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EXPERIMENTAL VALIDATION OF *PSIDIUM GUAJAVA* AND *MADHUCA INDICA* EXTRACTS AND FRACTIONS FOR PARKINSON'S DISEASE IN MICE

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Abstract
Parkinsonism is a neurodegenerative disease, mainly imbalance in dopamine neurotransmitter in mid brain, which manifestation of dysfunctions of extra pyramidal like akinesia, tremor, rigidity and catalepsy etc., even cognitive and memory loss. The current study is framed to evaluate the effect of *Psidium guajava* and *Madhuca indica* extract and fraction in Rotenone (RTN) induced Parkinson disease (PD) in mice. Further ,in vivo anti-PD activity has been evaluated by Rotarod, actophotometer, and chimney test to assess extrapyramidal, cognitive and memory function. Later, changes in biomarker level such as reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and dopamine level in the whole brain were measured. Finally, histopathology of cerebrum area was examined at 40x magnification to access restoring integrity and maintaining the architecture of neuronal cell in the treatment group compared to control group and L-DOPA as a standard treatment group.
The Rotarod, Actophotometer, and chimney test demonstrated that rotenone group administration declines performance time, decreases locomotion, cognitive and memory respectively. The treatment of *Psidium guajava* and *Madhuca indica* extracts and fractions(200, and 400 mg/kg p.o. and 50, 100mg/kgs significantly ($p < 0.05$ to $p < 0.0001$) reversed whole brain reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and dopamine level altered and significant pathological changes occur. The current study provides validation of *Psidium guajava* and *Madhuca indica* extracts and fractions for its anti-PD activity and could be a valuable source for the treatment of PD in future.

Introduction
Parkinson disease (PD) isa common, slowly progressive, and neurodegenerative disorder resulting from degeneration of dopaminergic neurons in the substantia nigra (SN),are in the region of the brain that controls the movement (Patil et al 2014), It was described by James Parkinsonin1817called as

'Shaking Palsy (Hardman et al 2001)The initial symptoms of PD include tremor at rest, muscular rigidity, bradykinesia, postural abnormalities and instability (DeMaagd and Philip 2015). The clinical manifestation of PD occurs when about 50% of nigral dopaminergic neurons and about 70% of straital dopamine fibres are lost (Yuste et al 2014). It has been widely reported that oxidative stress plays a pivotal role in the neurodegeneration associated with PD (Hwang 2013). The neuronal inflammation induces glial cell activity in SN of the brain which is a well known characteristic of PD pathology (Hardman and Limbird 2001). Oxidative sress is known to damage lipids, proteins and DNA along with decreased superoxide dismutase (SOD), catalase ,and glutathione levels. Oxidative stress and inflammatory pathways ultimately lead to neuronal death. The etiology of PD results from defect in mitochondrial function, dysregulation of brain iron, inflammatory responses and abnormalities of energy metabolism (Uttara et al 2009)

The conventional approach for the treatment, drug discovery and development for PD management is costly, time consuming, beside it a large number of volunteers are required for clinical trial. On other hand traditional medicines holistic approach from natural sources is comparatively easier, less time consuming in comparison to synthesis of drug in laboratory. Curcumin, flavonoid rich products like blueberries, green tea, jasmine tea red wine containing anthocyanin lowers the risk of PD. Phytochemical sources have been popular, particularly because of their remarkable pharmacological effects, low cost and minimal side effects (Fang et al 2014)

Psidium guajava (Myrtaceae) is a small evergreen sub deciduous tree. It is found in tropical and subtropical areas. Guava is rich in verity of phytoconstituents such as tannin, phenols, flavanoids, saponins, carotenoids, vitamins, fiber and fatty acids. Guava fruit is rich in Vitamin C (Nor, and Yatim 2010)

According to the literature there is no scientific evidence for use of *Psidium guajava* in treatment of Parkinson disease. The leaves specially contain polyphenolic compound having ability to reduce pathways involved to generate oxidative stress, that play a key role in pathogenesis of PD(Muhammad, and Abubakar 2009). The leaves contain essential oil with the main components viz *α*-pinene, *β*-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, *β*-bisabolene, caryophyllene oxide, *β*-selinene, cardinene and curcumene .Curcumene has anti-inflammatory effect that reduce neuroinflammation a key factor in pathogenesis of PD(Begum, and Hasan 2003)

Madhuca indica (Mahua) family Sapotaceae used to treat nerve disorder, cough and burning sensation. The bark of trunk contain lupeol acetate, quericitin, dihydroquericitin, beta amyrin acetate, alpha-spinasterol.The bark of drug show DPPH (1,1-diphenyl,2-picryl hydrazyl) free radical scavenging activity, nitric oxide radical scavenging activity, super oxide anion radical scavenging activity, inhibition of hydroxy radical, and lipid peroxidation activity(Mercadante et al 1999). These are oxidative stress marker in PD. In order to justify *In vivo* establishment *In vitro* activity should be performed.

Materials and methods

Chemicals and equipment's

Chemicals: Rotenone (PD [Catalepsy] Inducing agent), Syndopa (Syndopa, 275mg/kg, Standard molecule for PD), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dopamine, DTNB (Sigma USA) and other chemicals utilized in this study are analytical graded. Equipment's: Centrifuge (Remi), Morris Water Maze, Actophotometer, T-Maze, NOR apparatus, Rotarod, Rotary evaporator (Equitron Roteva, Media Instrument Mfg. Co., Mumbai), and many more

Collection, authentication, and preparation of plant material

Air-dried aerial parts of *Psidium guajava* (Myrtaceae) Leaves, *Madhuca indica* (Sapotaceae) Bark were procured from local areas of Haridwar in the month of march and identified by **Dr. Lal Babu Chaudhahary**, senior principal scientist and Professor of AcSIR, division of CSIR Department of Plant diversity, systmatics and herbarium division, **National Botanical Research Institute,**

Lucknow, India and the accession number of *Psiduim guajava* 104704 and *Madhuca indica* was 106870.
Fresh plant material was properly washed with running water to remove dust and then allowed to air dry in the shade at room temperature. The dried plant material was further processed with a grinder into a coarse powder before the extraction procedure started.

Extraction
Successive solvent extraction scheme was used for the preparation of different extracts. The leaves (500 g) were crushed to coarse powder and extracted with Petroleum ether (40-60ºC) using Soxhlet's extractor for 4 days. The extract was filtered through Whatmann filter paper and concentrated with rota-evaporator apparatus at room temperature and transferred to a pre-weighed china dish and dried in a vacuum desiccators until a constant weight of the extract was obtained. The marc obtained was then air-dried and used for further extraction with chloroform followed by ethyl acetate and methanol. After extraction with methanol the dried marc was then utilized for aqueous extraction by the process of cold maceration which was performed by macerating the marc with distilled water in a round bottom flask for 24 hrs. After 24 hrs, it was filtered through Whatmann filter paper and the extract was collected. This process was repeated using marc for another two times and all the three filtrates were collected and combined. The solvent was removed by distillation and concentrated in a rotary evaporator. The dried extract was placed in desiccators and used for further studies.

Experimental animals
To evaluate the anti-PD capacity of *Psedium gvajava* leavesand *Madhuca indica* bark the Adult male mice (40-50gm) were obtained from the Animal house, Animal House of **Acharya Narendra Deo College of Pharmacy, Babhnan (Gonda) Uttar Pradesh** housed in groups of 2-3 animals per cage and maintained at an ambient temperature of 25 ± 1º C and relative humidity of 50 ± 10%, a 12:12 dark: light cycle All the animals were housed in a clean and transparent polypropylene cage. The experiments were carried out during 10.00-1.00 h. Animals had free access to food and water. The overall study protocol was reviewed and approved by the Institutional Animal Ethical Committee, The experiments were conducted according to the guidelines of the Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), Government of India and approved by our Institutional Animal Ethics Committee their registration number is **1585/ PO/RE/ S/11/CPCSEA**, and. application no .is **IAEC/ANDCP/5/2022** The model used for PD The chronic administration of rotenone(3mg/kg i.p) causes symptoms like PD mainly catalepsy. In the current study, we administered roten one based on the literature survey (Naidu et al. 2003). The Rotenone solution was freshly prepared at 3 mg/kg. The Rotenone was dissolved in DMSO and adjusts to pH 7.4 with potassium hydroxide. Rotenone injected i.p at the dose of 3 mg/kg body weight, 7 days. The solution should be used immediately after preparation. Rotenone solution is stable only for a period of 24 hours at 250C.

In vivo experimental study design
The dose of the plant extract is selected based on the acute toxicity study reports (LD50: 2000 mg/kg,) Control group animals received normal food and water throughout the experiment. Negative control (NC) group received rotenone (3mg/kg i.p) The Rotenone was dissolved in DMSO and adjusts to pH 7.4 with potassium hydroxide. Rotenone injected *i.p* at the dose of 3 mg/kg body weight, for 7 days. The solution should be used immediately after preparation. Rotenone solution is stable only for a period of 24 hours at 250C. test drug treated group received ethyl acetate extract, flavonoid fraction, and tannin fraction of both plant (200,and 400 mg/kg p.o. and 50, 100 mg/kg) along with rotenone (3mg/kg i.p).Before initiating the study, all the animals were subjected to extero ceptive behavioral models using Rotarod, and Actophotometer for acquisition. After subjecting to the test agent, changes in the latency were examined on 0^{th} (first dose), 7th, 14th, and 21st day. After completion of the in

vivo studies, mice's were euthanatized, brains were isolated whole-brain Dopamine, reduced GSH, MDA, SOD, CAT, and Glutathione level were measured.

In vivo screening models for PD

Rotarod apparatus

The rotarod test is often used in rats to determine their "minimal extrapyramidal deficit," such as muscle control and balance. Before starting the therapy, each micewas given a training session to acclimate them to the rotarod apparatus. Mice's which were able to remain on the rotating rod, with a speed of 15 rpm for 3 minutes or more were selected and divided in different groups. The animals were placed on the rotarod after 50 min. of the drug administration and the latency to fall from the rotarod was noticed in a rotarod diameter of 7 cm and a speed of 25 rpm(rpm). (Krall et al1978).

Locomotors activity by actophotometer

The locomotors activity was assessed by using digital actophotometer (INCO, Ambala, India). Each animal was observed over a 5 min cut-off period in a square (30 cm) closed arena equipped with infrared light-sensitive photo cells and values expressed as counts per 5 min. The moving animal cuts off the light beam going through the photocell, and acount is taken. The apparatus was placed in a darkened, sound-attenuated and ventilated testing room (Adzu et al., 2002; Kulkarni and Dandiya, 1975). Each animal readings were taken on the 0th,7th, 14th, and 21st days. To stop the animal odour, the chamber was cleaned with 10% ethanol before the experiment began (Goverdhan et al. 2012).

Rotenone Induced Catalepsy

Rotenone causes dysfunctioning of various neurotransmitters such as acetylcholine, GABA, and serotonin. Pathology of RTN induced catalepsy underlying increased oxidative stress. It also produces a behavioral state in animals like mice and rats in which they fail to correct externally imposed postures (called catalepsy); thus, keeping the above fact in mind, the RTN induced catalepsy model was selected. The method described by Elliott et al(Elliott et al 1990).

Chimney test.

A Pyrex glass container of 30 cm height with a 3 cm of inner diameter and a label at 20 cm from its foundation was utilized in this process (Birhanie et al 2016). After treating animals with *Psidium guajava , Madhuca indica* extracts, flavonoids and tannins fractions(200, 400 mg/kg, and 50, 100 mg / kg), and ordinary saline (10 ml / kg) for about240, 120, 60 and 30 minutes, all the mice were tested. At one bottom of the container, every mice was released freely and permitted to travel from the foundation to the label at 20 cm height. Immediately after the animal reaching the point, the container was shifted to vertical site, and then we noticed that the animals attempted to climb the container with a reverse motion. We recorded the moment it took for every mice to rise back to the point. The mice that managed to achieve the target inside 30 secs was treated as muscle relaxant activity Novel object recognition

Determination of biomarkers level

Determination of reduced GSH

GSH levels were measured by using the method described by Ellman (1959). Firstly, brain homogenates (20 μL) were mixed with 10% (w/v) TCA solution and centrifuged at 1000×*g* for 10 min at 4 ◦C. Then, the obtained supernatants were mixed with 10 mM DTNB solution. After incubating for 1 h, absorbance values were measured at 412 nm. GSH levels were calculated from an analytical curve of reduced glutathione, and the results were expressed as nmol SH mg 1 protein (Ellman 1959)

Determination of MDA

MDA levels were estimated as described by Ohkawa et al. (1979). Briefly, brain homogenates (50 μL) were mixed with 100 μL of 15% (w/v) trichloroacetic acid (TCA) and centrifuged at 2500×*g* at 4 ◦C for 10 min. Then, 0.6% (w/v) TBA solution was added, and the mixtures were incubated in a boiling water bath for 30 min. The tubes were cooled at room temperature and extracted with *n*-butanol followed by centrifugation at 3000×*g* for 10 min. The MDA levels were calculated from an analytical curve of MDA. Absorbance values were measured at 532 nm (Ohkawa et al 1979).

Determination of SOD

The capacity of SOD to antagonize the auto oxidation of epinephrine to adrenochrome in the presence of alkaline pH. To the 25 µl of homogenate sample, added 0.1 mM of epinephrine in carbonate buffer (pH 10.2). At 295 nm, the adrenochrome formation in the above mixture was measure dusing the ELISA plate reader. Further, the SOD level was determined from the total protein value and expressed in U/mg of protein (Misra and Fridovich 1972).

Determination of catalase

CAT activity was measured according to the method reported by Aebi (1984) by using H2O2 as substrate. Firstly, potassium phosphate buffer solution (50 mM; pH 7.0) was mixed with brain supernatants (10 μL). Then, 3 mM H2O2 solution was added, and the absorbance was monitored at 240 nm for 2 min at a 30-s interval. the total protein value and expressed in U/mg of protein (Aebi, 1984)

Determination of dopamine level

At the end of experiment, rats were sacrificed and the whole brain was dissected out. 0.25 g of tissue was weighed and was homogenized in 5 mL HCl–butanol with motor driven Teflon coated homogenizer for about 1 min. The sample was then centrifuged for 10 min at 2000 rpm. An aliquot supernatant phase (1 mL) was removed and added to centrifuge tube containing heptane (2.5 mL) and 0.1 M HCl (0.31 mL). After 10 min of vigorous shaking, the tube was centrifuged under the same conditions as above in order to separate the two phases, and the overlaying organic phase was discarded. The aqueous phase was then taken either for DA assay (Manoharan et al. 2016).

Statistical analysis

The results were expressed as Mean ± SEM/SD. One-way and two-way ANOVA followed by Bonferroni and Tukey's comparison tests were used to determine the difference between the groups in GraphPad Prism v5.0. All statistical analysis was performed at 95% confidence level.

Results

Extract yield

The chloroform and ethyl acetate extracts ware obtained from leaves and bark part of *Psidium guajava and Madhuca indica* is 28 g and 23gm from 500 g of raw material. The percentage yield (w/w) of extracts by successive solvent extraction method was found to be in chloroform extract and ethyl acetate extract was 5.6%w/w and 4.6%w/w

In vivo studies

Effect of Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on performance time (grip strength) using rotarod

Administration of vehicle (CMC 1.0 %w/v, *p.o.*) once daily for 7 days did not produced any significant change on time spend on rotarod model. Administration of *Psedium gvajava* extracts/fractions (200, 400 mg/kg, and 50, 100 mg/kg, *p.o*) produced significantly effect on rotarod activity as compared to vehicle (1.0 % CMC, *p.o*) control and Rotenone (3 mg/kg) control.

Administration of vehicle (CMC 1.0 %w/v, *p.o.*) once daily for 7 days did not produced any significant change on time spend on rotarod model. Administration of *Madhuca indica* extracts/fractions (200, 400 mg/kg, and 50, 100 mg/kg, *p.o*) produced significantly effect on rotarod activity as compared to vehicle (1.0 % CMC, *p.o*) control and Rotenone (3 mg/kg) control. However Rotenone (3 mg/kg) significantly reduces muscle grip strength as compare to vehicle control.

Table 1: Effect of ***Psedium gvajava*** on muscle grip strength

Groups	Time spent on Rotarod (Sec)		
	Day 3	Day 5	Day 7
Control	184.2±13.9	183.8±13.9	184.8±14.04
Rotenone 3 mg/kg	65.4±4.9$^{###}$	58.9±3.2$^{###}$	39.3±2.1$^{###}$
Standard	73.9±4.9***	129.4±9.3***	179.4±13.6***
EAPG 200	64.8±4.2	86.1±6.9*	119.4±8.3*
EAPG 400	67.8±4.3	96.4±7.9**	149.4±11.4***
FFPG 50	66.0±4.8	93.6±8.2*	148.3±11.4**
FFPG 100	68.4±4.4	121.4±9.2***	174.8±12.3***
TTPG 50	65.9±4.6	71.9±3.8	79.4±3.8
TTPG 100	64.8±3.9	82.7±5.1	97.4±6.9

Effect of ***Psedium gvajava*** **extract and fraction** on grip strength using rotarod *p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is compared to rotenone induced PD group

Table 2: Effect of *Madhuca indica* on muscle grip strength

Groups	Time spent on Rotarod (Sec)		
	Day 3	Day 5	Day 7
Control	172.6±14.3	171.6±13.2	173.3±13.9
Rotenone 3 mg/kg	52.8±3.6$^{###}$	43.6±3.9$^{###}$	27.4±1.6$^{###}$
Standard	68.4±4.2***	114.3±7.9***	169.4±12.4***
EAMI 200	52.8±3.0	69.4±5.8	104.6±8.3*
EAMI 400	55.8±3.1	86.7±6.4*	148.3±12.5***
FFMI 50	55.0±4.2	84.6±6.9**	132.6±12.0**
FFMI100	57.4±4.7	119.4±8.2***	165.8.8±13.2***
TTMI 50	53.7±4.4	62.9±5.0	68.5±5.3
TTMI 100	55.9±4.1	71.3±6.3	82.6±7.3

Effect of *Madhuca indica* extract and fraction on grip strength using rotarod *p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is compared to rotenone induced PD group

Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on locomotion using actophotometer

Administration of *Psediumgvajava* and *Madhucaindica* extracts/fractions (200, 400 mg/kg, and 50, 100 mg/kg, *p.o*) produced significant effect on locomotor activity as compared to velicle (1% CMC, *p.o*) control.

Table 3 : Effect of **Psediumgvajava** on Spontaneous locomotor activity

Groups	Locomotive score (Sec)		
	Day 3	Day 5	Day 7
Control	483.9±35.6	472.1±35.9	497.4±35.1
Rotenone 3 mg/kg	227.4±18.4$^{###}$	173.6±15.3$^{###}$	112.9±10.3$^{###}$
Standard	184.3±14.6***	296.4±20.4***	473.9±32.8***
EAPG 200	152.9±10.1	179.4±12.2	264.6±20.0**
EAPG 400	158.4±9.6	221.5±16.3*	397.4±13.4**
FFPG 50	164.9±10.7	217.4±12.6**	394.8±24.8***
FFPG 100	168.4±10.3	258.4±20.5***	468.4±35.1***
TTPG 50	161.7±10.8	172.4±10.3	93.6±5.7
TTPG 100	164.9±10.4	179.4±10.7	203.7±14.6*

Effect of *Psediumgvajava* extract and fraction on Spontaneous locomotor activity *p < 0.05, **p < 0.01, ***p < 0.001, iscompared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is compared to rotenone induced PD group.

Table 4: Effect of ***Madhuca indica*** on Spontaneous locomotor activity

Groups	Locomotive score (Sec)		
	Day 3	Day 5	Day 7
Control	451.6±31.6	447.5±30.8	463.9±34.6
Rotenone 3 mg/kg	196.8±16.4###	142.6±11.9###	87.5±5.7###
Standard	157.2±12.7***	269.4±19.0***	428.4±36.2***
EAMI 200	119.5±14.6	147.9±11.8*	231.7±23.8**
EAMI 400	120.3±11.6	193.7±14.8*	364.8±28.1**
FFMI 50	131.6±11.8	183.5±13.9*	358.4±28.9***
FFMI 100	139.1±9.4	221.4±19.3***	431.4±37.2***
TTMI 50	130.9±9.3	147.8±11.9	64.7±3.7
TTMI 100	138.3±8.3	142.8±11.3	175.6±12.4*

Effect of ***Madhuca indica*extract and fraction** on **Spontaneous locomotor activity** *p < 0.05, **p < 0.01, ***p < 0.001, iscompared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is compared to rotenone induced PD group

Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on Catalepsy

Result ofrotenone induced catalepsy indicate that ethyl acetate extract (200mg /kg, 400mg/kg) and flavonoid fraction (50mg/kg, 100mg/kg) of *Psedium gvajava and Madhuca indica* significantly lower the catalepsy score.

Table 5 : Effect of Psedium gvajava on rotenone induced catalepsy

No. of Minutes	**15 min**	**30 min**	**45 min**	**60 min**
Group	**No. of catalepsy score**			
Control	7.83±0.5	5.37±0.17	5.88±0.1	5.61±0.1
rotenone(RTN) treated	5.83±0.2	136.81±9.6###	158.62±9.3###	184.97±8.4###
Standard + RTN	6.83±0.6	26.83±1.3***	38.61±1.9***	40.81±1.8***
EAPG 200 + RTN	5.92±0.2	86.92±7.5	106.72±8.4*	128.6±9.5*
EAPG 400 + RTN	5.22±0.1	57.51±2.4**	63.8±3.8**	79.4±5.7**
FFPG 50 + RTN	5.06±0.6	47.41±2.8**	57.4±2.6**	68.51±3.0***
FFPG 100 + RTN	5.17±0.3	30.71±1.9***	42.83±1.6***	47.51±1.7***
TTPG 50 + RTN	5.38±0.2	118.5±9.5	137.2±8.9	159.4±10.4
TTPG 100 + RTN	5.28±0.0	97.51±5.7	104.71±9.5	128.5±9.4

Effect of *Psedium gvajava* extract and fraction on RTN induced catalepsy
*p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is compared to RTN induced PD group.

Table 6 : Effect of *Madhuca indica* on RTN induced catalepsy

No. of Minutes	**15 min**	**30 min**	**45 min**	**60 min**
Group	**No. of catalepsy score**			
Control	5.92±0.5	6.82±0.1	6.91±0.1	5.29±0.1
RTN treated	6.70±0.1	157.4±11.3###	174.6±10.9###	201.8±15.3###
Standard + RTN	5.8±0.6	47.2±2.9***	59.6±2.4***	65.8±3.5***
EAMI 200 + RTN	6.2±0.2	103.8±7.9	121.9±8.3**	149.5±10.2*
EAMI 400 + RTN	5.8±0.1	76.8±4.8**	89.3±6.3**	98.2±8.4***
FFMI 50 + RTN	6.2±0.6	68.3±4.6**	75.2±5.2**	88.6±7.3***
FFMI 100 + RTN	5.71±0.3	48.2±2.7***	61.4±4.8***	69.4±4.2***
TTMI 50 + RTN	5.85±0.2	127.5±10.8	154.8±10.6	178.3±10.2
TTMI 100 + RTN	6.7±0.0	118.4±9.6	138.4±9.4	155.8±10.3

Effect of *Madhuca indica* extract and fraction on RTN induced catalepsy *p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is compared to RTN induced PD group.

Effect of *Psedium gvajava and Madhuca indica* extracts and fractions onchimney test

In chimney test, Effect of *Psedium gvajava*the inability of the mice to climb backward out of the tube within 30 s was considered as the end point to assess muscle relaxant action. At a dose of 200, 400 mg/kg, and 50, and 100 mg/kg dose) mice failed to climb out of the tube.None of the mice were able to climb with diazepam. All the mice given distilled water could climb out of the tube. Statistical analysis done using Chi-square test showed that dose of both plant 200 and 400 mg/kg at extract level and at fraction level show significant body weight showed a significant muscle relaxation activity. A dose dependent muscle effect is clearly produced by the extract and flavonoid fraction.

Table 7: Effect of *Psedium gvajava* on muscle relaxant effect in chimney test

Time (min) post administration	Treatment	Response time (Sec)
30	Control	2.17±0.13
	EAPG 200	4.32±0.12
	EAPG 400	4.45±0.18
	FFPG 50	4.68±0.20
	FFPG 100	4.86±0.21
	TTPG 50	4.10±0.22
	TTPG 100	4.12±0.27
60	Control	3.84±0.18
	EAPG 200	7.83±0.48**
	EAPG 400	10.72±0.19**
	FFPG 50	10.04±0.27**
	FFPG 100	12.83±0.18***
	TTPG 50	5.82±0.10
	TTPG 100	7.72±0.18
120	Control	4.26±0.18
	EAPG 200	8.62±0.21
	EAPG 400	11.29±0.82**
	FFPG 50	10.73±0.14**
	FFPG 100	13.84±0.21***
	TTPG 50	6.83±0.18
	TTPG 100	8.35±0.13
240	Control	4.99±0.12
	EAPG 200	10.62±0.11**
	EAPG 400	12.58±0.12**
	FFPG 50	12.73±0.16**
	FFPG 100	14.85±0.12***
	TTPG 50	4.82±0.11
	TTPG 100	7.84±0.18

Effect of *Psedium gvajava* extract on muscle relaxant effect in chimney test*p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group;

Table 8: Effect of *Madhuca indica* on muscle relaxant effect in chimney test

Time (min) post administration	Treatment	Response time (Sec)
30	Control	3.18±1.03
	EAMI 200	5.23±1.02
	EAMI 400	5.54±1.07
	FFMI 50	5.86±1.02
	FFMI 100	5.99±1.08
	TTMI 50	5.21±1.05
	TTMI 100	5.32±1.01
60	Control	4.82±1.04

	EAMI 200	8.42±1.04**
	EAMI 400	11.29±1.10***
	FFMI 50	11.03±1.04**
	FFMI 100	13.27±1.10***
	TTMI 50	6.37±1.01
	TTMI 100	8.62±1.04
120	Control	5.38±1.21
	EAMI 200	9.61±1.03**
	EAMI 400	12.18±1.62**
	FFMI 50	11.83±1.10**
	FFMI 100	14.28±1.03***
	TTMI 50	7.93±1.21
	TTMI 100	9.06±1.00
240	Control	5.73±1.03
	EAMI 200	11.28±1.07**
	EAMI 400	13.29±1.04**
	FFMI 50	13.58±1.10**
	FFMI 100	15.73±1.2***
	TTMI 50	5.37±1.04
	TTMI 100	8.83±1.82

Effect of *Madhuca indica* extract and fraction on muscle relaxant effect in chimney test*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, is compared to Normal group;

In vitro biochemical estimation

c) Effect of *Psedium gvajava and Madhuca indica* extract on reduced GSH

The treatment of Rotenone 3 mg/kg in the Negative control (NC)group remarkably ($p < 0.0001$) reduced level in the whole brain compared to the control group. However, treatment with *Psedium gvajava and Madhuca indica* ethyl acetate group 200, 400 mg/kg ($p < 0.0001$),and flavonoid fraction ($p < 0.0001$) 50, and 100mg/kg significantly increased the level of GSH compared to the Negative control(NC) group in both plants' extracts. Further, no significant change in GSH level is observed in the chloroform extract treated group 9200, 400mg/kg and tannin fraction group (50, 100mg /kg) as compared to the NC group in both plants' extracts.

d) Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on MDA level

MDA level is a marker of oxidative stress and the antioxidant status. The treatment of Rotenone 3 mg/kg in the Negative control (NC)group remarkably($p < 0.0001$) raised MDA level in the whole-brain compared to the control group. However, treatment with ethyl acetate group 200, 400 mg/kg ($p < 0.0001$),and flavonoid fraction ($p < 0.0001$) 50, and 100mg/kg remarkably decreased the level of MDA compared to the NC group in both plant extracts. Further, no significant change in GSH level is observed in the chloroform extract treated group 9200, 400mg/kg and tannin fraction group (50, 100mg /kg) as compared to the NC group in both plant extracts.

f) Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on SOD

The treatment of Rotenone 3 mg/kg in the Negative control (NC)group decreased the SOD level in the whole cerebrum compared to normal However, treatment with ethyl acetate group 200, 400 mg/kg ($p < 0.0001$),and flavonoid fraction ($p < 0.0001$) 50, and 100mg/kg remarkably decreased the level of MDA compared to the NC group in both plant extracts. Further, no significant change in GSH level is observed in the chloroform extract treated group 9200, 400mg/kg and tannin fraction group (50, 100mg /kg) as compared to the NC group in both plant extracts.

g) Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on CAT

The treatment of Rotenone 3 mg/kg in the Negative group (NC) group show significant low in CAT level in the whole cerebrum. However, L-DOPA treatment group showed its effect on the CAT level significantly, whereas, treatment with ethyl acetate group 200, 400 mg/kg ($p < 0.0001$), and flavonoid fraction ($p < 0.0001$) 50, and 100mg/kg have remarkably increased the level of CAT compared to the NC group in both plants extracts. Further, no significant change in CAT level is observed in the chloroform extract treated group (200, 400mg/kg and tannin fraction group (50, 100mg /kg) as compared to the NC group in both plant extracts.

Table 9: Effect of *Psedium gvajava* on oxidative stress and antioxidant activity

Treatment group	SOD (U/mg protein)	MDA (µM/mg protein)	CAT (Micromoles of H2O2 decomposed/mg protein/min)	GSH (µM/mg protein)
Control	16.27±0.93	35.82±0.27	31.92±2.5	29.73±1.4
Rotenone 3 mg/kg	7.93±0.27	102.63±5.38	10.82±1.9	10.26±1.0
Standard	16.04±0.91***	37.82±1.2***	30.71±2.8***	29.65±1.3***
EAPG 200	10.80±0.87*	82.89±4.7*	16.22±1.5*	15.37±1.2*
EAPG 400	12.84±0.82**	59.61±4.2**	22.76±1.5**	21.24±1.8**
FFPG 50	12.33±0.94**	57.51±5.2**	20.83±1.0**	19.27±1.2**
FFPG 100	15.26±0.99***	40.92±2.6***	28.61±1.4***	27.57±1.8***
TTPG 50	9.62±0.51	90.62±8.4	13.28±1.0	12.31±1.0
TTPG 100	11.72±0.62*	62.17±4.8	21.28±1.2*	20.94±1.2*

Effect of *Psedium gvajava* on oxidative stress and antioxidant activity on Rotenone induced oxidative extract *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, is compared to rotenone group;

Table 9: Effect of ***Madhuca indica*** on oxidative stress and antioxidant

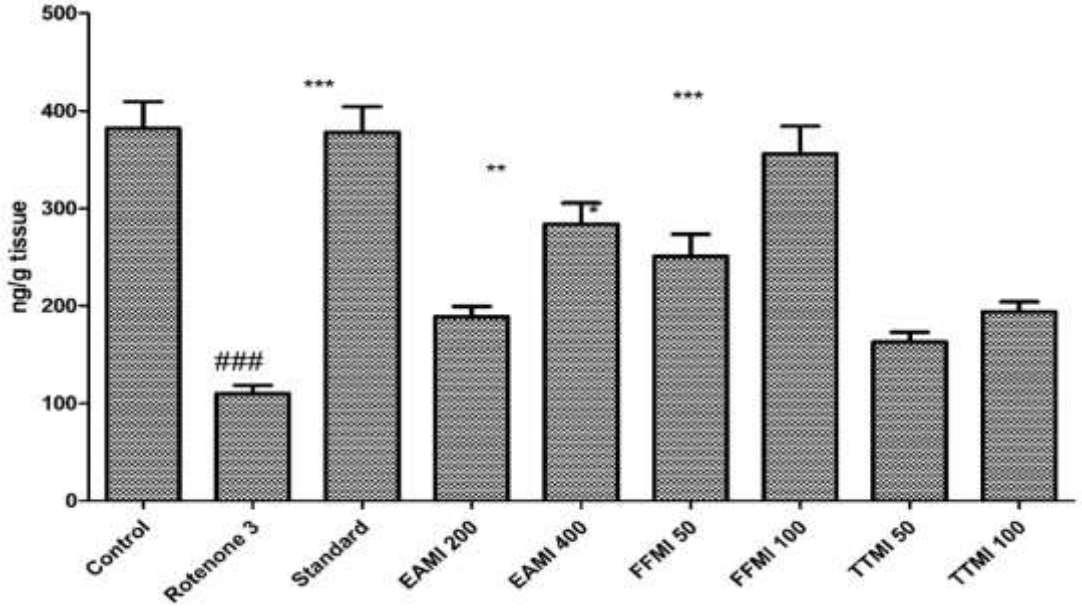
Treatment group	SOD (U/mg protein)	MDA (µM/mg protein)	CAT (Micromoles of H2O2 decomposed/mg protein/min)	GSH (µM/mg protein)
Control	18.93±	37.58±	29.61±	26.68±1.4
Rotenone 3 mg/kg	9.62±	105.32±	8.61±	7.58±1.0
Standard	18.51±***	39.61±***	28.68±***	26.48±1.3***
EAMI 200	12.73±*	84.26±*	14.26±*	12.28±1.2*
EAMI 400	14.66±**	61.28±**	20.94±**	18.28±1.8**
FFMI 50	14.58±**	59.55±**	18.32±**	16.62±1.2**
FFMI 100	17.31±***	43.27±***	26.58±***	24.68±1.8***
TTMI 50	11.27±	92.36±	11.04±	9.04±1.0
TTMI 100	13.28±*	64.61±	19.60±*	17.73±1.2*

Effect of *Madhuca indica* on oxidative stress and antioxidant activity on Rotenone induced oxidative extract *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, is compared to rotenone group;

(e) Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on dopamine level

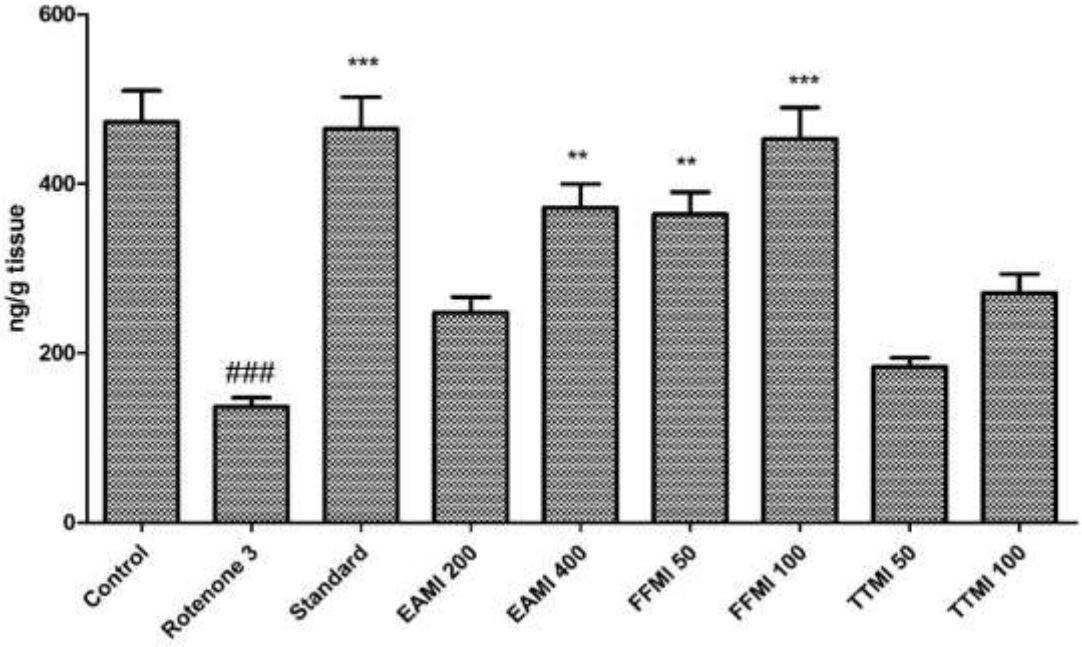
The treatment of Rotenone 3 mg/kg in the Negative control(NC) group remarkably ($p < 0.0001$) decreased the Dopamine level in the whole cerebrum compared to the control, group. However, treatment with L-DOPA group ($p < 0.01$),100 ($p < 0.0001$), EAMI 400 ($p < 0.05$) and FFMI (50,100 mg/kg) treated animals ($p < 0.0001$) remarkably increased the level of Dopamine compared to the Negative control (NC) group. The effect of *Psedium gvajava and Madhuca Indica*on whole-brain Dopamine level is shown in Supplementary figures.

Figure 1: Effect of *Psediumgvajava* on dopamine level in brain



Effect of *Psedium gvajava* extract and fraction on dopamine level *p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is dopamine level, compared to rotenone induced PD group

Figure 2: Effect of *Madhuca indica* on dopamine level in brain



Effect of *Madhuca indica* extract and fraction on dopamine level *p < 0.05, **p < 0.01, ***p < 0.001, is compared to Normal group; #p < 0.05, ##p < 0.01 ###p < 0.001, is dopamine level, compared to rotenone induced PD group

(f)Effect of *Psedium gvajava and Madhuca indica* extracts and fractions on histopathology of hippocampus and cortex

The histology of the control group cerebrum indicated mildcerebral odema, cerebral congestion, neuronal eosinophilia, meningeal congestion, and no change in anatomy was visualizedin RBC extravasation, macrophage influx, neuronalmicro vacuolisation, neuronal nuclear pyknosis, neutron philicinfiltration, neuronal karyorrhexis, reactive gliosis, and vascular proliferation. However, the Negative group (NC) group histogram indicated severe damage in the above-mentioned parameters. The EAGI,EAMI at extract dose of 200, 400 and FFGI, FFMI at fraction dose (50,100 mg/kg)group reversed the Rotenone damaged parameters. The histopathology of the brain (hippocampus and cortex) at 10x and 40x magnification is shown in Figure 3 and 4.

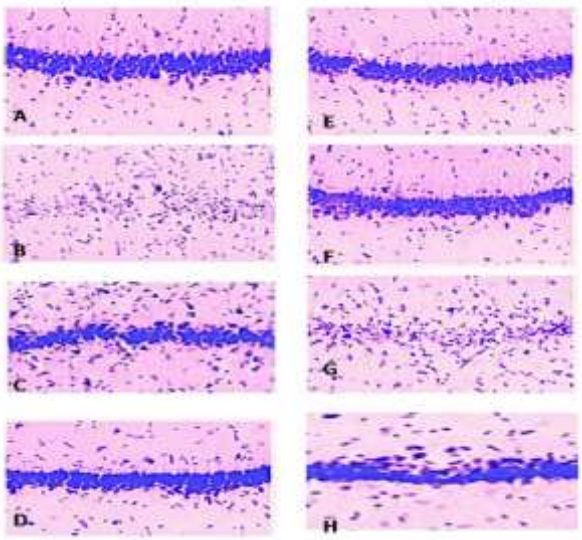


Figure 3: Histopathological analysis of rat treated with *Psediumgvajava*

A, control; B, Rotenone 3; C, EAPG 200; D, EAPG 400; E, FFPG 50; F, FFPG 100; G, TTPG 50; and H, TTPG 100.

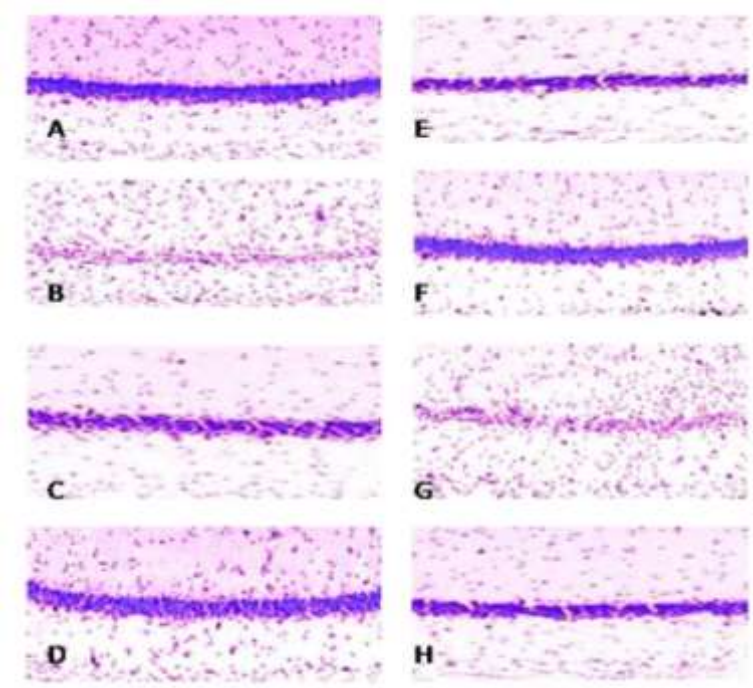


Figure 4: Histopathological analysis of rat treated with *Madhuca indica*

A, control; B, Rotenone 3; C, EAMI 200; D, EAMI 400; E, FFMI 50; F, FFMI 100; G, TTMI 50; and H, TTMI 100.

Discussion

This study carried outon anti-PD activity of *Psedium gvajava and Madhuca indica* extracts and fractions in RTN induced PD in rats through the analysis of motor and memory function. In vivo studies were carried out through Rotarod, Actophotometer, apparatus and Chimney test. The cerebrumbio chemicals (GSH, MDA, SOD, CAT, Dopamine) level were estimated. Allthe animals were trained for their acquisition and training toeach apparatus previously before study.

The rotarod apparatus was utilized to assess neuromuscular motor coordination and grip strength of muscle. In this study, inducing agents Rotenone 3 mg/kg antagonistic remarkably declined the performance time which indicated by the reduced grip strength and extrapyramidal activity of PD. Treatment with L-DOPA and *Psedium gvajava and Madhuca indica* ethyl acetate extract sand flavonoid fractionsat dose of 200 mg/kgand 400 mg/kg and 50 and 100mg/kg exhibited a remarkable decline in retention time.

Further, Locomotion activity using actophotometer was remarkably declined from3 to 7 day in Rotenone 3 mg/kg treatedmices, further reduce the locomotion and were reversed by L-DOPA and *Psedium gvajava and Madhuca indica* extracts and fractions.

The Administration of test drug extracts/fractions (200, 400 mg/kg, and 50, 100 mg/kg, *p.o*) produced significant effect on locomotor activity as compared to velicle (1% CMC, *p.o*) control. So, it is suggested that and *Psedium gvajava and Madhuca indica* ethyl acetate extract sand flavonoid fractions changes the rat motor activity.

Further, Chimney Test was used to investigate cognitive function, primarily a response to conditioned stimulito access the coordination of movements and motor deficits in mice(Kamila et al. 2014) Extracts and Fractions in compared with Diazepam treated group, exhibited a significant percent negative effect at the doses of200 mg/kg and 400 mg/kg and 50 and 100mg/kg. The most significant skeletal muscle relaxation was observed against diazepam which was used as positive control. The effect of diazepam was foremost among the tested doses. Further, through the in vitro biochemical analysis methods, GSH, MDA, SOD, CAT, and Dopamine level in the cerebrum were examined. In the present study, administration of *Psedium gvajava and Madhuca indica* ethyl acetate extract sand flavonoid fractions acts as a scavenging actions and antioxidant markers viz., GSH, MDA, SOD, and CAT level in the whole brain.

The results suggest, *Psedium gvajava and Madhuca Indica* ethyl acetate extracts and flavonoid fractions as a potent anti-oxidan tplants and pose a potential ROS neutralizing capacity.

Dopamine, a neurotransmitter involved in reward motivated actions and aids in the regulation of expression and the formation of motor coordination and new memories (Triarhou 2013). The SN's Dopamine neurons deteriorate over time in PD, reducing the quantity of DA available for neurotransmission in the corpus striatum. Resting tremor, stiffness, bradykinesia, poor balance, and motor coordination are all common clinical signs of a PD (Triarhou2013).

In the present study investigation, Rotenone 3 mg/kg in the Negative control(NC) group remarkably ($p < 0.0001$) significantly decreased the level of Dopamine and treatment with L-DOPA group ($p < 0.01$),100 ($p < 0.0001$), EAMI 400 ($p < 0.05$) and FFMI (50,100 mg/kg) treated animals ($p < 0.0001$) increased the level of Dopamine compared to the Negative control (NC) group. Histopathology examination of the brain of the normal animals showed no significant changes in the normal group. While, Rotenone treated rats brain showed moderate cerebral congestion, edema, microvacuolisation, Gliosis and moderate meningeal congestion, nuclear pyknosis, karyorrhexis, neutrophilic infiltration, RBC extravasation, and vascular proliferation. However, treatment with in the *Psedium gvajava and Madhuca indica* ethyl acetate extract sand flavonoid fractions reversed the damage induced by Rotenone. Treatment with L-DOPA and ethyl acetate extracts and flavonoid fractions groups-maintained neurons of posterior part of limbic system integrity, morphological and cellular architecture of neuronal. Also, various investigations demonstrated of *Psedium gvajava and Madhuca*

indica extract, believes the bioactivephy to constituents mainly of flavonoids will ameliorate the Rotenone induced PD in rats. Herein, we suggest proposing the molecular mechanism of *Psedium gvajava and Madhuca indica* against PD as using network pharmacology and molecular docking which is the future scope of this study.

Conclusion

The current study identified *Psedium gvajava and Madhuca indica* ethyl acetate extract and flavonoid fractions act as a potent herb for PD through in vivo and in vitro experiments, PD symptoms like neuromotorco-ordination by Rotarod, movement by locomotion, recognition i.e., Memory and cognitive function through MWand so on. In vitro studies, *Psedium gvajava and Madhuca indica ethyl acetate extract* Phytoconstituents, increases Dopamine level, and possess a potent antioxidant potency. The current study data were comparable to a standard molecule L-DOPA. Herein, based on the current study reports, *Psedium gvajava and Madhuca indica* can be takenfor further studies to identify key component responsible for enzyme inhibition, to explore the possible mode of action and protein expression analysis, which helps to explore *Psedium gvajava leaves and Madhuca indica* bark as an herbal therapy for PD.

Supplementary Information

The data are not publicly available due to their containing information that could compromise the privacy of research participants, but are available on request from the author.

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Author's contribution

Seema Tomar and Ashutosh Mishra oversaw the experiments, confirmed the findings, helped with data analysis, and helped write the manuscript while Mudita Mishra designed the study, carried out the experiment, gathered the data, and authored the manuscript. statistical analysis and supported the creation of the manuscript.

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Declarations

Ethical statement

The Institutional Animal Ethical Committee evaluated and approved the study protocol and Application No. **IAEC/ANDCP/5/2022**. and Reg No.**1585/PO/RE/S/11/CPCSEA**. The CPCSEA rules were followed for conducting the animal research

Conflict of interest

All the authors of this manuscript confirm that they do not have any conflict of interest.

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