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Neuroprotective Effect of Hydro Alcoholic Extract of Annonasquamosa Linn Against 6- OHDA-Lesion Model of Parkinson's Disease in Male Sprague-Dawlely Rats: A Behavioral, Biochemical and Histological Study

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Abstract - Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of substantianigra pars compacta in the ventral midbrain leads to the reduction of dopamine being released into the striatum. These processes are then responsible for the clinical features of PD including bradykinesia, resting tremor, rigidity, and difficulty in initiating movements.

Keywords : *Parkinson disease; 6-OHDA; Dopamine; Monoamine oxidase enzyme; Acetyl Cholinesterase; Annonasquamosa Linn.*

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Neuroprotective Effect of Hydro Alcoholic Extract of Annonasquamosa Linn Against 6-OHDA-Lesion Model of Parkinson's Disease in Male Sprague-Dawley Rats: A Behavioral, Biochemical and Histological Study

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Abstract - **Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of substantianigra pars compacta in the ventral midbrain leads to the reduction of dopamine being released into the striatum. These processes are then responsible for the clinical features of PD including bradykinesia, resting tremor, rigidity, and difficulty in initiating movements.**

a) Aim

Aim of the present study is to investigate the neuroprotective effect of hydro alcoholic extract of Annonasquamosa Linn (HAEA) in male Wistar Sprague-Dawley rats subjected to unilateral 6-OHDA lesion model.

b) Materials and Methods

6-OHDA is the most commonly employed agent for the induction of experimental Parkinsonism in rodents. The locomotion, muscular strength and behavioral response were significantly reduced in 6-OHDA lesioned group. Gait analysis and beam walk test were assessed to evaluate gait abnormalities, motor-coordination and postural balance. Pole test and catalepsy test was performed to evaluate the measure for bradykinesia. 6-OHDA injected rats were subjected to Rotarod, Open field spontaneous activity, Force swim test and Grid test to evaluate the motor coordination, locomotion, muscular strength, and catching reflex.

The results showed that HAEA significantly ameliorated high degree of bradykinesia and also showed improvement in gait and balance dose dependently.

HAEA significantly restored the motor deficits and behavioral changes caused by 6-OHDA. HAEA at both the dose level of 200mg/kg and 400mg/kg significantly ($p<0.01$) restores the dopamine level in the basal ganglia and antagonizing the excitatory effect of cholinergic neurons by increasing brain dopamine and Acetyl Cholinesterase (AchE) level. It was further observed that HAEA showed significant decreases in Monoamine oxidase enzyme (MAO) and Glutamate level when compare to 6-OHDA lesioned group. HAEA showed potent antioxidant activity by increasing the brain anti oxidant enzyme level, thus reestablishing the correct the balance between dopamine and acetylcholine and also to antagonize the damage due to oxidative stress.

c) Conclusion

Our results suggested that the HAEA have promising neuroprotective activity against 6-OHDA lesioned experimental Parkinsonism. Ameliorating effect of HAEA may be due to its potential antioxidant activity and might provide an opportunity to management neurological abnormalities in Parkinson's disease conditions.

Keywords : Parkinson disease; 6-OHDA; Dopamine; Monoamine oxidase enzyme; Acetyl Cholinesterase; Annonasquamosa Linn.

I. Introduction

The primary deficit in Parkinson's disease (PD) is a loss of the dopaminergic neurons in the substantianigra pars compacta which provides dopaminergic innervations to the striatum i.e. caudate and putamen. The present understanding of the pathophysiology of PD traced to neurochemical investigations which demonstrates that striatal dopamine content is reduced to 80%, which leads to the loss of neurons from substantianigra [1].

In Parkinson's disease, destruction of cells in the substantianigraresults in the degeneration of neurons responsible for secreting dopamine in the neostriatum. Thus the normal modulating inhibitory influence of dopamine on cholinergic neurons in the neostriatum is significantly diminished, resulting in overproduction or a relative overactivity of acetylcholine by the stimulatory neurons. This triggers a chain of

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abnormal signaling, resulting in loss of the control of muscle movements resulting in the Parkinsonism degeneration of the control of muscle movement [2].

The prevalence of Parkinson's disease in industrialized countries is estimated at 0.3% of the general population and about 1% of the population older than age 60 years [3,4] .People of all ethnic origins can be affected, and men are slightly more prone to the disorder [5,6].

In India about two thousand medicinal plants are found. Annonasquamosa Linn belonging to family Annonaceae, is a tree, cultivated throughout India [7]. Plant possesses antidiabetic [8] and anti-mycrobial property [9].The purpose of the study was to evaluate for the anti-parkinsonism activity of leaves of the plant. Anonasquamosa Linn leaves are found to have Cardiotonic and several alkaloids, quinoline, squamone and bullatacinone, terpene derivatives, flavanoids, polyphenols, dopamine, squamoline and a novel diazepine, squamolone as major active constituents and have Strong anti-oxidant properties.

Hence the present study was designed to study the neuroprotective effect of hydro alcoholic extract of Annonasquamosa Linn (HAEA) in male Wistar Sprague-Dawley rats subjected to unilateral 6-OHDA lesion model.

II. Materials and Methods

a) *Plant Material*

The leaves of Annonasquamosa Linn .were collected from Anna university campus in chennai in the month of August 2010 and identified by Dr.Sasikala Ethirajulu, Asst.Director, Pharmacognosy department, Siddha central research institute, Arumbakkam, chennai and a voucher specimen was deposited at C.L.Baid Metha College of Pharmacy for future reference.

b) *Preparation of Extract of HAEA.*

Fresh leaves were collected, shade dried and grinded to get a coarse powder with an electric blender and about 1000g of the powder was subjected to hot solvent extract using water and ethanol (4:10)in soxlet extractor at 40-50°C. The filtrate collected after extraction was evaporated to dryness at 45°C in hot air oven till liquid to semisolid mass was obtained. It was then stored in airtight containers in a refrigerator below 100 °C, the resulted extract yield was 17.61%, the appearance of extract was gummy resin in nature with dark brown color.

c) *Phytochemical screening*

Phytochemical screening of the HAEA extract was performed using the reagents and chemicals as follows.

- Alkaloids with Mayer's, Hager's and Dragendorffs reagent [10]
- Flavonoids with the use of sodium acetate, ferric chloride and amyl alcohol
- Phenolic compounds and tannins with lead acetate and gelatin
- Carbohydrate with Molish's, Fehling's and Benedict's reagent [11]
- Proteins and amino acids with Millon's, Biuret Xanthoprotein test
- Saponins test using the hemolysis method
- Sterols with 5% potassium hydroxide
- Steroids with Libermann Burchard's test [12]
- Saponins with foam test
- Terpenes with thionyl chloride
- Glycosides with ferric chloride, acetic acid and concentrated sulphuric acid
- Gum tested using Molish's reagent and Ruthenium red
- Coumarin by 10% sodium hydroxide and Quinones by concentrated sulphuric acid.

These were identified by characteristic color changes using standard procedures [13]

The screening results were as follows:

carbohydrate, flavonoid, flavones, tannins, protein, phenol and glycoside

Alkaloids +; Carbohydrates +; Proteins and amino acids +; Steroids -; Sterols -; Phenols +, Flavonoids +; Flavones+ Gums and mucilage +; Glycosides +; Saponins -; Terpenes -, and Tannins +ve. where + and - indicates the presence and absence of compounds.

d) *Acute toxicity study [14]*

This was performed for the extracts to ascertain safe dose by the acute oral toxic class method by the Organization of Economic Cooperation and Development (OECD). A single administration of starting dose of 2000 mg/kg body weight/po of the HAEA was administered to three female and male rats, and the rats were observed for three days to evaluate considerable changes in body weight and other signs of toxicity.

Repeating the experiment with the same dose level of HAEA for more seven days, we observed the body weight change and toxicity sign for totally fourteen days.

e) *Experimental Animals*

Colony inbreed strains of Male Sprague-Dawley rats of 250-300 g body weight were used for pharmacological studies, four different groups with 6 animals in each, were housed at controlled temperature (25±2°C) and light dark cycle (12/12 hr); on a standard rodent diet(Hindustan lever pvt ltd., banglore) and water ad libitum. Animals were handled carefully and acclimatized to laboratory conditions at least 1 week prior to experimentation. The experimental protocol was approved by Institutional animal ethics committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

IAEC Reference number: IAEC/XXX/02/CLBMCP/2010 dated 22.09.2010

f) Induction of Parkinson's disease in rodents [15].

Rats were weighed and anaesthetized with Xylaxine (5mg/kg) and Ketamine (100 mg/kg). Hair depletory was applied to head and scrubbed to remove hair. 1unit adrenaline was injected by IP route using insulin syringe to prevent the bleeding. Each rat was placed in a stereotaxic instrument (David Kopf Instruments, Tujunga CA, USA). A midsagital dorsal skull incision was made through the skin and the skin was retracted. The soft tissues overlaying on the skull were then removed. The landmark of the skull, bregma and lambda were identified and the skull was oriented such that both points were positioned at the same horizontal level. After cleaning the underlying fascia, a small hole was made using a driller with sharp end, by rotary movements of the needle through the skull over coordinates corresponding to the site of interest. The coordinates were estimated from the rat brain atlas (Franklin and Paxinosie AP – 5.0, ML + 1.5, DV – 8.0). ICV (Intra cerebro ventricular injections were given using micro syringe (Hamilton Company, Nevada, USA) connected by PE-10 polyethylene tubing to a 29 gauge internal cannula (Plastics One). Projected 8mm below the guide cannula.

The internal cannula was inserted into the guide cannula and a volume of 4 μl was delivered over a period of 4 min and the needle left in place for an additional 5 min before retraction (8 µg 6-hydroxydopamine in 4 µl saline containing 0.02% ascorbic acid in the left substantianigra). An air bubble was created into the polyethylene tube to avoid the mixing of drug solutions with disttiled water. The movements of air bubble inside the polyethylene tubing confirmed the drug administration. The internal cannula was held in position for another 1 min to prevent backflow and complete dispersal of the drugs or peptides.

After surgery, the animals were then allowed to recover for a week under antimicrobial cover of cefotaxime (50 mg/kg/day, s.c.) once a day for three days. After recovery from anesthesia, wound sites were inspected and cleaned on regular basis. Following surgical intervention animals were treated with extract for 1 week by Oral route. Rats with any neurological and motor deficits were excluded from the study. The animals were housed individually in home cages during recovery period. To avoid infection, Soframycin antibiotic ointment (Aventis Pharma Ltd., Pune) was applied on the wound. During this period, rats were habituated to the experimental protocol to minimize non-selective stress.

g) Experimental procedure

Male Sprague dawley rats of body weight 250-300g were randomized into 4 groups. Each group have 6 animals (n= 6 per group). The groups and treatment are designated as follows.

GROUP I : Animals (Control) treated with (0.9 % saline, p.o)
GROUP II : Animals (Negative control) with 6-OHDA (8 μg 6-hydroxydopamine)(I.C.V) and treated with saline (p.o)
GROUP III : Animals with 6-OHDA (I.C.V) lesion and treated with 200mg/kg of HAEA (p.o)
GROUP IV : Animals with 6-OHDA (I.C.V) lesion and treated with 400mg/kg of HAEA (p.o)

h) In-vivo behavior Examination

i. *Rota rod test [16].*

Rota rod unit consists of a rotating spindle (diameter 7.3cm)d individual compartments for each rat with varying rotational speeds. Initially animals were trained for four training sessions on consecutive days (each constituted by a maximum of 10 trails) to achieve the maximal performance. The animals were exposed on a rotating rod at 10r.p.m at 5 min intervals with the cutoff period of 180 seconds. Average retention time spent on rod was calculated.

ii. *Open field spontaneous activity [17].*

Animals were placed in the corner of an open field (33×33×30cm) chamber with floor grated into squares under weak light. Number of square crossed (in 5 mins) was counted manually in triplicates as forepaw criterion (horizontal activity). Number of observations including grooming and rearing (vertical activity) was also measured.

iii. *Pole test [18].*

Animal was placed head upward on the top of a vertical rough surfaced pole (diameter 1cm and height 55cm). Each rat was habituated to the apparatus on the day prior to testing, then allowed to descend five times. The total time taken by the animal to turn completely downward and climb down to the floor was recorded with the maximum duration of 120 seconds. Even if the rat descended part away and fell the rest of the way, the behavior was scored until it reached to the floor. When the rat was not able to turn downward and instead dropped from the pole, TLA was taken as 120 seconds because of maximal severity.

iv. *Catalepsy test [19].*

Catalepsy was measured by placing the animal on a flat horizontal surface with both hind limbs on square wooden block of 3cm in height and the latency in seconds required to move the limbs from the block to the ground was measured.

v. *Beam walking test [20].*

Motor coordination and balance were assessed by measuring the ability of the rat to transverse a narrow beam to reach dark goal box in beam walking test. The beams consisted of stationary wooden narrow flat beam (L 100cm × W1cm) placed at a height of 100cm from

the floor. The animals were trained in beam for ten trials with one minute interval. During experiment animals were monitored and rewarded with food pellets in the goal box. Time taken to transverse the beam from start box to goal box and number of stepping errors were measured.

vi. *Grid test [21].*

The grid apparatus consisted of a horizontal mesh (total size 12×2cm, openings 0.5×2cm) mounted 20cm above a hard surface, thus discouraging falling, but not leading to the injury in case of falling. The apparatus consists of a 3 inch wall that made up of any opaque sturdy material. Rat were lifted by their tail and slowly placed in the centre of the horizontal grid and supported until they grabbed the grid with both their fore and hind paws. The grid was then inverted so that the rat was hanging upside down for 30seconds and the maximum hanging time was measured.

vii. *Gait analysis [22].*

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 fore limbs and hind limbs were brushed with non toxic paint. Animals were kept at the beginning of the alley. As they walked through that alley into their home cage, they left their paw prints on the paper. By measuring the distance between paw prints stride length was determined.

viii. *Force Swim-test [23].*

Force swim test was carried out in water tubs (40cm length×25cmwidth×16cm height). Water was kept at the depth of 12cm and the temperature was maintained at 27±2 ◦C. The animals were wiped dry immediately after the experiment using a dry towel and returned to cages kept at 27±2 ◦C.

i) Estimation of Neurotransmitters and Metabolic Enzymes

i. *Acetylcholine esterase Estimation [24].*
ii. *Estimation of Monoamine Oxidase B [25].*
iii. *Estimation of Dopamine [26].*
iv. *Estimation o f Glutamate [27].*
v. *Estimation of Total Proteins*

Total protein was estimated in brain using the method described by (Lowry et al., [28].

j) Estimation of Anti Oxidant Enzymes

i. Assay of Superoxide dismutase (SOD) [29].
ii. *Estimation of Catalase (CAT) [30].*
iii. *Estimation of Lipid Peroxidation [31].*
iv. *Estimation of Glutathione Peroxidase (GPx) [32].*
v. *Estimation of Glutathione Reductase (GR) [33].*
vi. *Estimation of ascorbic acid [34].*
vii. *Methods for Histopathological sectioning staining*

The rats from each group were anesthesized by intra peritoneal injection of Ketamine and xylazine. The brain was carefully removed without any injury after opening the skull. The collected saline was washed with ice cold normal saline and fixed in 10% formal saline (10 ml of formaldehyde in 90 ml of physiological saline). Paraffin embedded sections were taken 100 μm thickness and processed in alcohol –xylene series and stained with Haematoxyli- Eosin dye. The sections were examined microscopically for histopathological changes.

k) Statistical Analysis

The statistical analysis was carried by one way ANOVA followed by Dunnet's "t" test. (P values < 0.001) was considered statistically significant, it was calculated using graph pad prism 5.

III. Results

a) Effect of HAEA on Rotarod test (Motor co-ordination)

The motor co-ordination in the control group was significantly ($p<0.001$) high when compared with the 6-OHDA treated animals. Which was found by noting the time taken by the animals to stay on the rotating rod? One-way ANOVA indicated that the decrease in motor coordination was improved with HAEA. Treatment at dose level of 200mg/kg and 400mg/kg showed the significant ($p<0.001$) increase in the motor co-ordination when compared with 6-OHDA injected group. Results are shown in Table 1.

b) Effect of HAEA on Open field spontaneous activity (Locomotor)

The significant ($p<0.001$) decrease in the number of square crossed, grooming and rearing (Locomotor) in the 6-OHDA injected animals were observed. With the treatment of 200mg/kg of HAEA, animals showed the significant ($p<0.01$) increase in the no. of sq crossed and grooming ($p<0.001$), but less significant ($p<0.05$) increase in rearing. With the treatment group 400mg/kg dose of HAEA, animals showed significant ($p<0.01$) increase no. of square crossed, grooming and rearing when compared with 6-OHDA treated animals. Results are shown in Table 2.

c) Effect of HAEA on Pole test (Bradykinesia)

The significant ($p<0.001$) increase in the time taken by the animal to turn downward and time taken to reach floor was observed in 6-OHDA treated animals. With the treatment of 200mg/kg and 400mg/kg dose of HAEA, animals showed the significant ($p<0.01$ and $p<0.001$) decreased time to turn downward and also showed less significant ($p<0.05$) decrease in time taken to reach the floor. Whereas, on treatment with 400mg/kg dose of HAEA, animals showed significant ($p<0.001$) decreased time to reach the floor when compared with 6-OHDA treated group. Results are shown in Table-8 and Histogram 3:- 3.1 and 3.2

d) Effect of HAEA on Catalepsy

In the control group catalepsy was not observed. When 6-OHDA injected group compared with control group, it showed a significant ($p<0.001$) rise in cataleptic rigor. Group treated with 200mg/kg and 400mg/kg dose of the HAEA showed significant ($p<0.01$ and $p<0.001$) decrease in cataleptic rigor when compared with 6-OHDA treated group. Results are shown in Table 4.

e) Effect of HAEA on Beam walking (Motor co-ordination and Balance)

There is a significant ($p<0.001$) increase in the number of foot errors and time taken to travel was observed in 6-OHDA treated animals. With the treatment of 200mg/kg and 400mg/kg dose of HAEA, animals showed the significant ($p<0.01$ and $p<0.001$) decrease in number of foot errors and time taken to travel when compared with the 6-OHDA treated group. Results are shown in Table 5.

f) Effect of HAEA on Grid test (Muscular strength and catching reflex)

6-OHDA injected group compared with control group, showed a significant ($p<0.001$) decrease in hanging time. Group treated with 200mg/kg and 400mg/kg dose of HAEA showed significant ($p<0.01$ and $p<0.001$) improvement in hanging time when compared with 6-OHDA treated group. Results are shown in Table 6.

g) Effect of HAEA on Gait

The stride length and fore paw stance width in 6-OHDA treated animals was significantly ($p<0.001$) decreased when compared with the control group while hind paw stance width in 6-OHDA treated animals was significantly ($p<0.001$) increased when compared with the control group. With the treatment of 200mg/kg and 400mg/kg dose of HAEA, animals showed significant ($p<0.01$ and $p<0.001$) increase in stride length and fore paw stance width and significant ($p<0.05$ and $p<0.01$) decrease in hind paw stance width. When compared with the 6-OHDA treated group. Results are shown in Table 7.

h) Effect of HAEA on Forced swim test (motor impairment)

6-OHDA injected group on comparison with control group, showed a significant ($p<0.001$) decrease in swim time. Group treated with 200mg/kg and 400mg/kg dose of HAEA showed significant ($p<0.001$) increase in swim time when compared with 6-OHDA treated group. Results are shown in Table 8.

i) Effect of HAEA on Acetylcholine esterase activity

The Acetylcholine esterase activity was found to be significantly ($p<0.001$) decreased in 6-OHDA treated animals when compared with control group. Treatment with HAEA 200mg/kg and 400mg/kg significantly ($p<0.01$ and $P<0.01$) increased activity of acetylcholine esterase whem compared to 6-OHDA treated group. Results are shown in Table 9.

j) Effect of HAEA on Monoamino oxidase B

The monoamino oxidase B levels in 6-OHDA treated group was found to be significantly ($p<0.01$) high than the control group. HAEA 200mg/kg and 400mg/kg treatment significantly ($p<0.01$) decreased the Monoamino oxidase-B in 6-OHDA lesion animals. Results are shown in Table 10.

k) Effect of HAEA on Dopamine

When compared to the control group, 6-OHDA treated group showed significant ($p<0.001$) decrease in the dopamine level in striatum. 200mg/kg and 400mg/kg administration showed significant ($p<0.01$ and $p<0.001$) increase in dopamine content when compared to 6-OHDA lesion animals. Results are shown in Table 11.

l) Effect of HAEA on Glutamate

The Glutamate content in 6-OHDA injected group was significantly ($p<0.001$) higher than control group. On treatment with 200mg/kg and 400mg/kg showed significant ($p<0.05$ and $p<0.001$) decrease in glutamate level. Results are shown in Table 12.

m) Effect of HAEA on Total protein

Group treated with 6-OHDA had shown significant ($P < 0.001$) lower brain total protein level when compared with control group animals Brain total protein level of those animals treated with HAEA 200mg/kg and 400mg/kg significantly ($P<0.01$ and $p<0.001$) increased when compared with the 6-OHDA lesion animals. Results are shown in Table 13.

n) Effect of HAEA on Superoxide Dismutase (SOD) Activity

A significant ($p<0.001$) decrease in the brain tissue Superoxide dismutase (SOD) was observed in 6-OHDA lesion animals when compared to control animals. Treatment with HAEA at doses of 200mg/kg and 400mg/kg showed significant ($p<0.05$ and $p<0.01$) increase in 6-OHDA lesion animals. Results are shown in Table 14.

o) Effect of HAEA on Catalase (CAT) Activity

A significant ($p<0.001$) decrease in the brain CAT was observed in 6-OHDA lesion animals when compared to control animals. Treatment with HAEA at doses of 200mg and 400mg/kg showed significant ($p<0.01$) when compared to 6-OHDA induced 6-OHDA lesion animals Results are shown in Table 15.

p) Effect of HAEA on Lipid Peroxidation (LPO)

The content of Lipid peroxidation in the brain tissue was significantly increased ($P<0.001$) in 6-OHDA injected animals when compared with control group. Treatment with HAEA at doses of 200mg/kg and 400mg/kg p.o showed significant ($p<0.01$and $p<0.001$)

decrease in LPO in 6-OHDA lesion animals. Results are shown in Table 16.

q) Effect of HAEA on Glutathione Peroxidase (GPx)

The activity of Glutathione Peroxidase (GPx) in brain was significantly decreased ($p<0.001$) in 6-OHDA administered animals when compared with control group. HAEA at200mg/kg and 400mg/kg doses showed significant ($p<0.01$ and $p<0.001$) increase when compared to 6-OHDA lesion animals. Results are shown in Table 17.

r) Effect of HAEA on Glutathione reductase (GR)

The activity of Glutathione reductase (GR) in brain was significantly decreased ($p<0.001$) in 6-OHDA administered animals as compared to control group. HAEA at 200mg/kg and 400mg/kg doses showed significant ($p<0.01$ and $p<0.001$) increase in GR when compared with 6-OHDA lesion animals. Results are shown in Table 18.

s) Effect of HAEA on ascorbic acid (vitamin c)

When compared with control animals the amount of ascorbic acid was significantly ($P<0.001$) reduced for the 6-OHDA lesion groups. HAEA at doses of 200mg/kg and 400mg/kg showed significant ($p<0.01$ and $P<0.001$) increase in ascorbic acid amount when compared with 6-OHDA lesion animals. Results are shown in Table 19.

t) Effect of HAEA on Neuronal Degeneration

There was a increase in neuronal degeneration and decrease in the number of neuronal cells in brain striatal region in 6-OHDA lesion group (negative control) group when compared with the control group .The group treated with 200mg/kg and 400 mg/kg HAEA showed the significant decrease in brain cell edema and improves the neuronal configuration when compared with negative control group as shown in Fig no: 1.

Table 1 : Effect of HAEA on Rotarod test.

Groups	Control I	Negative Control (6-OHDA) II	HAEA(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Retention time (Sec)	108.8 ± 2.839	$33.28 \pm 1.797^{a***}$	$61.00 \pm 2.739^{b***}$	$84.25 \pm 2.689^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 2 : Effect of HAEA on Open field spontaneous activity.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
No. of Sq. crossed	22.44 ± 1.078	$8.770 \pm 0.253^{a***}$	$12.47 \pm 0.3373^{b**}$	$16.62 \pm 0.4845^{b***}$
Rearing	9.650 ± 0.432	$4.963 \pm 0.413^{a***}$	$7.268 \pm 0.176^{b*}$	$13.621 \pm 1.006^{b***}$
Grooming	6.853 ± 0.434	$2.728 \pm 0.229^{a***}$	$5.138 \pm 0.237^{b***}$	$5.203 \pm 0.137^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 3 : Effect of HAEA on Pole Test.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Time taken to turn downward	4.655 ± 0.206	9.548 ±0.240[a]***	8.553 ± 0.166[b]**	8.343 ± 0.148[b]***
Time taken to reach the floor	3.513±0.192	11.46 ±0.354[a]***	9.853 ± 0.357[b]*	7.463± 0.463[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 4 : Effect of HAEA on Catalepsy.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Latency period (Sec)	0.0 ± 0.0	9.163 ±0.561[a]***	7.493 ± 0.214[b]**	6.490 ± 0.209[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 5 : Effect of HAEA on Beam walking.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Time taken to travel (Sec)	10.25 ± 0.812	25.65 ± 0.484[a]***	21.47 ± 0.914[b]**	12.25 ± 0.952[b]***
No. of foot errors	0.0 0.0	6.688 ± 0.186[a]***	5.228 ±0.337[b]**	3.863 ± 0.252[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 6 : Effect of HAEA on Grid test.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Hanging time(Sec)	2.61 ± 0.331	$6.713 \pm 0.179^{a***}$	$9.510 \pm 0.374^{b**}$	$14.26 \pm 0.723^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 7 : Effect of HAEA on Gait.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Stride length(cm)	6.290± 0.267	$1.490 \pm 0.113^{a***}$	$3.00 \pm 6.296^{b**}$	$5.510 \pm 0.196^{b***}$
Forepaw stance width (cm)	1.990 ±0.053	$1.490 \pm 0.113^{a**}$	$1.918 \pm 0.050^{b**}$	$1.938 \pm 0.051^{b**}$
Hindpaw stance width (cm)	2.863 ±0.045	$3.343 \pm 0.092^{a***}$	$3.088 \pm 0.004^{b*}$	$3.03 \pm 0.011^{b**}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 8 : Effect of HAEA on Forced Swim Test.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Time (Sec)	84.17 ±1.138	$62.33 \pm 0.494^{a***}$	$66.50 \pm 0.428^{b***}$	$70.00 \pm 0.365^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 9 : Effect of HAEA on Acetylcholine esterase activity.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg + 6-OHDA IV
nmoles acetyl thiocholinehydrolysed/min/ mg protein	2813 ±122.6	$1698 \pm 49.90^{a***}$	$2293 \pm 955.8^{b**}$	$2415 \pm 72.40^{b**}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 10 : Effect of HAEA on Monoamino Oxidase- B.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles/min/ mg	5845 ± 245.5	7238 ± 119.4[a]**	5995 ± 288.7[b]**	5845 ± 277.2[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 11 : Effect of HAEA on Dopamine.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
dopamine (ng/mg tissue)	664.3 ± 9.772	391.2 ± 15.30[a]***	441.9 ± 18.27[b]**	570.3 ± 14.47[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 12 : Effect of HAEA on Glutamate.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles/gm	72588 ±394.8	87605 ± 301.5[a]***	83510 ± 1535[b]*	82713 ± 304.2[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 13 : Effect of HAEA on Total Protein.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
mg/gm of tissue	1.868 ± 0.109	0.8775 ±0.0442[a]***	1.463 ± 0.743[b]**	1.543 ±0.106[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 14 : Effect of HAEA on Superoxide Dismutase (SOD) Level.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
unit/mg protein	10.81± 0.5070	6.668± 0.2244[a]***	7.890± 0.09656[b]*	8.740± 0.2074[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 15 : Effect of HAEA on Catalase (CAT) Level.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
n moles/H2O2/ deconsumed /min/mg protein	2813±122.6	1698±49.90[a]**	2293±155.8[b]**	2415±72.40[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 16 : Effect of HAEA on Lipid peroxidation (LPO).

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles of TABRS/ mg protein	2.343± 0.07192	3.935± 0.1028[a]***	3.310± 0.1646[b]**	2.915±0.7735[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 17 : Effect of HAEA on Glutathione Peroxidase (GPx).

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
units/min/mg protein	32.84±0.3824	22.12±0.6462[a]**	25.16±0.6525[b]**	29.34±0.3424[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 18 : Effect of HAEA on Glutathione reductase.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles of NADPH oxidised	33.12±0.4908	24.49±0.3512[a]***	27.24±0.4923[b]**	28.98±0.6632[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 19 : Effect of HAEA on Ascorbic acid.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
ng/mg protein	890.8±10.14	450.8±10,53[a]**	534.5±26.99[b]*	661.0±23.82 [b] ***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Histopathology of Nigro striatal region in rat brain

Figure 1a : Group - I

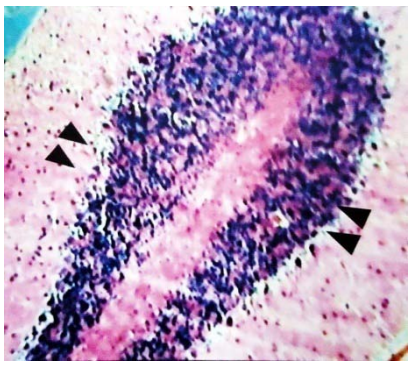


Figure 1b : Group - II

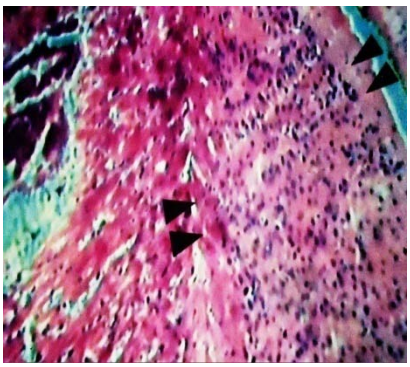


Figure 1c : Group - III

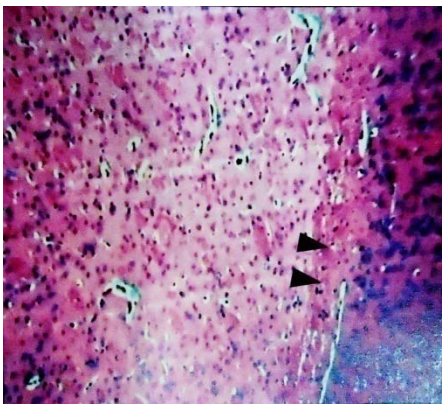
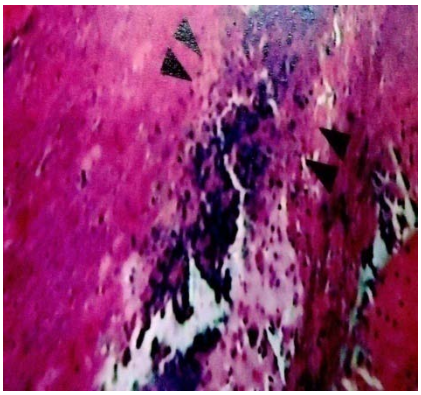


Figure 1d : Group - IV



IV. Discussion

From long time plants have been used as source of drugs for the treatment of Parkinsonism in developed as well as developing countries. Many species have been reported to have anti-parkinsonism effect. Working on the same line, we have undertaken a study on Annonasquamosa for its Anti-parkinsonism property along with its anti oxidant potential.

Acute oral toxicity studies of HAEAS were performed by using OECD 423 guidelines. Studies did not exhibit any lethality or any profound toxic reactions at a dose of 2000mg/kg/p.o. According to the (OECD) 423 guidelines for acute oral toxicity study LD50 dose of 2000mg/kg/p.o of HAEA was found to be safe.

6-OHDA is the most commonly employed agent for the induction of experimental Parkinsonism animal models. It is a selective catecholaminergic neurotoxin as it is thought that it is taken up by the dopamine transporter (DAT), that selectively destroys catecholaminergic neurons and it is typically injected unilaterally, since bilateral injections cause high mortality Furthermore, intra cerebral injection of 6-OHDA into the rat nigrostriatal pathway has been shown to permanently degenerate virtually all dopaminergic neurons in the substantia nigra pars compacta leading to stable motor deficits over time [35]. It is reported that the underlying mechanism of 6-OHDA on dopaminergic neurons is believed to be through a number of mechanism that includes its uptake through a dopamine transporter and leading to mitochondrial dysfunction by inhibiting complexes I and IV of the mitochondrial respiratory chain and increases free radicals formation. As a final effector of Dopamine neural cell death. It seems to induce a caspase 3-dependent apoptotic mechanism [36].6-OHDA is also attributed to the formation of various oxidants and impairs antioxidant enzyme levels which leads to the increase in oxidative stress by the production of ROS 6-OHDA can auto-oxidize to semiquinone and superoxide radical which is considered to be key pathogenesis of Parkinsonism [37]. Currently many evidences suggest the role of antioxidants as a neuroprotective compound by preventing 6-OHDA -induced dopamine depletion in rat.

The loss of dopaminergic neurons due to 6-OHDA administration leads to behavioral changes which are associated with an onset of motor deficits such as Bradykinesia, muscular rigidity, resting tremor and gait

abnormalities with poor postural balance are characterized symptoms of PD [38]. Our study found that when 6-OHDA injected rats were subjected to Rotarod, Open field spontaneous activity, Force swim test and Grid test they reveals poor motor coordination, locomotion, muscular strength, and catching reflex. In 6-OHDA lesion animal's motor impairment was prevented and the latency period was increased by HAEAS. The locomotion, muscular strength and behavioral response were significantly reduced in 6-OHDA lesion group where HEA significantly restored the motor deficits dose dependently. Gait analysis and Beam walk test were assessed to evaluate gait abnormalities, motor-coordination and postural balance. Nigrostraital damage gives the measure for gait abnormalities and also balance. This study revealed that 6-OHDA lesion group had poor gait and balance, where the treatment with HAEAS significantly improved gait and balance dose dependently. Pole test and catalepsy test were assessed to evaluate the measure for bradykinesia.Our results showed a high degree of bradykinesia in 6-OHDA lesion group and treatment with HAEA significantly ameliorated high degree of bradykinesia.

Dopamine, a neurotransmitter plays a key role in parkinson's disease. Degeneration of dopaminergic neurons from substantianigra results in the progressive loss of neostriatal nerve endings which are associated with the body movements and motor control [39]. Our study results showed a marked decrease in the dopamine level and neuronal cell death in brain of 6-OHDA lesioned group, when treated with HAEA it showed significant restoration of dopamine level and also prevention of neuronal cell death in brain.

In neostriatum, dopamine makes the synapses with cholinergic neurons through its inhibitory influence on its activity. In PD the loss of dopaminergic neurons leads to its diminished inhibitory activity on cholinergic neurons and overproduction of acetylcholine. Acetylcholine is generally degraded within the synapse by acetylcholinesterase (AChE).For decades AChE has been recognized for decades as a marker for cholinergic pathways in brain located on both pre and postsynaptic membranes. Overall AChE activities are also affected in the brains of patients with PD [40].Our study results showed that the 6-OHDA induced group showed the decrease in AChE activity and when treated with HAEAS it showed significant amelioration.

Monoamine oxidases (MAO) are belonging to the class of enzymes involved in the oxidative deamination of biogenic amines such as the neurotransmitters dopamine, norepinephrine and serotonin. The MAO- B form is predominant in the brain. In pathogenesis of PD MAO-B is well known for its property to generate free radicals. Increased expression of MAO-B leads to the deficiency of the neurotransmitter dopamine. MAO-B concentration and activity have been reported to be increased in several regions of the central nervous system in association with Parkinson's disease [41]. In the present study, it was observed that there was an increase in activity of MAO in 6-OHDA lesioned group and when it was treated with HAEAS, significant inhibition of MAO-B enzyme activity in brain. It may be due to alkaloids and flavonoids constituents in present in HAEAS.As it was reported that MAO-B can be inhibited by some plant-derived alkaloids, anthraquinones, flavanoids and phenols [42].

Glutamate is an excitatory amino acids are the primary neurotransmitters that mediate synaptic excitation which plays a dual role as neurotransmitter and in excess it plays as neurotoxic in CNS. It has been reported as its role in the pathogenesis of the neuronal degeneration in PD ativation of NMDA receptor gated ion channels by excess glutamate leads to Ca2+ influx and facilitates the formation of ROS such as Nitric oxide [43]. In the present study, it was observed that there was an increased level of glutamate in 6-OHDA lesion group and when it was pretreated with HAEAS, significant reduction in glutamate level in striatum.

Oxidative stress is nothing but increased oxidation due to increased amount of potential oxidants and decreases in the antioxidant levels. Small amounts of the neurotoxin are formed from the metabolism of dopamine itself by auto oxidation or enzymatic catabolism via MAO deamination leads to production of HVA and DOPAC, hydrogen peroxide can be converted to highly toxic hydroxyl radicals via iron-mediated Fenton reactions causes' production of ROS, higher levels of free radical production and free radical damage. It was reported that changes in oxidative damage or in antioxidant status in nervous tissue are found in the pathogenesis of Parkinson's disease [44].

Certain studies points to oxidative stress as a component of the pathological mechanisms accompanying nigral cell death and increased lipid peroxidation (LPO), impaired glutathione metabolism, and enhanced superoxide activity in PD.6-OHDA in rat brain generate ROS as it can auto-oxidize to semiquinone and superoxide radical (the most common free radical in the body) and leads to the neurotoxicity. Superoxide Dismutase (SOD) is an enzyme that repairs cells and reduces the damage done to them by superoxide [45].In the present study, it was observed that there was a decreased activity of SOD in 6-OHDA lesion group and when it was treated with HAEAS, significant restoration of SOD level in striatum. The detoxification of free radicals prevents cell damage which generally prevents the progress of LPO, TBARS is a reliable marker of LPO. In our study we found the elevated level of LPO in 6-OHDA lesion group, and significant reduction in LPO on pre-treatment with HAEAS was observed. The oxidative stress also causes damage to proteins, which leads to impaired protein

synthesis [46] when compared to 6-OHDA lesion group, pre-treatment with HAEA restored the protein level in brain.

Glutathione is a tripeptide, which plays a major defense system against oxidative stress in the brain. Glutathione peroxidase (GPx) is an enzyme which plays an important role in removing excess of free radicals and hydroperoxidases and Glutathione reductase (GR) is an enzyme which provides pool for GSH that protects the membrane from toxification. Dysfunction of this system leads to the oxidative damage in PD [47]. We found the significant decrease in the GPx and GR levels in 6-OHDA lesion group and found the significant restoration on pretreatment with HAEA.

The catalase (CAT), which was found at a very low level of activity in the brain, detoxifies hydrogen peroxide to water and prevents ROS. Our studies found the significant increase in CAT levels in 6-OHDA lesion group and found the significant restoration on pretreatment with HAEA.

Due to the oxidative damage by ROS generation in neurons and tissues of brain, level of Ascorbic acid level get reduced in neurons and tissues in brain [48]. Our results found to have a significant increase in the activity of ascorbic acid which was restored on pretreatment with HAEAS when compared to 6_OHDA lesion group.

Our histopathological study revealed striatal neuronal damage and decrease in neuronal cell number in 6-OHDA lesion group, but on pretreatment with HAEA has significantly protected striatal neuronal damage against 6-OHDA toxicity and showed increase in neuronal cell number.

V. Conclusion

In conclusion the results obtained from the present study, indicates that HAEAS protective effect may be due to its activity against free radicals by its antioxidant effect, inhibiting activity of MAO-B and elevation in the dopamine level in the brain. Further studies are required to establish the anti-Parkinsonism activity of Annonasquamosa in terms of molecular mechanism(s) involved in the activity.

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