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Pharmacological Evaluation of Plant Origin Active Compound in Neurodegenerative Disease

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ABSTRACT

The present study was undertaken with the aim & objectives of exploring the neuroprotective and antiparkinsons activity of Embelin isolated from fruits of *Embelia ribes burm.* The phytochemical analysis and confirmatory test for Embelin showing presence of Proteins, Amino acids, Flavonoids, Alkaloids, Tannins. From the interpretations FTIR spectroscopy, the spectra of isolated embelin was almost identical with standard Embelin spectra. Isolated compound showed a significant free radical scavenging action in a concentration dependent manner against DPPH free radicals, showing good antioxidant activity. Also isolated compound showed a strong metal chelation activity on ferrous ions, playing important role in antioxidant activity of fruit isolation. Evaluation of neuroprotection activity of isolated compound of selected plant was done by using monosodium glutamate induced excitotoxicity model. Also evaluation of anti-Parkinson's activity using Haloperidol induced catalepsy model. The IE was administered orally to Rat and mice at dose 200mg/kg p.o. and 400mg/kg p.o. At that dose, the IE was showing significant reduction from disease group and vehicle group. It is compared with monosodium glutamate (2gm/kg i.p.); standard Dextromethorphan (30mg/kg p.o.) which was used as standard drug. IE also have property to restore effect caused from Haloperidol induced catalepsy. The result of *in-vivo* model suggested that IE reverse the action of monosodium glutamate & haloperidol; may exert its neuroprotective effect and antiparkinson's effect.

INTRODUCTION

Neurodegenerative disease includes a great variety of conditions which principally affect the neurons in the human brain. Parkinson's, Alzheimer's, and Huntington's disease are the well known examples of Neurodegenerative diseases. Neurodegenerative diseases are untreatable and making someone very weak as well as infirm that result in progressive degeneration and/or death of nerve cells. Thus it becomes reasons for movement problems or mental functioning.[1]

Ever since PD was first described in 1817, scientists have pursued the causes and treatment of the disease. In the early 1960s, researchers established the principal problem underlying the disease the loss of brain cells that produce a chemical called dopamine, which is important for coordination and control muscle activity. This discovery led to the first successful treatment for PD and suggested ways of devising new and even more effective therapies. Parkinson's research continues to be a very active and intriguing finding reported every day.[2]

In recent times there is an increase in global utilization of herbal medicines in the treatment of various disease affecting humans. *Embelia ribes* berries contain several chemical constituents like embelin, volatile oil, fixed oil, resin, tannin, christembine (alkaloid), phenolic acids like caffeic acid, vanillic acid, chrorogenic acid, cinnamic acid, o-cumaric acid. Embelin is the main constituent found in the plant E. ribes[3]. Embelin posses all suitable physical and chemical properties and its ability to cross the blood brain barrier making it an appropriate candidate for the treatment of neuroprotection in parkinsons disease[4]. No research has yet been carried out on the above mentioned active compound from plant for parkinsons disease treatment. Thus the reason for selecting the compound from plant is its probability of having a neuroprotection activity in Parkinson's disease. The pathogenesis of cell degeneration in neurodegenerative diseases are: dopamine depletion, oxidative stress, mitochondrial dysfunction, protein aggregation, misfolding, neuroinflammation, excitotoxicity, apoptosis and cell death pathway, loss of trophic factor. Immoderate release of glutamate from presynaptic nerve terminals and astrocytes into the extracellular spaces with resultant overstimulation of glutamate receptors mainly NMDA receptors which ultimately related to Excitotoxicity. Several neurodegenerative disorders, including Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, AIDS dementia complex, and Parkinson's disease observed because of neuronal injury which is mainly due to overstimulation of both inotropic and metabotropic glutamate receptors. The concept of

oxidative stress and antioxidants may be directly or indirectly involved in the pathogenic process of Parkinson's disease. Thus with this present study an attempt to evaluate neuroprotective and antiparkinson activity for neurodegenerative disease.

MATERIALS AND METHODS

Drugs and Chemicals:

Embelia ribes burm dried friut (Ekvira Ayurvedic shop, kalwa). Ethanol, Methanol & DPPH, Ferrozine were purchased from Molychem. n- Hexane (Merk). Monosodium glutamate (Himedia), Haloperidol injection IP (5mg/1ml) (syndopa sun pharma), Dextromethorphan (Lastuss ct FDC mfg.), Trihexyphenidyl HCl (Pacitane Pfizer mfg).

Animals:

Animal house conditions:

The wistar rat (150-200gm) and swiss albino mice (25-30)gm were purchased from Bombay veterinary college, Parel, Mumbai- Maharashtra 400012 and from National Institute of Bioscience, Pune. The animals were brought to animal house of Dr. L. H. Hiranandani College of Pharmacy, opposite to Ulhasnagar railway station, CHM campus, Ulhasnagar-03. These animals were acclimatized in animal house under standard husbandry conditions, i.e. room temperature of 24 ± 100C, relative humidity 45-55% and 12:12 hr. light/dark cycle. The institutions animal house is registered with Govt. of India, having registration number 879/PO/Re/S/05/CPCSEA and confirms to the CPCSEA guidelines for the use and care of experimental animal research. The animals were housed in standard propylene cages with wire mesh top and husk as bedding. The animals had free access for food and water supplied *ad libitum* under strict hygienic condition. Each experimental group had separate set of animals and care was taken to ensure that animals used for one response were not employed elsewhere. The approval of the Institutional animal ethical committee (IAEC) of Dr. L. H. Hiranandani College of Pharmacy was taken prior to the start of experiments. All the protocols and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by committee for the purpose of control and supervision of experiments on animals (CPCSEA) and with the protocol no. PCOL06/IAEC/2017.

Collection, Authentication and Extraction of plant:

The dried fruits of *Embelia ribes burm* was collected from Ekvira Ayurvedic shop, kalwa (W), and dried fruits were authenticated from former HOD botany of Khalsa College, Matunga, Dr. Harshad M. Pandit, Ph.D. (Botany), Andheri West, Mumbai 400058.

Physico-chemical & Preliminary Phytochemical analyasis: Physicochemical analysis of crude drug was performed, parameters such as total ash value, water soluble & acid insoluble ash value, loss on drying etc. was performed.[5] Preliminary phytochemical test was carried out to identify different constituents and other compound present in the extract.

***In-Vitro* studies**

DPPH Assay: The free radical scavenging activity of the extract (Test drug) and ascorbic acid as standard were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. Different concentrations were prepared for test (2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml, 10µg/ml) and Standard (20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml). Freshly DPPH solution was prepared in methanol. Reaction mixture was allowed to stand for 10 min. and absorbance was taken at 517nm. The percentage inhibition of DPPH free radical scavenging activity and IC50 was calculated.[6,7]

Metal chelation Assay:

Metal chelation of test compound was tested for their antioxidant activity. It is apparent that the antioxidative activity of medicinal plant plays an important role in the healing of various diseases. The mixture of ferrozine and ferric chloride shows a strong absorbance at 562nm. The Ferrous ions are known to stimulate the free radical reaction decomposing the lipid peroxides to chain propagating alkoxyl radicals and also reacting with hydrogen peroxide to produce and other highly reactive species.[8]

Acute toxicity study:[9]

According to OECD guideline 423, acute toxicity study of test drug was performed on Wistar rat (male) weighing around 150-200gm. Animals were fasted overnight and then test drug (IE) was suspended in calculating volume of 0.5% CMC solution, administered orally with a single dose of 2000mg/kg. Rats were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24hours, with special attention given the first

4 hours and daily thereafter up to 14 days. Parameter to be observed: Alertness, Grooming, Hyperactivity, Convulsion, Hypoactivity, Ataxia, body weight, other behavioral changes.

***In-Vivo* studies:**

To study the neuroprotective and antiparkinson effect of the test drugs, two models namely, Monosodium glutamate for neurotoxicity and Haloperidol induced catalepsy model were utilized.

Monosodium glutamate induced Neurotoxicity:[10,11,12,16,17]

Monosodium glutamate (MSG) is the sodium salt of the glutamic acid, a nonessential amino acid. The MSG is widely used as a flavouring agent in food industry as well as at homes and restaurants. It acts through the activation of both ionotropic and metabotropic glutamate receptor (iGluR and mGluR) found in the central nervous system (CNS). Hyperactivation of these receptors has been reported to produce excitotoxicity and neuronal death. It is also known that MSG or sodium salt of glutamate exerts excitotoxicity by over activation of glutamate receptors namely N-methyl D-aspartate(NMDA).

And overactivation of NMDA receptors by the MSG or sodium salt of glutamate exerts excitotoxicity. Overactivation of NMDA along with other glutmate/glycine receptors disturb the calcium homeostasis, which is the key mediator of glutamate-induced excitotoxic neuronal damage. Dextromethorphan, a synthetic opioid agonist functions additionally as an NMDAR antagonist. Hence in the present study, we have used dextromethorphan against MSG-induced neurotoxicity.

The main objective of this study was to evaluate the neuroprotective effect of IE in Monosodium glutamate induced neurotoxicity.

Parameters to be studied are as follows:

- Muscle rigidity using Rotarod test
- Locomotor activity using Actophotometer
- Catalepsy by Blok test

Biochemical parameter evaluation:

- Lipid peroxide level
- Nitric oxide level

Study design: Overnight fasted wistar rats were randomly divided into five groups of six animal each. Following was the experimental protocol:

Groups	Dose	No. of animals (Rats)
Standard (Dextromethophan)	30mg/kg (p.o)	6
Vehicle control	1% cmc	6
Toxic (Monosodium glutamate)	2gm/kg i.p.	6
Test Group 1	200mg/kg	6
Test Group 2	400mg/kg	6
Total		30

Preparation of drug:

The dose of IE i.e. 200mg/kg and 400mg/kg was prepared by suspending IE in the required volume of 1% sodium carboxyl methylcellulose in water. Dose of dextromethorphan 30mg/kg was prepared by suspending it in cmc. Vehicle containing 1% cmc in water was used as control. All the solutions were freshly prepared every day prior to the administration.

Induction of neurotoxicity:

Induction of catalepsy was done by the administration of monosodium glutamate 2gm/kg in double distilled water i.p. once daily for 7 days.

Dosing schedule:

The animals in the respective groups were administered IE suspended in 1% sodium carboxymethyl cellulose orally, and the standard drug group was received dextromethorphan p.o. daily half hour after the administration of monosodium glutamate for 7 days.

Behavioral parameters:

- **Rotarod apparatus:**

The rotarod test is used to assess motor coordination and balance in rodents. Rats have to keep their balance on a rotating rod. It is measured the time (latency) it takes the rat to fall off the rod rotating at under continuous acceleration i.e. 4rpm.

Before the beginning of the study, the animals were trained at the rotarod apparatus for 5 days. On the day of testing, rats in their home cages and allowed to acclimatize to the testing room for at least 15min. (Acclimation phase). For case of identification at later trials, the rats were marked, using non toxic ink, with respective stripes at the base of the tails before testings. After induction of catalepsy with monosodium glutamate, the animals were placed on the rod in the apparatus. The rod was rotated under continuous acceleration of 4 rpm. The time (latency) when the rat falls off the rod was recorded. If a rat was found to be clinging on the rod and completed a full passive rotation, the timer was stopped and the latency was recorded. After the day's study, the rats were placed back in their home cage. The readings were recorded on 0, 2^{nd}, 5^{th}, 7^{th} days.

- **Actophotometer:**

One of the cardinal features of PD is bradykinesia which refers to slowness of movement, which will results in a decrease in locomotor activity. Assessment of locomotor activity was done using an actophotometer. An actophotometer consists of infrared sensors and a digital counter. The animal's movement will interrupt the infrared beam, which will get recorded and displayed digitally. This principle is used to count total locomotor activity of an animal. Before subjecting the animal to cognitive task they were individually placed in actophotometer and the total activity count was registered for 10 minutes. The locomotor activity was expressed in terms of total count /5min per animal. In the present study, the locomotor activity was assessed on the 0, 2^{nd}, 5^{th}, 7^{th} days.

- **Muscular rigidity by Bloke test**

This scoring method followed is in 3 steps.

Step 1: The rat was taken out of the home cage and placed on table. If the rat failed to move when touched or pushed gently on the back, a score of 0.5 was assigned.

Step 2: The front paws of the rats were placed alternately on a 3cm high bloke. If the rat failed to correct the posture within 15 sec., a score of 0.5 for each paw to be added to step 1.

Step 3: The front paws of the rats were places alternately on a 9cm high bloke, if the rat failed to correct the posture within 15 sec., a score of 1 for each paw was added to the score of step 1 and step 2.

Thus, the highest score for any animal was 3.5 (cut off score) and that reflects total catalepsy.

Biochemical parameters:

Evaluation of Antioxidant Enzymes as follows:

On 8th Day after behavioral assessments, animals were sacrificed by cervical dislocation and brains were removed. The cerebellum was discarded and the remaining brain tissue was weighed and preserved at -20^{0}C in deep freezer till further analysis. The known weight of brain tissue was homogenized for the estimation of antioxidant enzymes.

Lipid peroxidation

Lipid peroxidation was estimated spectrophotometrically in brain tissue by quantifying TBARS according to the methods of NIEHAUS and SAMUELSON. In brief for the estimation of TBARS the supernatant of the tissue was homogenate was treated with tertiary butanol tricholoro acetic acid-hydrochloric acid, (TBA-TCA-HCL) reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15minutes. After cooling the tubes were centrifuged for 10 minutes and the supernatant taken for measurement. The developed color was read at 535nm using a UV spectrophotometer against a reagent blank and expressed as 100mM/100 gm tissue.

Nitric oxide[13]

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as described by Green et al (1982). Equal volumes of supernatant and Greiss reagent were mixed, and then the mixture was incubated at 25°C for 10 min in the dark. The concentration of nitrite was

assayed at 540 nm and calculated with reference to the absorbance of the sodium nitrite standard curve.

Haloperidol induced catalepsy:[14,15]

Activity I: Locomotor activity By Actophotometer

Rational: Haloperidol works by antagonizing dopamine D2 and to a lesser extent, D1 receptors in medium spiny neurons that comprise the indirect and direct pathways of the motor circuit respectively. The resultant block of strital dopamine transmission results in abnormal downstream firing within the basal ganglia circuits that is manifest as symptoms of muscle rigidity and catalepsy within 60 min of haloperidol (1mg/kg,i.p.) injection.

Trihexphenidyl HCl (standard): Trihexyphenidyl is a selective M1 muscarinic acetylcholine receptor antagonist. It is able to discriminate between the M1 (cortical or neuronal) and the peripheral muscarinic subtypes (cardiac and glandular). Trihexyphenidyl partially blocks cholinergic activity in the CNS, which is responsible for the symptoms of Parkinson's disease. It is also thought to increase the availability of dopamine, a brain chemical that is critical in the initiation and smooth control of voluntary muscle movement.

Study design: Overnight fasted wistar rats were randomly divided into five groups of six animals each. Following was the experimental protocol:

Groups	Dose	No. of animals (Rats)
Standard (Trihexphenidyl HCL)	1.5mg/kg (p.o)	6
Vehicle control	Saline	6
Toxic(Haloperidol)	1mg/kg/kg i.p.	6
Test Group 1	200mg/kg	6
Test Group 2	400mg/kg	6
Total		30

Preparation of drug:

The dose of IE i.e. 200mg/kg and 400mg/kg was prepared by suspending IE in the required volume of 1% sodium carboxyl methylcellulose in water. Dose of trihexyphenidyl HCl 1.5mg/kg was prepared by suspending it in cmc. Vehicle containing saline used as control. All the solutions were freshly prepared every day prior to the administration.

Induction of catalepsy:

Induction of catalepsy was done by the administration of Haloperidol 1mg/kg in double distilled water i.p. once daily for 12 days.

Dosing schedule:

The animals in the respective groups were administered IE suspended in 1% sodium carboxymethyl cellulose orally, and the standard drug group was received trihexyphenidyl HCl p.o.daily half hour before the administration of Haloperidol for 12 days.

Behavioral parameters:

- **Rotarod apparatus:**

The rotarod test is used to assess motor coordination and balance in rodents. Rats have to keep their balance on a roating rod. It is measured the time (latency) it takes the rat to fall off the rod rotating at under continuous acceleration i.e. 4rpm.

Before the beginning of the study, the animals were trained at the rotarod apparatus for 5 days. On the day of testing, rats in their home cages and allowed to acclimatize to the testing room for at least 15min. (Acclimation phase). For case of identification at later trials, the rats were marked, using non toxicink with resepective stripes at the base of the tails before testings. After induction of catalepsy with monosodium glutamate, the animals were placed on the rod in the apparatus. The rod was rotated under continuous acceleration of 4 rpm. The time (latency) when the rat falls off the rod was recorded. If a rat was found to be clinging on the rod and completed a full passive rotation, the timer was stopped and the latency was recorded. After the day's study, the rats were placed back in their home cage. The readings were recorded on 0, 2^{nd}, 5^{th}, 7^{th} days.

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Before subjecting the animal to cognitive task they were individually placed in actophotometer and the total activity count was registered for 10 minutes. The locomotor activity was expressed in terms of total count/5min per animal. In the present study, the locomotor activity was assessed on the 0, 2^{nd}, 5^{th}, 7^{th} days.

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Lipid peroxidation

Lipid peroxidation was estimated spectrophotometrically in brain tissue by quantifying TBARS according to the methods of NIEHAUS and SAMUELSON. In brief; for the estimation of TBARS the supernatant of the tissue was homogenate was treated with tertiary butanol tricholoro acetic acid-hydrochloric acid, (TBA-TCA-HCL) reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15minutes. After cooling the tubes were centrifuged for 10 minutes and the supernatant taken for measurement. The

developed color was read at 535nm using a UV spectrophotometer against a reagent blank and expressed as 100mM/100 gm tissue.

Statistical Analysis

The result of neuroprotectant and antiparkinson's activity for *in vivo* model were significantly analyzed using one way analysis of variance (ANOVA) by Dunnett's test.

RESULTS AND DISCUSSION

The present study was performed to carry out the phytochemical and pharmacological evaluation of isolated compound from the dried fruits of *Embelia ribes burm.* The results are presented in tables and graph format. The result displayed include physicochemical, phytochemical evaluation and confirmatory test for IE, acute toxicity study for IE, *in vitro* antioxidant activity and neuroprotective activity of IE and pharmacological effect of IE on msg induced neurotoxicity and haloperidol induced catalepsy.

PHYSICO-CHEMICAL EVALUATION

The fruits powder of *Embelia ribes burm* (crude powder) was subjected to physicochemical parameter evaluation such as total ash value, water soluble & acid insoluble ash value, loss on drying was performed. Result obtained mention in table no. 5.1.

Table No. 1.1: Total ash value, acid insoluble & water soluble ash value, Loss on drying for ER fruit powder

Total ash value	Acid insoluble ash value	Water soluble ash value	Loss on drying
12.5%	5%	1%	1%

PLANT EXTRACTION & ISOLATION:

The fruits powder of *Embelia ribes burm* was subjected to soxhlet extraction method followed by isolation using n-hexane as solvent system. The % yield, color of extract, solubility, melting point and consistency of extract was shown in table no. 1.1.

Table No. 1.2: % yield, color, solubility and consistency of extract

Plant part used	Solvent	% yield	Color of isolated compound	Solubility	Melting point	Consistency
Fruits of *Embelia ribes burm*	n-hexane	0.93%	Brown golden colored crystals	DMSO Ethanol Methanol	143-145	Fine, solid crystals

Table No. 1.3: Confirmatory test for Embelin

Test	Observation	Inference
Compound dissolved in pet. ether & then diluted ammonia solution was added to it.	Formation of bluish violate precipitate.	Embelin confirmed.

Table No. 1.4: TLC Profiling

Indication	Wavelength	Solution	Volume	Rf value
Test sample	523	IE	10□l	0.68

Instrumental Analysis:

FTIR spectroscopy

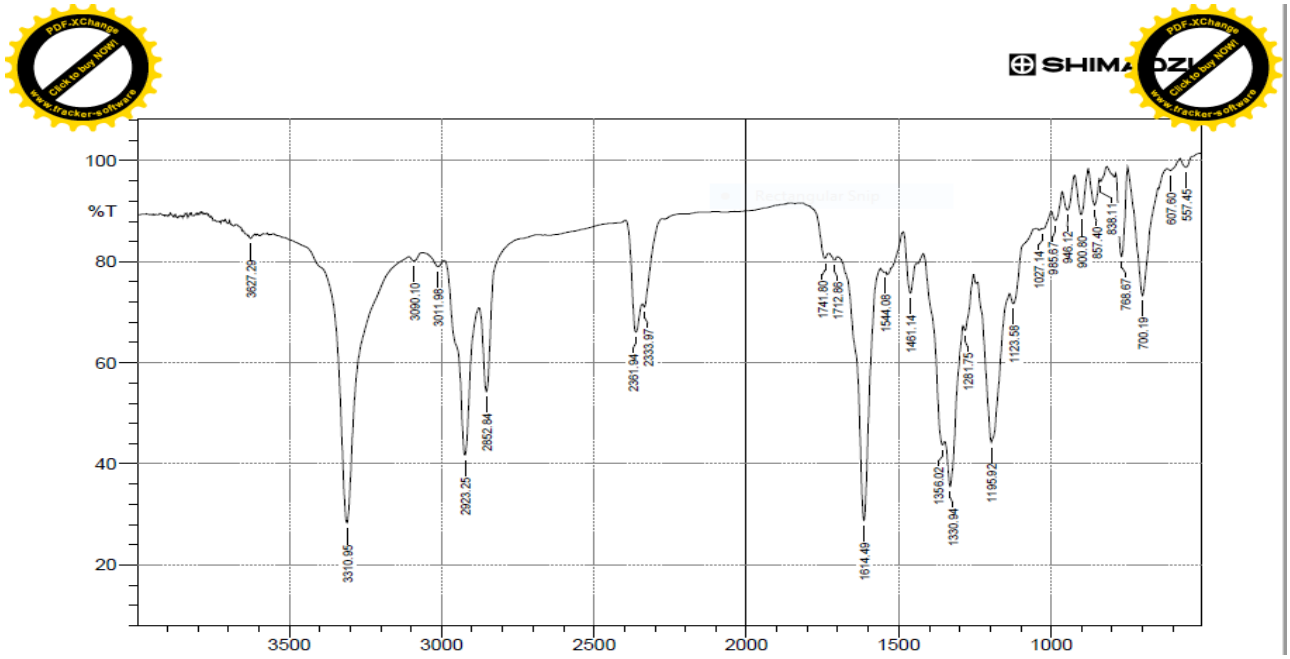

Figure No. 1.1: FTIR Spectra

Table No. 1.5: Peak positions obtained in the FTIR

No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)
1	557.45	98.675	1.973	576.74	539.13
2	607.6	98.037	0.944	620.14	576.74
3	700.19	73.29	23.412	749.38	650.04
4	768.67	81.006	17.082	787.96	749.38
5	838.11	95.86	0.943	841.96	816.89
6	857.4	91.236	6.086	878.61	841.96
7	900.8	89.332	8.465	922.98	878.61
8	946.12	90.204	5.01	963.48	922.98
9	985.67	88.148	3.307	1000.13	963.48
10	1027.14	86.513	0.562	1031.96	1000.13
11	1123.58	71.678	4.026	1136.12	1057.04
12	1195.92	44.262	30.683	1241.25	1136.12
13	1281.75	66.394	2.912	1288.5	1254.75
14	1330.94	35.544	15.773	1349.26	1288.5
15	1356.02	43.764	4.225	1419.67	1349.26
16	1461.14	73.754	8.691	1484.29	1442.82
17	1544.08	77.812	0.1	1547.94	1541.19
18	1614.49	28.763	50.806	1696.47	1559.51
19	1712.86	80.317	0.079	1729.26	1711.9
20	1741.8	80.611	2.913	1792.91	1729.26
21	2333.97	71.064	2.566	2341.68	2276.1
22	2361.94	66.041	11.716	2396.66	2341.68
23	2852.84	54.255	18.504	2876.95	2687.92
24	2923.25	41.693	33.006	2991.72	2876.95
25	3011.98	79.019	1.689	3061.16	2991.72
26	3090.1	80.134	1.157	3107.46	3061.16
27	3310.95	28.268	54.425	3487.45	3107.46
28	3627.29	84.56	0.799	3634.05	3611.86

***IN- VITRO* ANTIOXIDANT ACTIVITY**

- **DPPH free radical scavenging activity :**

IE showed free radical scavenging effect on DPPH radical in concentration dependent manner. Antioxidant activity of IE (test drug) and standard drug ascorbic acid is shown by graph in which percentage of scavenging activity plotted against various concentrations. The IC50 value of IE was found to be 32.75µ/ml. The correlation coefficient (R^2) was calculated from graph and was found to be 0.971. Then was compared with ascorbic acid which was used as standard antioxidant having IC50 value 2.29µ/ml and the correlation coefficient (R^2) was calculated from graph and was found to be 0.9856.

Table No. 1.6: % of DPPH free radical & IC50 value for IE (Test drug)

Sr. No.	Concentration (µ/ml)	% inhibition of DPPH radical	IC 50 value
1.	10	39	
2.	20	40	32.75µ/ml
3.	40	59	
4.	60	61.20	
5.	80	74.7	
6.	100	86	

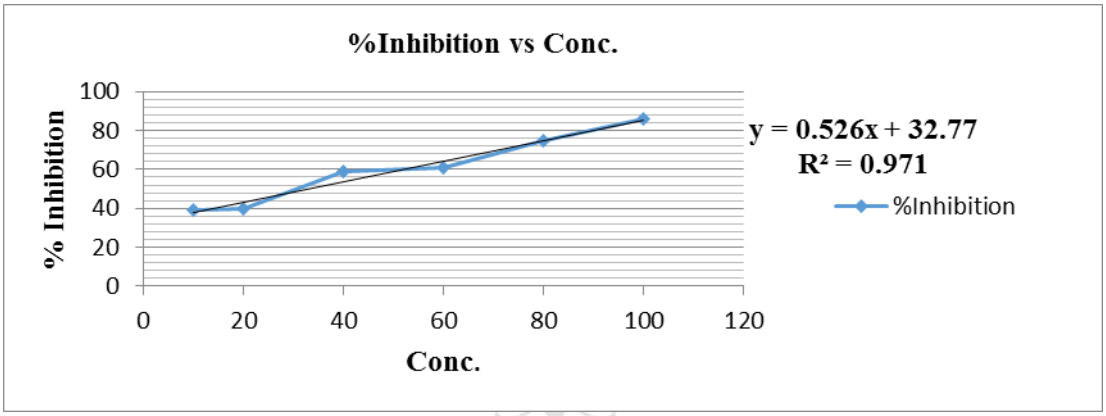

Figure No. 1.2: Graphical presentation of DPPH free radical scavenging activity of IE (Test drug)

Table No. 1.7: Percent inhibition of DPPH free radical & IC50 value for Ascorbic acid (Standard drug)

Sr. No.	Concentration (µ/ml)	% inhibition of DPPH radical	IC 50 value
1.	2	48.33	2.29 µ/ml
2.	4	61.59	
3.	6	67	
4.	8	80.23	
5.	10	93	

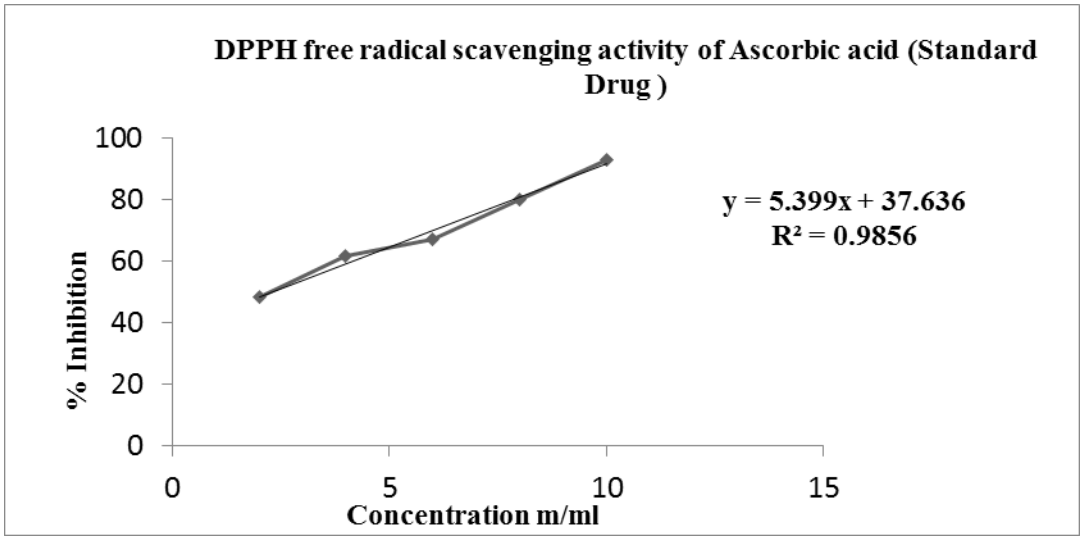

Figure No. 1.3: Graphical presentation of DPPH free radical scavenging activity of Ascorbic acid (Standard drug)

***IN-VITRO* METAL CHELATION ACTIVITY:**

Table No. 1.8: Metal chelating activity of IE (Test drug)

Concentration (µ/ml)	% Chelating activity of IE test sample
10	42.68
20	56.67
30	68.52
40	74.21
50	85.59

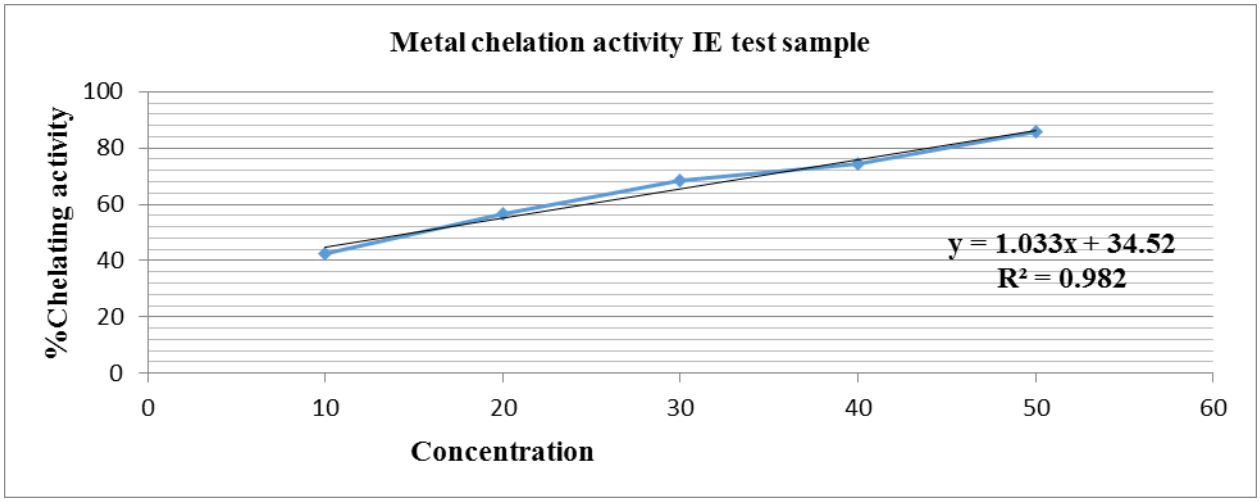

Figure No. 1.4: Metal chelation of IE test sample

IE showed a significant effect from above graphical representation it can be seen that IE inhibits the metal complex formation in concentration dependent manner up to 50µg/ml and thus inhibit metal complex formation mechanism that lead to degenerative diseases.

Table No. 1.9: Metal chelation activity of standard EDTA

Concentration (µ/ml)	% Chelating activity of IE test sample
5	55.51
10	69.10
15	75.26
20	91.75
25	94.19

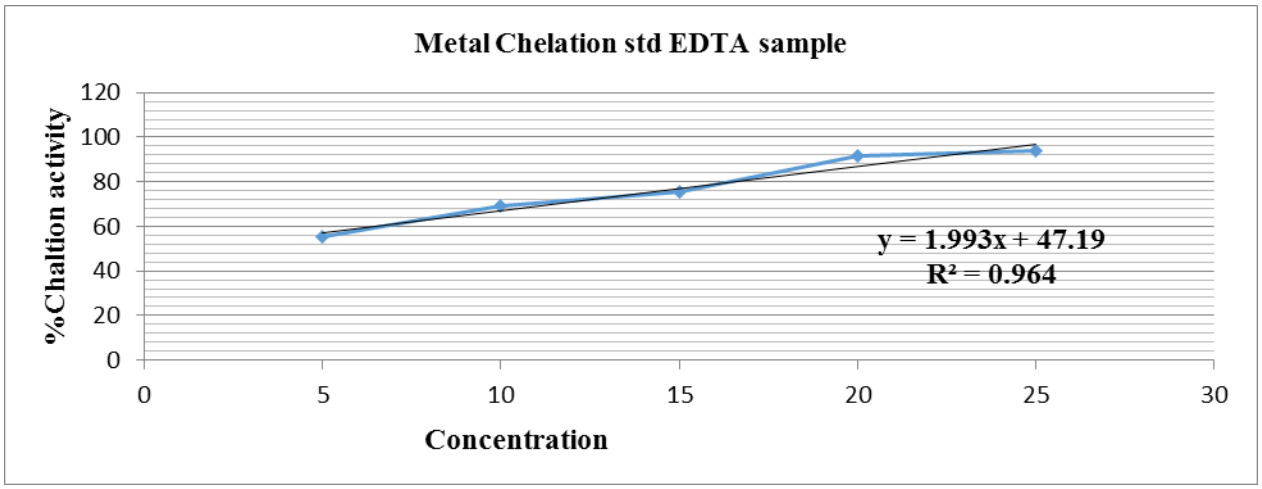

Figure No. 1.5: Metal chelation of standard EDTA sample

EDTA showed promising metal chelating activity in concentration dependent manner up to 25µg/ml. The IE extract was compared with EDTA which was used as standard metal chelating agent. IE can be used as a metal chelating agent in degenerative diseases as per *in vitro* study it shows nearly same metal chelating activity as the standard EDTA.

ACUTE TOXICITY STUDY

The test drug was found safe at 2000mg/kg body weight. There was no death of animals during or after 14 days. The parameter such as alertness was found to be normal in mice. Parameter such as grooming, hyperactivity, convulsion, hypoactivity and ataxia were found to be absent in mice. Autonomic (eye, salivation) parameter and bodyweight of mice were

found to be normal in mice. 1/10th as minimum dose and 1/5th as maximum dose of test drug (IE) used in acute toxicity testing was considered as therapeutic dose for experiment.

***In-Vivo* study:**

Monosodium glutamate induced excitotoxicity: Parameters to be studied are as follows:

- Behavioral changes:
  - Locomotor activity by actophotometer
  - Muscle rigidity by rotarod
  - Catalepsy by block test
- Biochemical parameters
  - Lipid peroxidation
  - Nitrite level

Muscle rigidity by Rotarod:

Table No. 1.10: Muscle rigidity by Rotarod

Day	Vehicle control	Disease (Monosodium glutamate)	Standard drug (Dextromethorphan)	Test 1 IE 200mg/kg	Test 2 IE 400mg/kg
Day 0	84.16± 1.35	84.83± 0.90	83.16± 1.4**	81.16± 0.83**	83.33± 1.2**
Day 3	85.66± 1.22	38± 0.96*	79.5± 0.34**	61.83± 0.90**	67.33±1.49**
Day 5	82.83± 1.30	36± 0.68*	77.5± 0.84**	60.5± 0.34**	65.16±1.30**
Day 7	84.16± 1.35	28.66± 0.33*	77.5± 0.84**	60.16± 0.16**	64.83±1.13**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * $p \leq 0.05$ when compared with vehicle control group, ** $p \leq 0.05$ when compared with disease group.

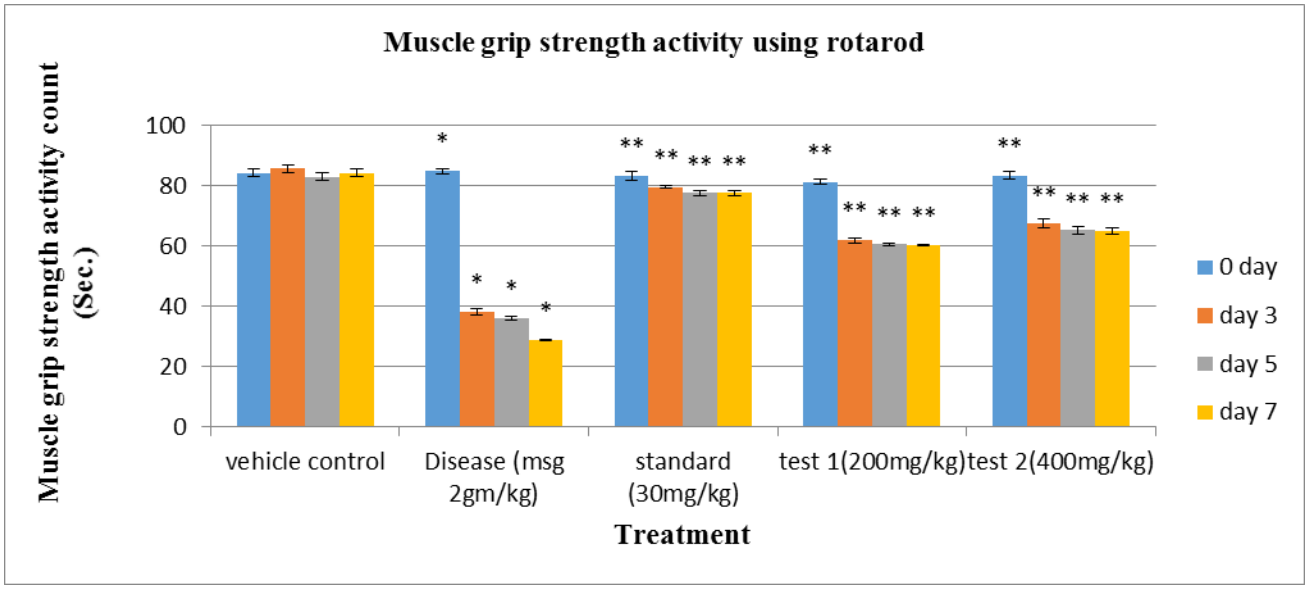

Figure No. 1.6: Muscle grip strength activity using rotarod

Vehicle control group showed significant differences in muscle rigidity as compared to disease control group. Standard group (Dextromethorphan 30mg/kg + Monosodium glutamate 2 gm/kg) and Test group IE (200mg/kg) and IE 400mg/kg showed a significant difference when compared to Disease control group (Monosodium glutamate 2 gm/kg). From above result it is seen that test drug IE 200mg/kg and400mg/kg decreases muscle rigidity in Parkinson disease induced rats as compared to Disease control Monosodium glutamate group.

Locomotor activity by actophotometer:

Table No. 1.11: Locomotor activity by actophotometer

Day	Vehicle control	Monosodium glutamate (Disease)	Standard drug (Dextromethorphan)	IE 200mg/kg	IE 400mg/kg
Day 0	201.16±1.24	201.33±1.02*	202.83±1.30**	202.66±1.68**	203..5±1.62**
Day 3	200.83±0.83	80±1.43*	186.33±1.49**	148.33±0.47**	158.16±1.3**
Day 5	199.5±0.34	74.66±0.98*	184.16±0.40*	148.5±0.34**	156.66±0.55**
Day 7	199.66±0.21	69±0.36*	183.5±0.50**	147±0.57**	153.33±1.22**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

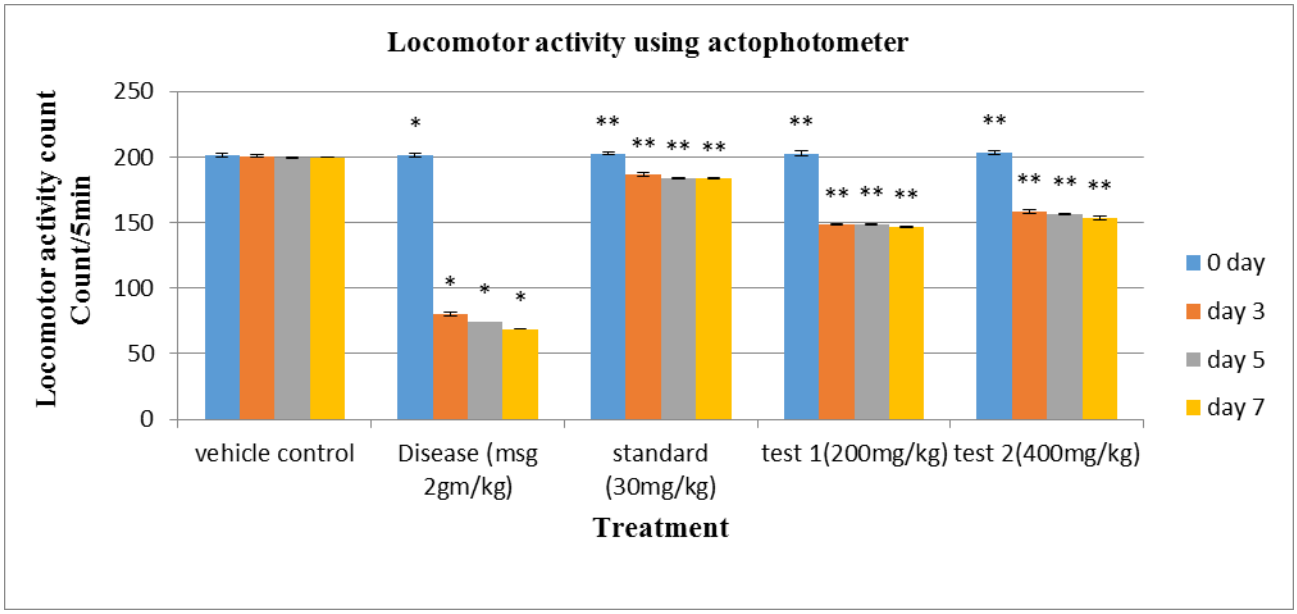

Figure No. 1.7: Locomotor activity using actophotometer

The locomotion of the animals belonging to the Monosodium glutamate treated group was decreased significantly when compared to the vehicle treated group. Standard group (Dextromethorphan 30mg/kg + Monosodium glutamate 2 gm/kg) and Test group IE (200mg/kg) and 400mg/kg showed a significant difference when compared to Disease control group (Monosodium glutamate 2 gm/kg). From above result it is seen that Test drug IE 400mg/kg increases locomotion in Parkinson disease induced rats as compared to Disease control Monosodium glutamate group.

Catalepsy by block test:

Table No. 1.12: Catalepsy by block test

Day	Vehicle control	Monosodium glutamate (Disease)	Standard drug (Dextromethorphan)	IE 200mg/kg	IE 400mg/kg
Day 0	0	0	0	0	0
Day 3	0	1.25±0.11*	0.33±0.10**	0.41±0.08**	0.33±0.10**
Day 5	0	1.33±0.10*	0.33±0.10**	0.5±0.0**	0.33±0.10**
Day 7	0	1.33±0.10*	0.33±0.10**	0.41±0.08**	0.33±0.10**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * $p \leq 0.05$ when compared with vehicle control group, ** $p \leq 0.05$ when compared with disease group.

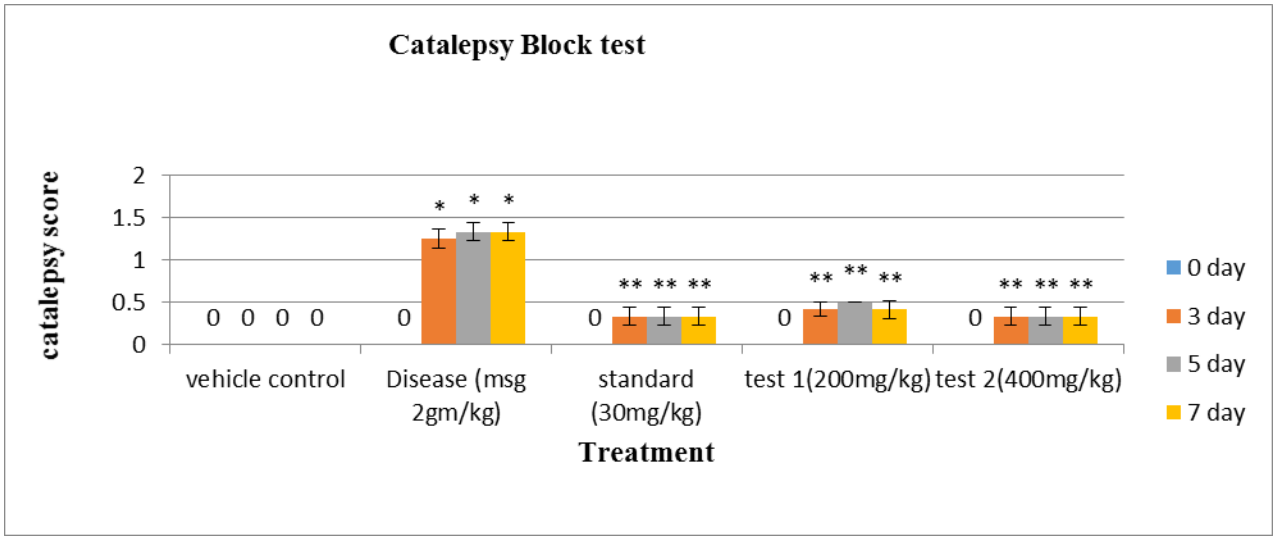

Figure No. 1.8: Catalepsy Block test

Catalepsy score of 3.5 shows maximum catalepsy. The vehicle control group showed no catalepsy. The IE (200 mg/kg and 400mg/kg) group showed significant decrease in the catalepsy score when compared with the disease control group. Also, the standard Dextromethorphan 30mg/kg + Monosodium glutamate 2 gm/kg showed significant decrease in the catalepsy score when compared with the disease control group. From above result it is seen that Test drug IE 400 mg/kg decreases catalepsy in Parkinson disease induced rats as compared to Disease control Monosodium glutamate group.

Biochemical estimations:

Table No. 1.13: Biochemical estimations

Groups	Lipid peroxidation level	Nitrite
Vehicle control	0.77±0.22	91.96±1.19
Disease (Monosodium glutamate)	0.77±0.22*	180.95±1.37*
Standard group (Dextromethorphan+ msg)	2.91±0.14**	116.18±1.7**
Test 1 (200mg/kg)	3.81±0.41**	150.95±3.42**
Test 2 (400mg/kg)	2.80±0.15**	138.76±1.50**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * $p \leq 0.05$ when compared with vehicle control group, ** $p \leq 0.05$ when compared with disease group.

Lipid peroxidation

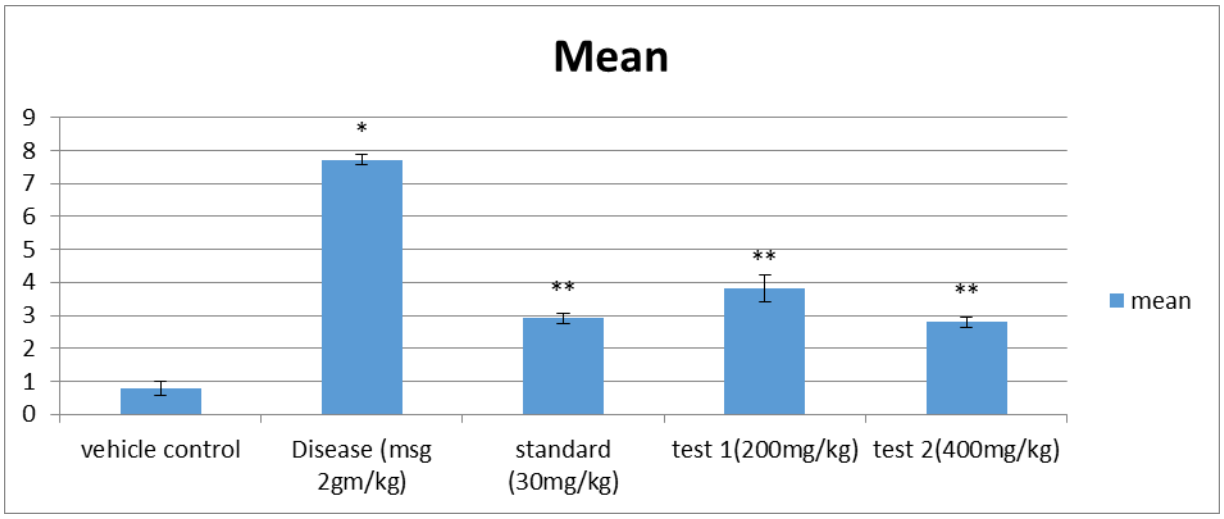

Figure No. 1.9: Lipid peroxidation estimation

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

Nitrite level

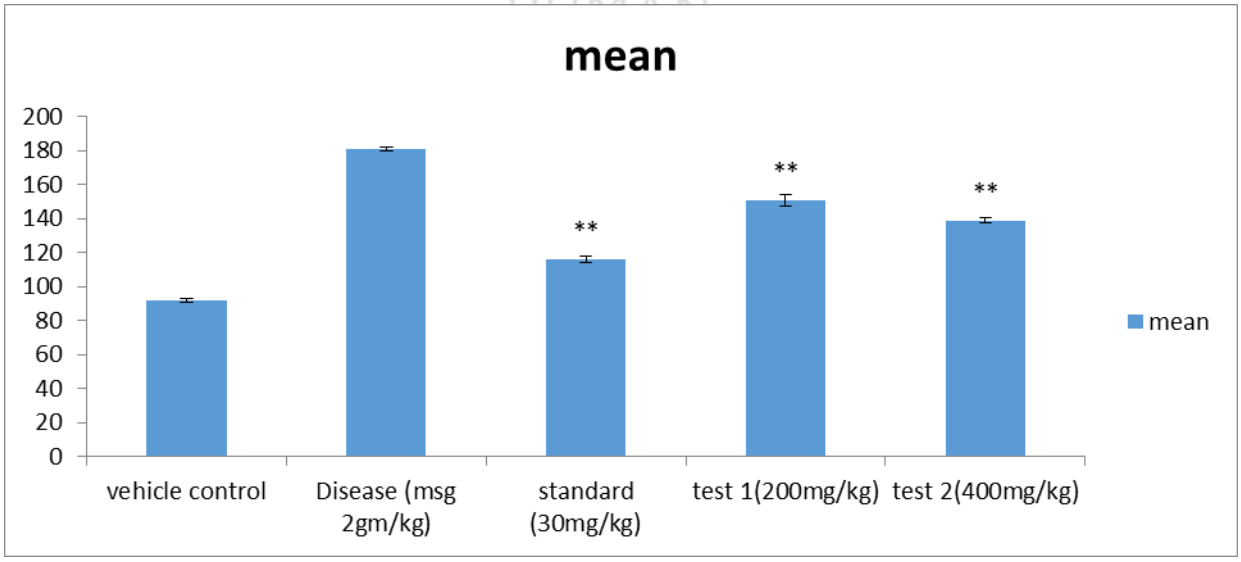

Figure No. 1.10: Nitrite level estimation

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

Haloperidol induced catalepsy:

Parameters to be studied are as follows:

- Behavioral changes:
  - Locomotor activity by actophotometer
  - Muscle rigidity by rotarod
  - Catalepsy by block test
- Biochemical parameters
  - Lipid peroxidation

Muscle rigidity by Rotarod:

Table No. 1.14: Muscle rigidity by Rotarod

Day	Vehicle control	Disease Haloperidol	Standard drug (Trihexphenidyl HCl)	Test 1 IE 200mg/kg	Test 2 IE 400mg/kg
Day 4	92±0.68	68±1.2*	85.83 ± 0.70**	67.83±1.42**	78.16±0.70**
Day 8	95.66±0.88	53.16±0.94*	71.16 ± 1.19**	63.83±1.93**	69.66±0.760**
Day 12	94.66±0.98	17.5±1.60*	70.66 ±1.08**	63.66±1.20**	68.16±1.07**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

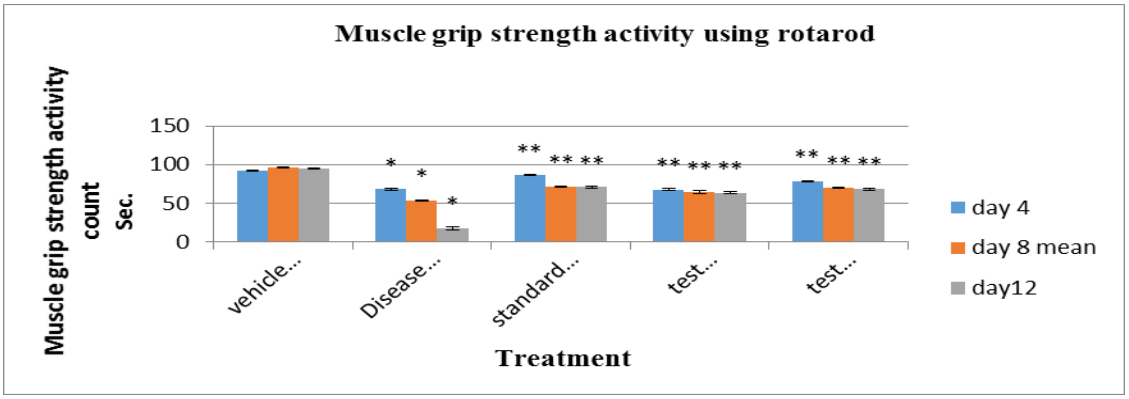

Figure No. 1.11: Muscle grip strength activity using rotarod

Vehicle control group showed significant differences in muscle rigidity as compared to disease control group. Standard group (Trihexyphenidyl HCl 15mg/kg + Haloperidol 1mg/kg) and Test group IE (200mg/kg) and IE 400mg/kg showed a significant difference when compared to Disease control group (Haloperidol 1mg/kg). From above result it is seen that test drug IE 200mg/kg and 400mg/kg decreases muscle rigidity in Parkinson disease induced rats as compared to Disease control Haloperidol group.

Locomotor activity by actophotometer:

Table No. 1.15: Locomotor activity by actophotometer

Day	Vehicle control	Haloperidol (disease)	Standard drug (Trihexphenidyl HCl)	IE 200mg/kg	IE 400mg/kg
Day 4	103.16±2.0	73±2.87*	88±0.85**	70.83±0.60**	80.66±0.91**
Day 8	104.5±1.87	56.33±1.08*	87.66±0.66**	68.5±0.42**	82.5±1.0**
Day 12	104.5±0.76	23.83±1.35*	82.83±0.83**	70.33±0.95**	81±0.51**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

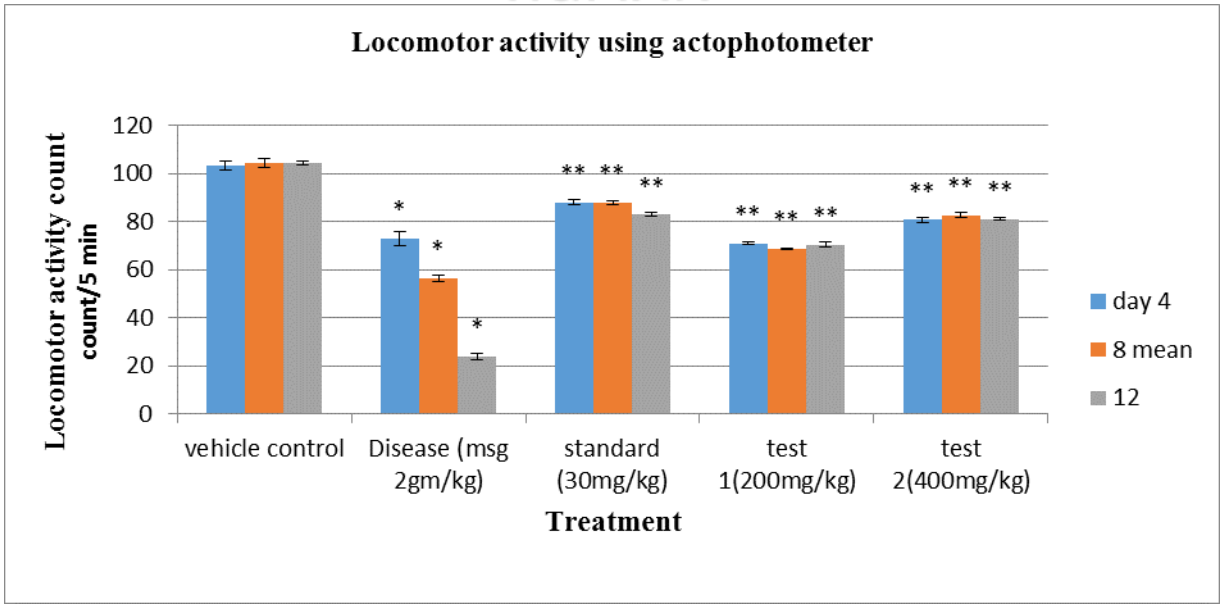

Figure No. 1.12: Locomotor activity using actophotometer

The locomotion of the animals belonging to the Monosodium glutamate treated group was decreased significantly when compared to the vehicle treated group. Standard group (Trihexyphenidyl HCl 15mg/kg + Haloperidol 1mg/kg) and Test group IE (200mg/kg) and 400mg/kg showed a significant difference when compared to Disease control group (Haloperidol 1mg/kg). From above result it is seen that Test drug IE 400mg/kg increases locomotion in Parkinson disease induced rats as compared to Disease control Haloperidol group.

Catalepsy by Block test:

Table No. 1.16: Catalepsy by Block test

Day	Vehicle control	Disease Haloperidol	Standard drug (Trihexphenidyl HCl)	Test 1 IE 200mg/kg	Test 2 IE 400mg/kg
Day 4	0	1.25±0.11*	0.33±0.10**	0.41±0.08**	0.33±0.10**
Day 8	0	1.33±0.10*	0.33±0.10**	0.5±0.0**	0.33±0.10**
Day 12	0	1.33±0.10*	0.33±0.10**	0.41±0.08**	0.33±0.10**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

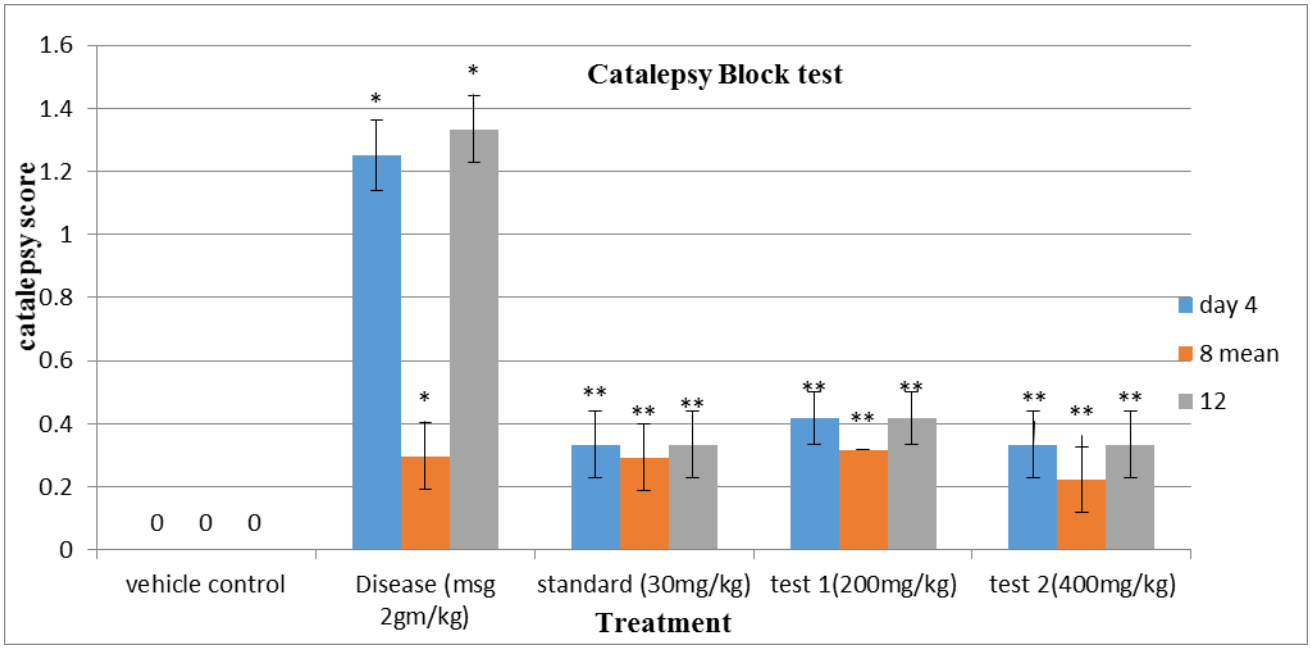

Figure No. 1.13: Catalepsy Block test

Catalepsy score of 3.5 shows maximum catalepsy. The vehicle control group showed no catalepsy. The IE (200 mg/kg and 400mg/kg) group showed significant decrease in the catalepsy score when compared with the disease control group. Also, the standard (Trihexyphenidyl HCl 15mg/kg + Haloperidol 1mg/kg) showed significant decrease in the catalepsy score when compared with the disease control group. From above result it is seen that Test drug IE 400 mg/kg decreases catalepsy in Parkinson disease induced rats as compared to Disease control Haloperidol group.

Biochemical estimations:

Table No. 1.17: Biochemical estimations

Groups	Lipid peroxidation level
Vehicle control	0.71±0.16
Disease (haloperidol)	7.15±0.29*
Standard group (Trihexphenidyl HCl+ Haloperidol)	2.09±0.29**
Test 1 (200mg/kg)	4.02±0.31**
Test 2 (400mg/kg)	2.80±0.22**

Values are expressed as Mean ± sem for 6 rats in each group. Significance was determined by One way ANOVA followed by Dunnett's multiple comparison test's * p≤0.05 when compared with vehicle control group, ** p≤0.05 when compared with disease group.

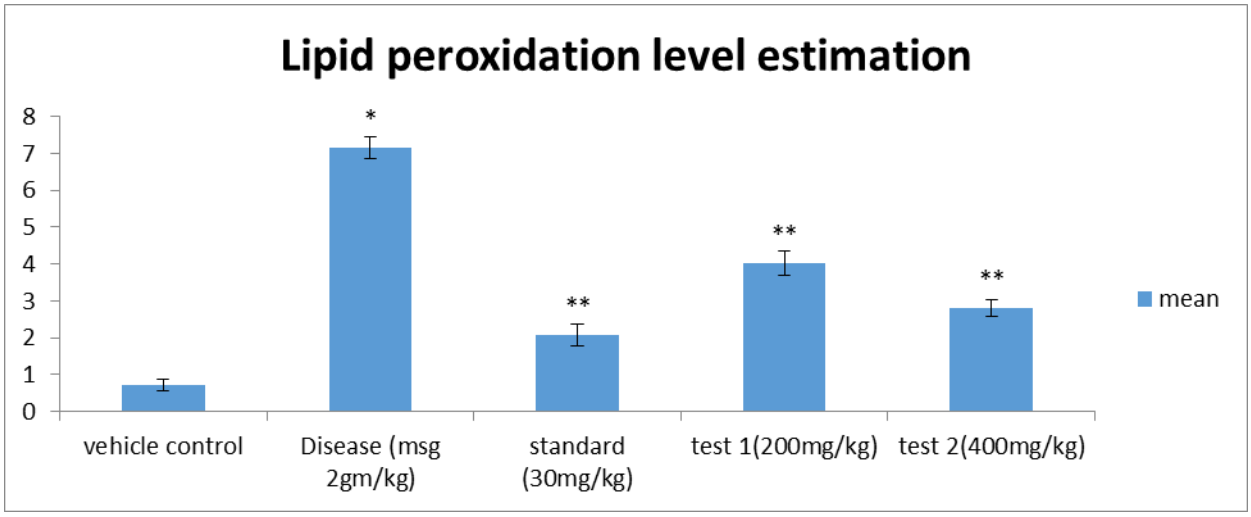

Figure No. 1.14: Lipid peroxidation level estimation

DISCUSSION

In this study monosodium glutamate induced excitotoxicity was used to study behavioral changes in rats accompanied by assessment of biochemical parameters, such as lipid peroxidation, nitrite level. The study further involved the assessment of the activity of the isolated embelin against catalepsy induced by haloperidol which was evaluated by the behavioural and biochemical parameters.

Acute toxicity of IE did not show any toxic or deleterious effects up to 2000 mg/kg oral dose indicating low toxicity of IE at high doses. Thereby suggesting non-loxic nature of IE. In the present study, the *in vitro* antioxidant potential of Embelin isolated from dried fruits of *Embelia ribes burm.* was evaluated with the help of DPPH free radical scavenging activity. The free radical scavenging activity of the IE was evaluated based on their abılity to scavenge the synthetic DPPH The bleaching of DPPH absorption is a representative of the capacity of the test drugs to scavenge free radicals independently. IE showed free radical scavenging activity against the stable DPPH free radical by binding with it and thereby showing reduction in absorbance in concentration dependent manner antioxidants play an important role in scavenging free radicals. The presence of quinine compound in isolation as seen in preliminary phytochemical analysis might have been responsible for the antioxidant effect.

Metal chelation activity is the basis to prove the neuroprotective potential of the plant. The IE showed considerable inhibition of the metal complex. The IE showed increase in the metal complex inhibition with increase in the concentration.

In the present study, we have focused upon exploring the potential of IE (200mg/kg & 400mg/kg) for the anti-Parkinsonian activity. Parkinson like effects was induced in rats with the help of Monosodium glutamate (2gm/kg) & Haloperidol (1mg/kg). Dextromethorphan and trihexyphenidyl HCl are the established neuroprotective agent was used as a standard in the present study.

Overactivation of NMDA receptors by the MSG or sodium salt of glutamate exerts excitotoxicity. Overactivation of NMDA along with other glutmate/glycine receptors disturb the calcium homeostasis, which is the key mediator of glutamate-induced excitotoxic neuronal damage. Dextromethorphan, a synthetic opioid agonist functions additionally as an NMDAR antagonist. Hence in the present study, we have used dextromethorphan against MSG-induced neurotoxicity. The accumulation of high intracellular calcium together with

increased level of sodium and decreased level of potassium intracellularly triggers a cascade of membrane, cytoplasmic and nuclear events leading to mitochondrial dysfunction and free radical generation resulting in neurotoxicity

In the present study, MSG resulted in behavioral alterations like decreased locomotor activity, loss of muscle strength and catalepsy. The administration of MSG resulted in aggressiveness, diarrhea and hyper activeness in all animals.

The MSG significantly decreased locomotor activity compared with normal animals. Treatment with IE at the dose of 200 and 400 mg/kg as well as standard drug, dextromethorphan significantly reversed the MSG-induced decrease in locomotor activity score. The MSG is known to impair locomotor activity by causing damage to the dopaminergic neurons by generating free radicals. Dextromethorphan and IE might be overcoming the deleterious effect of MSG by protecting the damage by free radicals. This is evidenced in the antioxidant effects of the treatment.

Treatment with MSG significantly decreased the time to fall from the rotating rod compared with normal group. This effect might be due to over activation of glutmate pathway leading to neurotoxocity. Treatment with IE at both doses as well as standard drug dextromethorphan significantly increased the time to fall from the rotating rod compared with MSG treated group. Thus, the reversal of MSG-induced loss of muscle grip activity with the treatment of IE may be attributed to its antioxidant activity, which provides protection against neurotoxicity. Treatment with MSG also shows cataleptic signs compared with normal vehicle group. The decrease in the level of dopamine is responsible for catalepsy. Treatment with IE at both doses as well as standard drug dextromethorphan significantly reversed the catalepsy when compared with MSG treated group.

Treatment with IE at both doses as well as standard drug dextromethorphan significantly reduced the levels of lipid peroxidation and nitrite levels.

Typical neuroleptic agents like haloperidol induce a cataleptic state in rodents. Neuroleptic induced catalepsy has been linked to a blockade of postsynaptic striatal dopamine D1 and D2 receptors. In the present study, we have focused upon exploring the potential of Isolated Embelin (200mg/kg & 400mg/kg) for the anti-Parkinsonian activity. Parkinson like effect was induced in mice Haloperidol 1mg/kg in Haloperidol induced catalepsy. Trihexyphenidyl hydrochloride, the establish anti-cholinergic agent was used as a standard in the present study

as a standard in the present study. In the present study involving mice, in haloperidol treatment group decrease in the level of Dopamine than vehicle control was observed. This decrease in the level of dopamine is responsible for catalepsy and other motor defects. IE treated group restored the dopamine level dose dependently in haloperidol. Dopamine deficiency in the brain is the major biochemical deficit in PD. Chronic administration of haloperidol for a period of 12 days in mice resulted in decrease in Rotarod task also there are decrease counts in Actophotometer activity along with cataleptic signs when Haloperidol treated group as compared to IE (200mg/kg & 400mg/kg) treated group. IE dose dependently increased the muscle grip strength, locomotor activity as well as cataleptic signs. When compared with haloperidol treated group lipid peroxidation level increased while treatment with IE at both the doses along with trihexyphenidyl HCL showed significant decreased in the level of lipid peroxidation.

CONCLUSION

Parkinson's disease belongs to group of conditions called motor system disorders neurodegenerative disorders which are the result of the loss of dopamine producing brain cells. The four primary symptoms of PD are tremor, or trembling in hands, arms, legs jaw, and face; rigidity or stiffness of the limbs and trunks Bradykinesia or slowness of movement and postural instability or impaired balance and coordination. This disease is characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta and a gradual accumulation of Lewy bodies which are deposits of specific cytoplasmic proteins such as ubithiquitin, ct-synuclein, and oxidized neurofilaments.

The present study provides sufficient evidence that IE; an antioxidant natural origin medicinal plant has an effective potential activity in the treatment of PD drug due to its neuroprotective effect. Treatment with IE exhibited a protective effect against DPPH free radical scavenging activity by *in vitro* antioxidant enzyme DPPH assay.

The neuroprotective property of IE further confirmed by the marked metal chelating activity. IE was effective in normalizing monosodium glutamate and haloperidol induced behavioral alterations (decreased locomotor activity and muscle rigidity) and decrease in brain antioxidant enzyme levels (LPO, NO) IE exhibited neuroprotective activity against Parkinson's disease. *In vitro* and *In vivo* models involving rats and mice models.

It can be concluded that IE exerts neuroprotective property, through its potent antioxidant property or NMDA receptor antagonizing property.

Further studies are required to get a proper mechanism and molecular pathways for the potential use of IE as a neuroprotective in Parkinson's disease.

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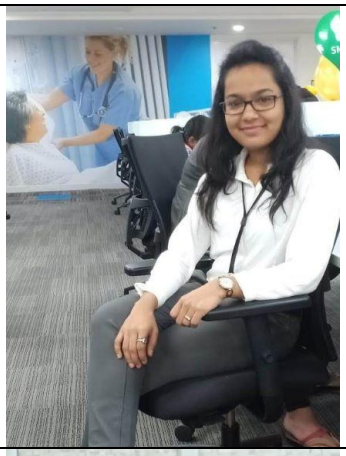	***Author Name – Darshana A.Patil**** *M.Pharm (Pharmacology)* *Dr. L. H. Hiranandani College of pharmacy, Ulhasnagar-03*
	***Author Name-Rachana D.Sarawade*** *Assistant Professor/H.O.D Pharmacology,* *Dr. L. H. Hiranandani College of pharmacy, Ulhasnagar-03*
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