reduced
the L-glutamate levels (p<0.001) and there was no significant change (ns) in L-glutamate at 200 mg/kg of EEHh when compared with haloperidol induced group.

7.6. Discussion

In the present scenario, research works on neuroprotective and neurorescue approaches using medicinal plants would be a promising one as it not only reduce the progression of neurodegeneration as well as convalesce the condition like PD. Previous study reports have confirmed that oxidative stress leads to inflammation which could be the main factors behind the degeneration of dopaminergic neurons in PD (Taylor et al., 2013). In the current study, the antiparkinson like effect using ethanolic extract of *H. hookerianum* (EEHh) and its glycosidic flavonoid enriched extract (GFHh) on haloperidol induced mice by behavioral and biochemical methods was examined. The effects of extracts were compared with standard drug L-Dopa (30 mg/kg).

L-Dopa, a D2- like receptor agonist is used as the standard drug for treatment of PD but it exhibits undesirable side effects like hallucinations, motor fluctuations and psychosis (Simpkins and Jankovic, 2003). To overcome this situation, natural phytoconstituents like flavonoids or flavonoid enriched extracts were used to treat PD due to their antioxidant potential and neuroprotective effects (Patil et al., 2014).

Haloperidol is recognized as a well known neuroleptic drug in the schizophrenia treatment, classically it acts as a D2 receptor antagonist in the pathway of mesolimbic- mesocortical sytem. It is non- selective in action and produces blockade of D2- receptors (post synaptic) in the pathway of nigrostraital in brain which causes extrapyramidal side effects such as dystonia, muscular rigidity, akasthisia, parkinsonism in long term treatment (Farde et al., 1992; Leucht et al., 2013).

The chronic use of haloperidol can cause extrapyramidal side effects like Tardive dyskinesia (TD) in humans and Orofacial dyskinesia (OD) in animals which includes the repetitive involuntary movements like Vaccum Chewing
Movement, Tongue Protrusions and Orofacial Burst (Peroza et al., 2013). In the current study, haloperidol induced group showed increased frequencies of VCM, TP and OB due to the neurotoxic effect of haloperidol which was associated with enhanced free radicals in brain specifically SN region (Rogoza et al., 2004; Sookram et al., 2011). While the EEHh, GFHh and L-Dopa administered groups exhibited reduced frequencies of VCM, TP and OB. This findings suggested that the antioxidant action and free radicals scavenging effect of flavonoidal constituents in these extracts might contribute to their neuroprotective effect. The results of the current study were similar to the extracts of *Withania somnifera* (Bhattacharya et al., 2002; Naidu et al., 2003), *Murraya koenigii* (Patil et al., 2012) and *Bauhinia forficata* (Peroza et al., 2013).

The loss of dopaminergic neurons due to enhanced production of free radicals caused by haloperidol leads to behavioral changes which are linked with an onset of motor dysfunction such as postural deformity, resting tremor, muscular rigidity, bradykinesia and akinesia (Sivaraman et al., 2012). Current findings indicated that haloperidol induced group exhibited high degree of these symptoms on 6\textsuperscript{th} day. But the treatment groups (EEHh, GFHh and L-Dopa) significantly ameliorated the high degree of postural deformity, resting tremor, muscular rigidity, bradykinesia and akinesia. The current findings are in concordance with the previous studies where they used *Plumbago scandens* (Morais et al., 2004) and *Annona squamosa* Linn (Sivaraman et al., 2012) extracts.

Catalepsy is the immobile posture failure to correct the external position and it is mainly caused by neuroleptics (Hubbard and Trugman, 1993). The administration of haloperidol in mammals generated free radicals and increased catalepsy scores (Sanberg et al., 1980) which has been confirmed by Rasheed et al (2010). Even in the current study, haloperidol induced group showed increased cataleptic scores in block and metal bar method as well as attenuated the ptosis. But the EEHh, GFHh and L-Dopa administered groups, significantly potentiated the catalepsy scores. The present findings corroborates with the previous reports of
Morus alba (Yadav et al., 2008) and Ageratum conyzoides L (Anitha et al., 2012) extracts.

Nigrostriatal damage is probably measured by gait analysis and muscular coordination using forepaw stride length and beam walk test respectively (Colin and Hernandez, 1991). Imbalance walk i.e., poor gait is also involved in clinical findings because it is the main symptom of PD (Pradeep et al., 2012). In the present study, poor gait that is decreased forepaw stride length was observed in haloperidol induced group. But mice treated with EEHh, GFHh and L-Dopa improved the gait performance when compared with haloperidol induced group. The results of present study are in agreement with the previous reports of H.perforatum (Mohanasundari et al., 2006) and bi-herbals of Plumbago zeylanica and Camellia sinesis (Ittiyavirah and Ruby, 2015) extracts.

The beam walk assay is a sensitive model to study the motor coordination ability (Stanley et al., 2005). Motor coordination deficit effect was confirmed in haloperidol induced group by the increase in errors occurred during walking (Meredith and Kang, 2006). But EEHh, GFHh and L-Dopa showed better improvement in motor coordination especially in balancing behavior. Similar pattern of results were also observed in Passiflora incarnata Linn (Narayanan et al., 2011) and Annona squamosa (Sivaraman et al., 2012) extracts.

Wire hang test is mainly used to find out the neuromuscular strength and advantage of this model is that it is inexpensive, self constructed and the evaluation is very useful to find out the grip strength of mice (Gomez, 1997). In the current study, the haloperidol induced group showed very less grip strength but in case of EEHh (400 mg/kg), GFHh (100 mg/kg) and L-Dopa (30 mg/kg) treated groups increased grip strength was observed by their motor coordination. These results were contrary to the previous findings of Wedelia chinensis (Suresh et al., 2010) and Mallotus peltatus (Chattopadhyay et al., 2003) extracts.

Depression and anxiety are common symptoms in PD (Pontone et al., 2009). With this background, stair case test was employed to study the anxiolytic effects of
plant extracts. Rearing is used as an index of anxiety while climbing of steps is an index of exploratory and locomotor behavior (Castiella et al., 1990). Therefore, the molecules that reduce rearing activity are said to possess anxiolytic effects (Abid et al., 2006). In stair case test, reduction in the number of rearings and steps climbed in haloperidol induced group was observed. Because of the anxiolytic like effects exhibited by EEHh and GFHh, there was increase in number of steps climbed and rearings. These findings were contrary with the previous reports of Securinega virosa (Magaji et al., 2012; Aiyelero et al., 2012).

Oxidative stress (OS) plays more prominent role in the ageing process, and it is one of the most important risk factor for PD (Cui et al., 2012). Pathological process of PD mainly includes mitochondrial dysfunction, damaged proteins and lipids owing to OS (Perier and Vila, 2012). Previous literature on haloperidol stated that the basal ganglia of brain area are specifically involved in motor disturbances which have high concentration of transition metals like Cu$^{2+}$ and Fe$^{2+}$. In addition, haloperidol is metabolized by various enzymes, including cytochrome 450 resulting in the generation of large quantities of oxyradicals and apotent neurotoxic metabolite further increasing the amount of free radicals present in the brain (Wright et al., 1998; Fang et al., 2001). In the present study, reduced activities of enzymic (SOD, CAT, GPx, and GST) and non-enzymic antioxidants (GSH, vitamin C and E) and increased LPO in haloperidol induced group have been observed.

SOD and CAT are the main enzymes which scavenge the excessive free radicals (Weydert and Cullen, 2009). From the previous studies, it is understood that, GPx is also involved in antioxidant defense mechanism (Kakkar et al., 1992; John et al., 2001; Limaye et al., 2003). These enzymes are parallely involved in reactions especially SOD and GPx is very important because SOD metabolizes excess O$_2^-$ and produces H$_2$O$_2$, which is converted to H$_2$O by GPx (Ghadrdoost et al., 2011).
Previous studies have confirmed that brain is highly vulnerable to oxidative stress as the organ is primarily composed of post mitotic cells. The CNS shows increased susceptibility to OS because of its high oxygen consumption rate (20% of the total oxygen inhaled by the body) that accounts for the increase in generation of free radicals like superoxide radical (O\(^{\cdot}\)), singlet oxygen (O\(_2\)), H\(_2\)O\(_2\) and hydroxyl radicals (OH\(^{\cdot}\)). Usually the balance is maintained between the oxidative attack of the free radicals and the antioxidative defense mechanism prevailing in cells and tissues of the body (Srinivasan, 2002).

In this study, EEH\(_h\) and GFH\(_h\) and L-dopa administered groups showed elevation in SOD, CAT, GPx, and GST indicating tremendous protection from Oxidative stress. Such regulation of oxidative markers by EEH\(_h\) and GFH\(_h\) correlates with the previous findings of Hibiscus sabdariffa calyx, Camellia sinensis (Oboh and Rocha, 2008) Hibiscus asper Hook (Hritcu et al., 2011) which may be due to their free radical quenching activity. Lipid peroxidation is the end product of oxidative stress in cell and this is mainly elevated in diseased condition especially in neurodegenerative or neurotoxic conditions (Balijipelli et al., 2001). Prokai et al (2005) reported that free radical generation leads to cellular structural damage and GSH protects against neurotoxicity.

This study also showed increased level of LPO and decreased level of GSH and GPx in haloperidol induced group. EEH\(_h\), GFH\(_h\) and L-dopa restored the enzymatic activities of SOD, CAT, GPx and GST and decreased the level of LPO (MDA level). The current findings support the hypothesis that increased enzymatic antioxidants led to reduced production of intracellular H\(_2\)O\(_2\) with the concurrent decreased level of peroxidation, protein oxidation which implicates that the plant extracts EEH\(_h\) and GFH\(_h\) acts against neurotoxicity. The reduced LPO levels can be associated to proficient free radical scavenging activity of flavonoids present in EEH\(_h\) and GFH\(_h\) (Patil et al., 2014).

Vitamin E and C levels were decreased in brain tissue homogenate of haloperidol induced group when compared with control group due to increased
oxidative stress in PD because of its increased consumption. Vitamin E ceases the free radicals and interrupts the reaction chain that damages the cells (Bhumik et al., 1993). The decreased activity of vitamin E and C might cause oxyradical mediated injury and may also contribute to neurodegeneration. But the EEHh, GFHh and L-dopa treated groups restored the activity of vitamin E and C due to their antioxidant nature.

Dopamine is primarily metabolized through oxidation by MAO to 3, 4-dihydroxyphenyl acetic acid. This reaction produces H$_2$O$_2$. Dopamine is also metabolized by auto oxidation yielding superoxide radical. H$_2$O$_2$ can further react with Fe or Cu ions to produce OH radical, which are the most toxic free radicals. Increased dopamine turnover by haloperidol could lead to excessive production by potentially damaging free radicals (Fleckenstein et al., 1994). ROS are also reported to diminish the dopamine transporter function further increasing the extracellular dopamine levels. The EEHh, GFHh and L-dopa treated groups confirmed the antiparkinson like effect by restoring the values of dopamine.

In this study, EEHh and GFHh treated animals showed decreased levels of glutamate than haloperidol induced group. Decreased glutamate levels may result in improved motor coordination (Dajas et al., 2002; Dajas et al., 2003; Tandon et al., 2005). Glutamate is one of the neurotransmitter which is classically known to excite amino acids, mediate synaptic excitation and its presence in excess leads to neurotoxic condition. The increased glutamate is one of the main factors in neuronal degeneration and cause PD by activation of NMDA receptor gated ion channels which leads to Ca$^{2+}$ influx and facilitates the formation of nitric oxide (ROS) (Akaikea et al., 1999). Thus, the antioxidant and neuroprotective flavonoids present in EEHh and GFHh might be the contributor for its antiparkinson like effect.

Thus in conclusion, EEHh and GFHh attenuated the behavioral and biochemical changes in haloperidol induced mouse model. In addition, GFHh possessed remarkable antiparkinson like effect than EEHh in all the behavioral and biochemical changes which is proposed to be the result of its high concentration of
flavonoids. Thus, GFHh was proved to be a potential candidate in treating neuroleptic induced Parkinson disease. However, whether these neuropsychopharmacological effects of EEHh and GFHh is contributed from their flavonoid contents is still needed to be explored.
Graphical summary - Antiparkinson like activity of EEHh and GFHh in haloperidol induced Swiss Albino mice

Swiss Albino mice

Haloperidol induction

Treatment with EEHh and GFHh

Involuntary movements
- VCM, OB, TP increased
- Ptosis
- Increased score

Catalepsy
- Increased score in block and metal bar method

WHT
- Decreased grip strength

GA
- Decrease in forepaw stride length

BWT
- Increased walking time
- Increased falling errors

SCT
- Decrease in steps climbed and rearings

Oxidative stress

Neuro protection by EEHh and GFHh

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

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