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Anti-Parkinson's activity and *in vitro* antioxidant activity of *Origanum majorana* plant extract

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ABSTRACT: Neurodegenerative diseases are increasing worldwide, and new drugs to treat them effectively with fewer side effects are in high demand today. Treatments available for these diseases are associated with significant side effects in the long term. Therefore, finding a therapy that inhibits disease progression with fewer side effects is crucial. *Origanum majorana* commonly called sweet marjoram was reported to have a wide range of health benefits particularly in treating neurological diseases. In this context, the leaf extract of *Origanum majorana* was evaluated for its antioxidant, antiparkinson's and neuroprotective properties. The antioxidant property was assessed by two in-vitro methods, nitric oxide radical scavenging assay and phosphomolybdenum assay. The antiparkinson's property was assessed in haloperidol-induced Parkinson's disease in mice using the catalepsy bar test and rotarod test. The results of this study show that *Origanum Majorana* has a powerful antioxidant property that can protect neurons from haloperidol-induced cognitive and motor impairments by reducing oxidation.

KEYWORDS: Neurodegenerative diseases; *Origanum majorana;* Parkinson's disease; antioxidant and neuroprotective properties.

1. INTRODUCTION

The emergence of neurodegenerative diseases is one of the most significant trends today. Approximately 1 billion people throughout the world suffer from neurological disorders, such as Alzheimer's, Parkinson's, epilepsy, and so on.

Parkinson's disease (PD) was first described by James Parkinson in 1817 and is the second commonest neurodegenerative disorder of the elderly. It is a progressive neurodegenerative motor disorder that involves degenerative changes to the dopaminergic neurons in the nigrostriatal cortex (1,2). Worldwide, approximately 1% of people above 65 have PD, and only a small number of people suffer from familial PD or parkinsonism. Males are believed to be more prone to PD than females (3).

In PD, motor skills and cognitive abilities are affected and can only be treated with conventional therapeutic approaches that alleviate symptoms. Levodopa (l-DOPA) is the most effective therapy for PD; however, the disease progresses with significant side effects in the long term, so finding a therapy that inhibits its progression is necessary. A wide range of current investigations are focused on finding novel substances that may provide therapeutic benefits for patients with PD (4).

There is no drug currently available for the treatment of Parkinson's disease that provides permanent benefits to patients. But Nutritive Scientists have found that natural products can reduce the incidence of Parkinson's drugs with a comparatively low rate of adverse events while attenuating the symptoms of the disease (5). Phytochemicals such as flavonoids, phenolic acids, stilbenes, and lignans, along with terpenes, are the most abundant types of phytochemicals with antiparkinsonian effects.

There are several mechanisms through which phytochemicals exert their anti-parkinsonian effect. Among them, widely accepted mechanisms are, suppression of apoptosis (by lowering Bax/Bcl-2 and caspase-3, -8, and -9, as well as the accumulation of α-synuclein), reduction in the loss of dopaminergic neurons, dopamine depletion and a reduction in proinflammatory cytokines (Eg; prostaglandin E2, interleukin-6, etc.,). The anti-parkinsonian effect can also be attributed to the modulation of cellular and nuclear inflammatory signaling elevation of neurotrophic and antioxidant factors. Thus, plant-derived natural products could potentially be used as pharmaceutical drugs or adjuvant treatments to conventional therapies (6).

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Origanum majorana commonly called sweet marjoram is a perennial herbaceous plant of the *Lamiaceae family*. There are numerous health benefits associated with this plant. In traditional medicine practice, it is used to treat migraines and headaches caused by depressive and anxious states (7). In this context, we aimed to evaluate the antioxidant activity, the neuroprotective activity, and the antiparkinson's activity of the leaves extract of *Origanum majorana* in albino mice.

2. RESULTS

2.1. Phytochemical Screening

Based on the preliminary phytochemical analysis of aqueous extract of *Origanum majorana* (AEOM) , various components such as alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, phytosterols, saponins, and terpenoids were identified, while components like tannins were absent. As a result of the extraction, the AEOM showed a percentage of extract yield of 39.2%. (Table 1).

In quantitative determination of total flavonoids was done with standard calibration curve of quercetin was achieved between 0.120 to 0.143 (Fig 1).

Concentration of Quercetin (r^2=0.998)

% of total Flavonoids in AEOM is = 33.22µg/g

In quantitative determination of total phenol was done by Folin Ciocalteu Method with standard calibration curve of gallic acid was achieved between 0.125 to 0.158 (Fig 2).

Concentration for gallic acid (r^2=0.996)

% of total Phenols in AEOM is = 67.3µg/g

Table 1. Preliminary Phytochemical test of aqueous extract of *Origanum majorana* (AEOM)

Phytoconstituent	Test	Observation	Inference
AlkaloidTest	1. Mayer's Test	White or Pale yellow ppt	+
	2. Hager's Test	Yellow ppt	+
	3. Dragendroff'sTest	Orange brown ppt	+
	4. Wagner's Test	Reddish-brown ppt	+
Saponins	Foam Test	Stable foam	+
Terpinoids	Liebermann-BurchardTests	Dark green colored	+
Flavonoids	(i) Alkaline Reagent Test	Formation of intense Yellow color which turns to colorless Addition of few drops of dilute hydrochloric acid	+
	(ii) Ferric Chloride Test	Blackish Red color	+
	(iii) Shinoda Test	Yellow precipitate	+
	(iv) Lead Acetate Test	Formation of YellowPrecipitate	+
Glycosides	(i) Legal's Test	Appearance of pinkto red color	+
	(ii) Baljet Test	Yellow to orange color	+
Carbohydrates	(i)Molish's Test	Formation of Brownviolet ring	+
	(ii)Fehling's Test(A) &(B)	Brick red Precipitate	+

+ indicates presence of particular phytochemical

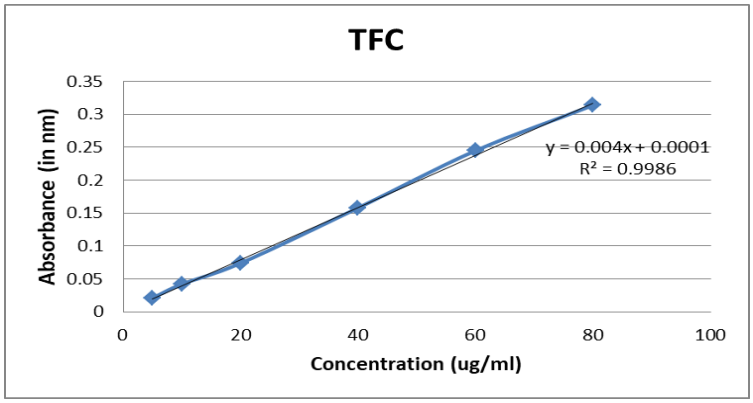

Figure 1. Calibration Curve of Total Flavonoid Content Quercetin (Standard)

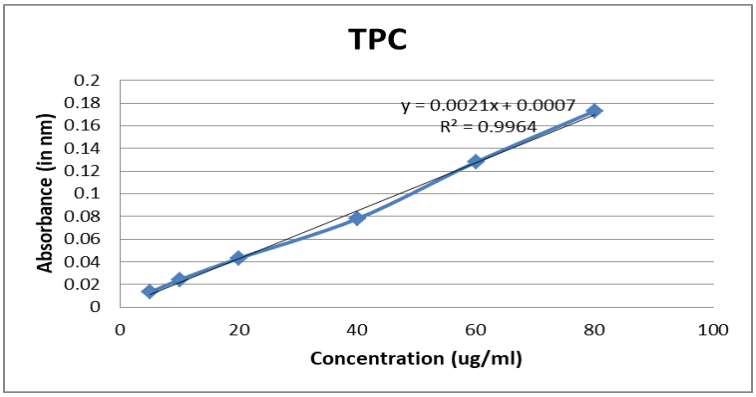

Figure 2. Calibration Curve of Gallic acid (Standard)

2.2. Antioxidant Activity

2.2.1 Nitric Oxide assay

The results of antioxidant activity by nitric oxide assay indicated a significant scavenging capability with an increase in the concentration when compared to the standard drug (Table 2 , Fig 3).

Table 2. Nitric Oxide assay of **AEOM (Aqueous extract of *Origanum majorana*-**test) compared with the standard

Concentration **(µg/Ml)**	Absorbance **(nm)**		% Scavenging	
	Standard	AEOM	Standard	AEOM
50	1.105	1.131	2.98	0.7
100	0.935	1.123	17.91	1.4
200	0.855	1.106	24.93	2.89
400	0.801	0.979	29.67	14.04
600	0.759	0.804	33.36	29.41

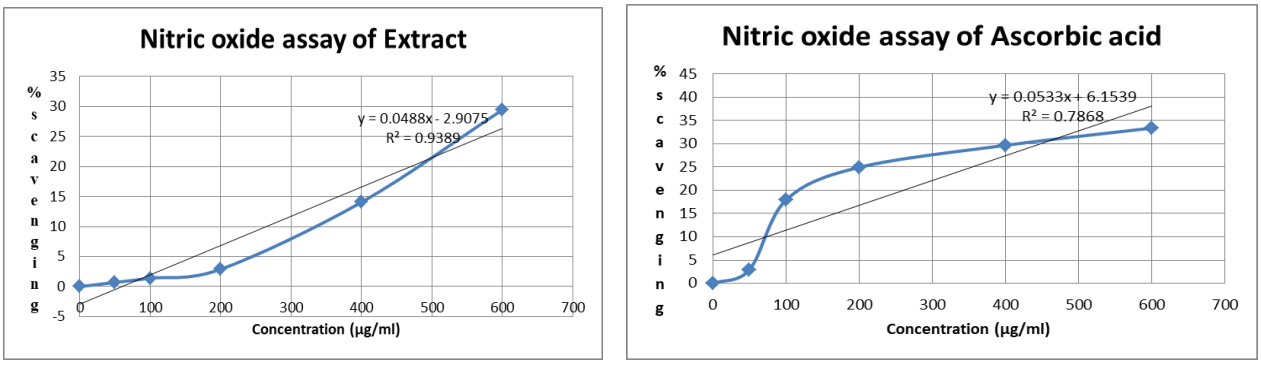

Figure 3. Nitric Oxide assay Standard compared with Aqueous extract of *Origanum majorana*

From figure 4 and Table 3, we know an increase in absorbance with an increase in the concentration of standard Ascorbic acid and AEOM. The increase in absorbance indicates an increase in reducing power both in standard and AEOM.

Table 3: Reducing Power Assay of Ascorbic acid (Standard compared with AMOE)

Concentration	Absorbance (nm)	
	Standard	AMOE
50	0.914	0.88
100	0.801	0.964
200	0.911	1.073
400	1.954	1.079
600	2.145	1.082

AEOM -Aqueous extract of *Origanum majorana*

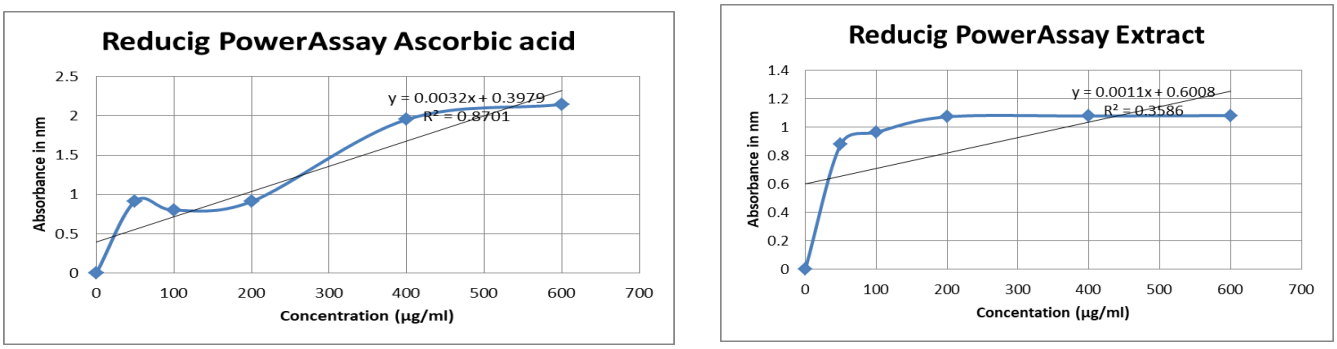

Figure 4. Reducing Power Assay of Ascorbic acid

2.2.2 Phosphomolybdenum Assay

The results of Phosphomolybdenum spectroscopic method for the quantitative determination of antioxidant capacity was depicted in Table 4 and figure 5.

Table 4. Phosphomolybdenum Assay of Ascorbic acid (Standard compared with AMOE)

Concentration	Absorbance (nm)	
	Standard	**AMOE**
50	0.163	0.028
100	0.517	0.141
200	0.641	0.221
400	0.652	0.306
600	0.627	0.295

AEOM -Aqueous extract of *Origanum majorana*

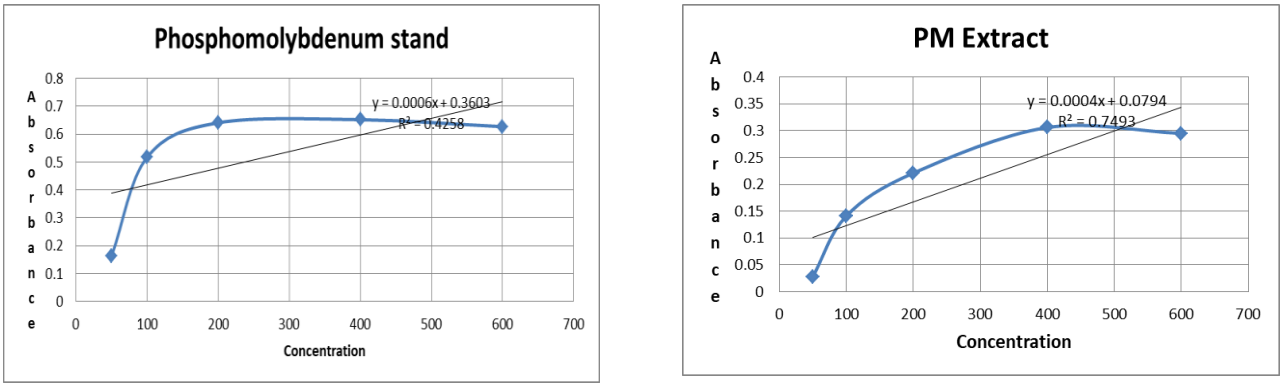

Figure 5. Phosphomolybdenum Assay of Ascorbic acid

2.3. Acute Toxicity Study of AEOM

AEOM was tested for acute toxicity in female mice according to the OECD guideline number 423. There were no significant effects on body weight development. Due to the lack of mortality, we were unable to estimate the lethal dose (LD50) of marjoram extract.

2.4. Anti-Parkinson's activity of AEOM

2.4.1 Haloperidol induced Parkinson's Disease (Cataleptic Study)

In Catalepsy test, the group which received only Haloperidol (Group II), significantly, progressive increased catalepsy (P<0.001) on 1st, 4th and 7th day, compared to the Normal group (Group I). In standard treated group (Group III), a significant decrease in catalepsy (P<0.001) was seen compared to the Haloperidol treated group (Group II). In extract treated group (Group IV) and (Group V) a significant decrease in catalepsy (P<0.001) was seen on compared to the Haloperidol treated group (Group II) (Table 5).

Figure 6 represents that the AEOM (400mg/kg) significantly decreased the cataleptic effect and better anticatalepsy effect as compared to other groups. However, the standard group with Benserazide (10mg/kg) is better anticatalepsy effect.

Table 5: Effect of Aqueous extract of *Origanum majorana* Catalepsy Bar test in Mice

Groups	Catalepsy (Sec) (Mean ± SD)			
	DAY = 1st	**DAY= 4th**	**DAY= 7th**	**P-Value**
Group 1	7.933 ± 0.704	6.833 ± 0.602	6.167± 1.137	>0.001
Group 2	133.5 ± 11.99*	143.6± 14.55*	155.3± 17.88*	<0.001
Group 3	65.16± 5.426***	63.16± 5.193***	62.83± 5.004***	<0.001
Group 4	82.33± 5.538**	80.66± 5.513**	79.83± 5.587**	<0.001
Group 5	73.83± 4.098**	71.66± 4.015**	69.33± 3.920**	<0.001

Group 1 (Normal Saline 2ml/kg); Group 2 (Haloperidol 2ml/kg); Group 3 (Benserazide 10mg/kg); Group 4 (AEOM 200mg/kg); Group 5 (AEOM 400mg/kg) ; SD- Standard deviation

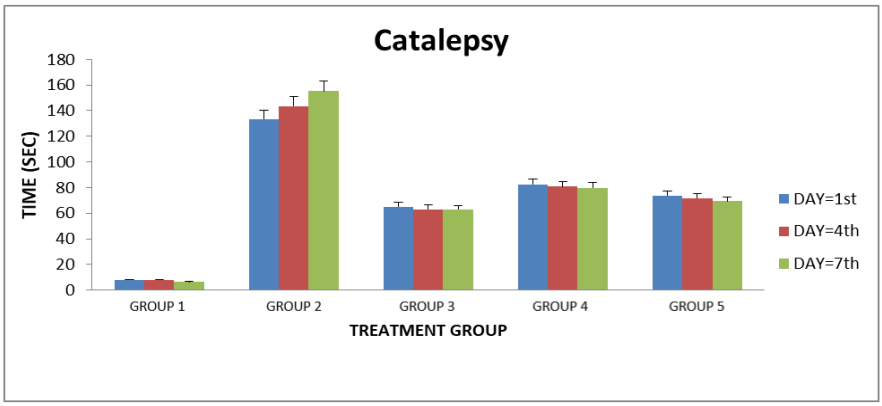

Figure 6. Effect of Aqueous extract of *Origanum majorana* on Catalepsy in Mice

2.4.2 Haloperidol induced Parkinson'sDisease used in Rota Rod Apparatus

In Rotarod test, the group which received only Haloperidol, significantly, decreases fall off time (P<0.001) compared to the Normal group (Group I). In standard treated group (Group III) a significant increase in fall off time was recorded, while in extract treated group (Group IV), is less significant increases in fall off time compared tothe Haloperidol treated group. Whereas no significant difference in fall off time was seen when 400mg/kg treated group (Group V) and standard treated group (Group III) (Table 6).

Figure 7 represents the difference in muscle strengths exhibited in all five groups. The standard group with Benserazide (10mg/kg) exhibited better muscle strength after Haloperidol administration followed test group administered with 400 mg AMOE.

Table 6: Effect of Aqueous extract of *Origanum majorana* on Rota Rod Test in Mice

Groups	**Rotarod Test (Sec) (Mean ± SD)**			
	DAY = 1st	**DAY= 4th**	**DAY= 7th**	**P Value**
Group 1	70.667 ± 3.613	71.500 ± 2.377	70.000 ± 2.520	>0.001
Group 2	25.833± 2.623*	24.833 ± 2.432*	21.833 ± 2.276*	<0.001
Group 3	51.000± 3.011***	53.667±2.826***	55.833 ±1.880***	<0.001
Group 4	27.000 ± 3.120**	31.500 ± 2.430**	33.167 ± 1.732**	>0.001
Group 5	41.167± 1.286**	43.500 ± 1.896**	45.333 ± 2.311**	>0.001

Group 1 (Normal Saline 2ml/kg); Group 2 (Haloperidol 2ml/kg); Group 3 (Benserazide 10 mg/kg); Group 4 (AEOM 200 mg/kg); Group 5 (AEOM 400 mg/kg); SD- Standard deviation.

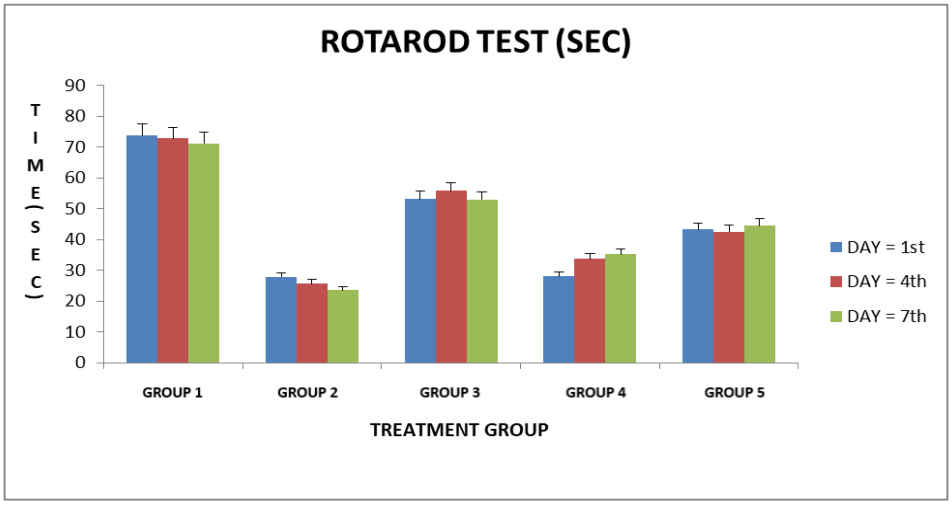

Figure 7. Effect of AEOM on Rotarod Test in Mice.

2.5. Evaluation of Neuroprotective activity of LPS induced NeuroinflammationDisease used in Rota Rod Apparatus

In Neuroprotective activity of LPS induced Neuroinflammation study observed in Rotarod test, the group which received only LPS (Group II), significantly, decreases fall off time (P<0.001). In standard treated group (Group III), extract treated group (Group IV), a significant increase in fall off time was observed compared to the LPS treated group (Group II) (Table 7, Fig 8)

Table 7: Neuroprotective activity of Aqueous extract of *Origanum majorana* in LPS induced Neuroinflammation Disease used in Rota Rod Apparatus

Groups	Rotarod Test (Sec) (Mean ± SD)			
	DAY = 1st	DAY= 4th	DAY= 7th	P Value
Group 1	73.777± 1.633	72.8± 3.479	71.234± 2.520	>0.001
Group 2	27.843± 2.742*	25.733± 3.635*	23.632± 3.568*	<0.001
Group 3	53.112±2.032***	55.767±4.928***	52.92±2.783***	>0.001
Group 4	28.098± 4.240**	33.666± 2.525**	35.268± 2.656**	>0.001
Group 5	43.277± 2.366**	42.476± 3.466**	44.432± 2.311**	>0.001

Group 1 (Normal Saline 2ml/kg); Group 2 (Haloperidol 2ml/kg); Group 3 (Benserazide 10 mg/kg); Group 4 (AEOM 200 mg/kg); Group 5 (AEOM 400 mg/kg); SD- Standard deviation

3. DISCUSSION

In this study, the in vitro and in vivo antioxidant and neuroprotective effects of *Origanum majorana* on haloperidol animal models were investigated. The antioxidant property evaluated by nitric oxide radical scavenging and phosphomolybdenum assay showed that AEOM has potent antioxidant property. The antioxidants contained in the plant exhibited radical scavenging and inhibiting properties. A number of previous studies conducted with the methanolic extract and essential oil of *Origanum majorana* showed significant antioxidant potential. They reported a decrease in oxidative stress markers such as MDA and FRAP and an increase in brain-derived neurotrophic factor (BDNF) expression in various in vivo mouse models (13-14). This activity was attributed to the phenolic compounds in the plant extract. Further research needs to be conducted to further purify the phenolics from AEOM (15).

AEOM was evaluated for its acute toxicity in Swiss/Albino mice to determine the lethal dose (LD50). However, this was not possible. In this study, the lethal dose (LD50) of marjoram extract could not be estimated because no mortality was observed after administration of the crude extract. This was also the case in the study by Amaghnouje A. et al (citation needed). In all experimental animals, the mice gained weight. They did not show significant behavioural changes, indicating that administration of the crude

extract has a negligible level of toxicity on the growth of the animals. Therefore, 1/10th dose (200mg/kg) and 1/5th dose (400mg/kg) were selected for further study (16).

Motor symptoms begin when the brain loses 80% of the dopamine. As the PD progresses, specific brain regions of the brain, like the striatum, selectively lose their dopaminergic cells. The loss of DA-producing neurons in the substantia nigra is thought to result from oxidative stress (17). Haloperidol produced Parkinson like syndromes and extrapyramidal symptoms in psychiatric patients (18). Haloperidol-induced catalepsy model of neuroleptic-induced Parkinsonism was used in the present study to assess the nigrostriatal function in rodents. Haloperidol being a dopaminergic antagonist, induces cataleptic immobility in the mice (19).

In the present study, two behavioral parameters were used to evaluate haloperidol-induced Parkinson's disease in mice: the catalepsy bar test and the rotarod test. In the catalepsy bar test, the group receiving haloperidol alone showed a significant increase in catalepsy. On the other hand, a significant difference in catalepsy was observed in the group treated with 400 mg/kg AMOE compared with the standard group. The present study shows that AMOE has a significant protective effect against haloperidol-induced catalepsy. The results of rotarod test measuring motor coordination showed that AEOM (400mg/kg) significantly increased muscle strength. A similar study reported that the loss of muscle contraction under haloperidol was improved by vinpocetine (20).

A major cause of neurodegeneration in PD is oxidative stress in substantia nigra pars compact dopaminergic neurons. The role of reactive oxygen species (ROS) in neurodegenerative disorders and ischemic injury has been suggested, and their neutralization by antioxidants may delay or minimize neurodegeneration.

4. CONCLUSION

The results of this study show that *Origanum majorana* possesses a potent antioxidant property that can protect neurons from haloperidol-induced cognitive and motor impairment by reducing oxidation and nitrosation. Therefore, it may be a promising tool for the treatment of Parkinson's disease. According to the study, well-designed clinical trials are essential to determine whether phytochemicals are helpful in treating neurodegenerative diseases.

5. MATERIALS AND METHODS

5.1. Experimental design

5.2. Preparation of Plant extract:

The Whole Plants of *Origanum majorana* were collected from the forest region of Bobbili, vizianagaram (Dt) Andhra Pradesh, India, were identified and authenticated by Dr. Madhava Chetty, Department of Botany, S.V. University, Chittoor Dist. Tirupati.

Origanum majorana leaves were separated, dried under shade for 45-50 days and further processed to reduce the size. An electrical grinder was used to grind *Origanum majorana's* leaves into powder. To macerate the powder, added 500ml distilled water to 5.0gm of powder. Three days later, the solid residue was removed by filtration using a muslin cloth, and the filtrate was collected. After the extraction to calculate the % of yield:

$$\% \text{ of extract yield} = \frac{\text{Weight of dried extract}}{\text{weight of dried leaves powder}} \times 100$$

5.2.1 Experimental animals

A female Swiss albino mouse (7-8 weeks old, 20-30 grams) was obtained from Saha Enterprises in Hyderabad. The animals were kept in polypropylene cages at a temperature of 25 ± 200c, RH (50 ± 5%) and 12h light and dark cycles. The laboratory animals were provided food, water, and libitum as standard laboratory animal care. Before the test, the animals were acclimatized to laboratory conditions.

5.2.2 Ethical Approval

The Institutional Animal Ethical Committee (IAEC) approved the experiments on animals. An experimental protocol (Approval Number 23) has been approved by the Institutional Animal Ethics Committee (Regd. No IAEC/ SIMS/ 2021/10) to be conducted at PG Department of Pharmacology, SIMS college of Pharmacy.

5.3. Preliminary phytochemical screening of aqueous extract of *Origanum majorana* (AEOM)

The dry extract of AEOM was screened for the presence of phytochemical components such as alkaloids, saponins, flavonoids, glycosides, and total phenolic content using standard screening procedures **(**8).

5.4. In-vitro Evaluation of Antioxidant Activity

5.4.1 Nitric oxide Radical Scavenging Assay

Materials: UV-Vis Spectrophotometer (SHIMADZU 4800-Japan)

Method: AEOM at varying concentrations were diluted in 2.5 ml test tubes. 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml of potassium ferricyanide were mixed with the above solution. After incubation for 20 minutes at 50°C, the mixture was cooled to room temperature. Then 2.5 ml of 10% trichloroacetic acid was added and centrifuged for 10 minutes at 3000 RPM. 2.5ml of supernatant was combined with 2.5ml of distilled water or deionized water, and 0.5ml of ferric chloride should be added. The absorbance at 700 nm was measured against a blank after incubation at room temperature for 10 minutes by using UV-Vis Spectrophotometer. The higher the absorbance, the greater the reducing power.

5.4.2 Phosphomolybdenum assay

The antioxidant analysis by phosphomolybdenum assay was conducted by measuring the absorption of compounds by using UV-Vis Spectrophotometer. 2.5 ml of plant extracts at varying concentrations were taken into a test tube. In 3mL of reagent solution, H2SO4, sodium phosphate, and ammonium molybdate are combined. After that, the tubes are incubated in a thermal block for 90 minutes at 95°C. All tubes were measured against a blank in the aqueous solution at 695nm after cooling at 28°C. Standard and test samples were compared (10).

5.5 Maximum Tolerated Dose/ Acute Toxicity Study

The powder of AEOM was dissolved in distilled water and administered orally to the animals by oral gavage. For the acute toxicity test, the OECD guidelines 423 for chemical testing were used. On the day before the drug was administered, the mice were fasted overnight and were allowed free access to water. AEOM in saline water was administered to each animal in single doses of 5 mg/kg, 50 mg/kg, 300 mg/kg, and 2000 mg/kg, used as oral gavage. The animals were observed for 30 minutes, then for the first 24 hours, with special attention being given to the first 4 hours and daily thereafter for a total of 14 days. Toxicological changes were observed daily in the animals' skins, furs, eyes, mucous membranes, respiratory, circulatory, autonomic, and central nervous systems, as well as their behavioral patterns.

5.6 Anti-Parkinson's Activity

Study by using haloperidol induced parkinson's animal

The mice were divided into five groups of six, each containing 20-30 g Swiss Albino mice. The animals were deprived of food, but not water for four hours before the experiment.

- Group -1 received normal saline (2ml/kg) (i.p) while served as control.
- Group-2 received Haloperidol (2mg/kg) (i.p) which served as standard.
- Group-3 received the standard drug, Benserazide (10 mg/kg, oral, once per day x 1 week), and Haloperidol (2 mg/kg) (i.p) served as a positive control.
- Group-4 received AEOM extract at 200 mg/kg (p.o) doses, which served as test-1.
- Group-5 received AEOM extract at 400 mg/kg (p.o) doses, which served as test-2.

Within 30 minutes of injection, each received a dose of Haloperidol (2 mg/kg i.p.) (standard drug) every day for a week. Animals were observed for the Catalepsy study (muscular rigidity and fixity) and the Rotarod apparatus muscle rigidity study on days 1, 4 and 7. Fig. 1 shows observations after 30 minutes of Haloperidol administration.

5.6.1 Measurement of Catalepsy (11)

Catalepsy is the inability to correct an externally imposed, unusual posture over a prolonged period of time. The cataleptic symptoms of mice are similar to Parkinson's extrapyramidal symptoms. In this procedure, the mouse was measured by how long it was able to maintain a resting position with both front limbs extended, resting on a 4 cm high (1 cm diameter) bar. When an animal held the imposed position for at least 30 seconds, it was considered cataleptic, and the time was recorded. The endpoint of catalepsy was considered when both front paws were removed from the bar or if the animal moved its head in an exploratory manner. A cut-off time of 300 seconds was used. The animals were returned to their individual cages between the determinations. The animals were tested for immobility on the first, fourth, and seventh day after receiving the drugs, and only the animals who remained immobile the longest were considered. The observations were made between 9.00 and 15.00 hrs in a quiet room at 23- 25°C.

5.6.2 Motor Co-ordination Test (Rota Rod Test) (12).

Motor Co-ordination test was conducted using rota rod apparatus. On the day before the first day of testing, the animal was placed on the rotating rod individually and trained for 3 minutes at 25 rpm. The animal naturally tries to stay on the rotating rod to avoid falling to the ground. Three separate trials were conducted by each animal at five-minute intervals with a fixed cut off time of 180s. To alleviate stress and fatigue, a 5-minute rest period was given after each trial. Motor coordination was tested by comparing the latency to fall on the very first trial between treatment groups. The time taken by animals to fall from the rotating rod was noted.

5.7. Neuroprotective Activity Study

LPS Induced Neuroinflammation Animal required: Swiss Albino mice weighing 20-30 g were divided into 5 groups containing 6 mice each. 4 hours before the start of the experiment, food but not water was withdrawn.

- Group -1 received normal saline (2ml/kg) (i.p) while served as control.
- Group-2 received LPS (250µg/kg) (i.p), which was standard.
- Group-3 received Ibuprofen (40mg/kg p.o once per day x 1 week) and LPS (250µg/kg) (i.p) served as positive control.
- Group-4 received AEOM extract at 200mg/kg (p.o) doses, which served as test-1.
- Group-5 received AEOM extract at 400mg/kg (p.o) doses, which served as a test.

The animals were observed for muscle rigidity study for Rotarod apparatus on day-1st, 4th & 7th-day study. For one week, each received a dose of LPS (250*g/kg) (i.p.) after 30min of drug administration. The animals were observed 30 mins after LPS administration. Results of treatment compared with that of control.

5.8. Statistical Analysis Data:

The data of all parameters were analyzed employing one way ANOVA followed by Dunnett's multiple comparison test using SPSS software version 16. The statistical results were expressed as Mean ±SEM. *P<0.05 was considered statistically significant.

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Conflict of interest statement: There are no conflicts of interest.

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