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Evaluation of anti-Parkinson's activity of *Uncaria rhynchophylla* in 6-hydroxy dopamine lesioned rat model

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Abstract
The present study was designed to evaluate the possible Anti-Parkinson's activity of aqueous extract of hooks with stem of *Uncaria rhynchophylla* which is showing monoamine oxidase-B (MAO-B) inhibition and anti-oxidant activity. On zero day wistar rats were treated with 6-hydroxy dopamine (2µg/µl) intra nigrally. After 48 hrs of induction, aqueous extract of *Uncaria rhynchophylla* (200 mg/kg/day, p.o.) was given to the treatment groups for 60 days and levodopa (6mg/kg) was used as the standard drug. The possible pharmacological actions of the drug was evaluated by grip strength test, striatal dopamine (DA) levels, mitochondrial complex-I activity and glutathion level. The results revealed that the treatment of *Uncaria rhynchophylla* aqueous extract significantly protected the dopaminergic neurons when compared with 6-OHDA control group. So, it is concluded that *Uncaria rhynchophylla* aqueous extract can exert a major neuroprotective effect and can be a future drug of choice for the treatment of clinical Parkinsonism.

Keywords: Parkinson's disease, 6-OHDA, levodopa, *Uncaria rhynchophylla*, complex-I**.**

1. Introduction
Parkinson's disease (PD) is a neurodegenerative disorder caused by the progressive loss of mesencephalic dopaminergic neurons in the substantia nigra innervating the striatum [1]. Research on the pathogenesis of PD has rapidly advanced due to the development of animal models. Neurotoxins like 6-OHDA and MPTP used to create PD models which act by inhibiting the electron transport chain (ETC) at complex-I [2]. The effectiveness of levodopa (L-DOPA) in Parkinson's treatment is remaining obscure and most of the PD patients experienced an intolerable adverse effects and the disease condition seemed to be progressed.
Monoamine oxidase (MAO), a flavin-containing enzyme, is widely distributed in both the central and peripheral nervous systems and plays a central role in the control of substrate availability and activity [3]. MAO is a key enzyme in catecholamine metabolism, and increased catecholamine metabolism seen in aging has been extensively studied. The control on MAO activity may alleviate symptoms and slow the progression of neurodegenerative disorders. In humans, MAO-B activity increases with age and is especially elevated in certain neurodegenerative diseases. Therefore, inhibition of MAO-B activity may improve the quality of life of the elderly and it is used as part of the treatment of Parkinson's patients.
Uncaria rhynchophylla [4] is a genus of flowering plants in the family Rubiaceae. It is also known as Cat's Claw. It has about 40 species. Their distribution is pantropical, with most species native to tropical Asia. The total alkaloid content in *Uncaria rhynchophylla* is about 0.2 %. The other trace components include catechin, hirsutine, hirsuteine, corynantheine, dihydrocorynantheine, isocorynoxeine, akuammigine, geissoschijine, and methylethe. The phytochemical catechin showed a potent in vitro inhibitory activity against human brain monoamine oxidase (MAO)-B enzyme. Cat's Claw herb, is used around the world for conditions including immune disorders, gastritis, ulcers, cancer, arthritis, rheumatism, rheumatic disorders, neuralgias and chronic inflammation of all kinds.
The purpose of the present study to evaluate the possible Anti-Parkinson's activity of *Uncaria rhynchophylla* plant which is showing monoamine oxidase-B (MAO-B) inhibition.

2. Materials and methods

2.1. Chemicals

The chemicals which were used for the present study were procured from Sd-Fine Chemicals Mumbai, Sigma Aldrich USA, Loba chemicals Mumbai, Merk chemicals Mumbai.

2.2. Plant material

Uncaria rhynchophylla was collected from local vender from Coimbatore district, Tamilnadu, India. The collected plants were authentified by Survey of Medicinal Plants & Collection Unit, Central Council for Research in Homoeopathy, Dept. of AYUSH, The Nilgiris, Tamilnadu. For further reference a voucher specimen has been deposited at Padmavathi College of Pharmacy herbarium, Dharmapuri, India.

2.3. Preparation of plant aqueous extract

Uncaria rhynchophylla aqueous extract was prepared by boiling plant materials in 10 volumes of water for 15 min [4]. The aqueous solution so obtained was filtered through a Whatman filter paper, concentrated on a water bath under vacuum, freeze dried to yield the aqueous extract (EUR) (yield 12.8% w/w), which was then stored at -20 ◦C until required.

2.4. High Performance Thin Layer Chromatography Standardization

EUR standardization was carried out by HPTLC method using catechin as a reference standard according to the method described earlier [5]. The amount of catechin present was determined using the calibration curve plotted between concentration and area of standard.

2.5. Animals

Healthy, adult Wistar rats of both sexes (180-220g) were obtained from the Central animal house facility of Padmavathi College of Pharmacy, Dharmapuri, India and maintained under standard laboratory conditions. All the experiments were performed after obtaining prior approval from CPCSEA and IAEC.

Approval No.:1143/ac/07/CPCSEA/PCP/IAEC/PhD/132/12.

2.6. Acute toxicity

The acute toxicity study of *Uncaria rhynchophylla* aqueous extract was performed using up and down procedure at a dose level of 2000 mg/kg body weight orally in rats, as per OECD 425 guidelines in two different groups of 3 female each and observed for mortality for 24 h [6]. The dose 2000 mg/kg was found to be safe for all animals. From this 1/10th of 200 mg dose was selected for further study.

2.7. Induction of Parkinsonism by 6-OHDA

The rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital and were fixed in a stereotaxic apparatus (USA) [7, 8]. The needle was inserted into the substantia nigra with the following coordinates: anterior/ posterior: -4.8 mm; medial/lateral: -2.2 mm; ventral/dorsal: -7.2 mm−3.5mm from bregma and injection of 6-OHDA (20 µg of 6-OHDA hydrobromide in 4µl 0.9% saline with 0.02 µg/ml ascorbic acid) was then made over 5 min and the needle was left in place for a further 5 min. Then the skull was secured with stainless metallic screws and the wound area was covered by dental cement. Each rat was housed individually following the surgical procedure.

2.8. Experimental design

Animals were divided into four groups of 6 rats in each group. Group I served as sham operated, received normal saline (10 ml/kg, p.o.), Groups II to IV were induced with Parkinsonism as follows: group II served as a 6-OHDA control, received normal saline (10 ml/kg, p.o), Group III served as a L-DOPA control, received L-DOPA (6 mg/kg, p.o.) [9] and Group IV served as EUR treated, received 200 mg/kg, p.o. of EUR respectively. The treatment of animals were started after 48hr of induction with 6-OHDA according to their respective group once a day for 60 days.

2.9. Parameters evaluated

The following parameters were evaluated, after the 60th day of treatment.

2.10. Rotarod (Grip strength)

The main symptom of the Parkinsonism disease is muscle rigidity. This effect can be easily studied in animals by using rotarod apparatus. Rotarod test was carried out similar to a reported method [10]. Briefly the apparatus consist of a 70 cm long rod with diameter 3 cm placed at a height of 50 cm and divided into four sections. Five trials were taken before the main reading to all the groups by adjusting the rate of rotation at 30 rpm. The animal was placed individually one by one on the rotating rod. Then the fall off time from the rotating rod of animal in control and all treated group was compared.

2.11. HPLC measurement of dopamine

Dissected striata were immediately frozen on dry ice and stored at -80 °C. Striatal tissues were sonicated in 0.1 M of perchloric acid (about 100 µl/mg tissue). The supernatant fluids were taken for measurements of levels of dopamine by HPLC [11]. Briefly, 20 µl supernatant fluid was isocratically eluted through an 4.6-mm C18 column containing paracetamol (100 mg/ml) as the internal standard with a mobile phase containing 50 mM ammonium phosphate pH 4.6, 25mM hexane sulfonic acid pH 4.04, 5% acetonitrile and detected by a UV detector. The flow rate was 1 ml/min. Concentrations of dopamine was expressed as nenogram per milligram of brain tissue.

2.12. Isolation of mitochondrial fractions and Complex I activity assay

Brain tissue was homogenized in a Dounce tissue grinder in mitochondrial isolation buffer and suspensions were centrifuged at 800 g, 4 ºC, for 10 min. The supernatant fluids were centrifuged at 13,000 g, 4 ºC, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at 13,000 g, 4 ºC, for 10 min to obtain the crude mitochondrial fraction. NADH: ubiquinone oxidoreducase (Complex I) activity was measured in the SN as described earlier [12].

2.13. Analysis of GSH/ Glutathion

GSH was measured enzymatically by the method described earlier [13]. The striata were homogenized in ice-cold perchloric acid (0.2 M) containing 0.01% EDTA. The homogenate was centrifuged. The enzymatic reaction was started by adding 200 µl of clear supernatant in a spectrophotometric cuvette containing 500 µl of 0.3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 µl of 6 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 10 µl of 25 units/ml glutathione reductase (all the above three reagents were freshly prepared in phosphate buffer at pH 7.5). The absorbance was measured over a period of 3 min at 412 nm at 30 °C. The GSH

level was determined by comparing the change of absorbance of test solution with the standard GSH.

2.14. Statistical analysis
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test, using statistical package PRISM 5.0 version. The significance of difference between and within various groups was determined. Differences were considered to be significant when $P < 0.01$.

3. Results
Standardization of herbal extracts is required in terms of quality and quantity before subjecting to any pharmacological evaluation. EUR was standardized by HPTLC using catechin, one of the major marker compounds present in *Uncaria rhynchophylla*.

The effect of EUR on rotarod, dopamine estimation using HPLC, Complex I activity and glutathion estimation was given in Table 1.

Table 1: Effect of EUR on rotarod, dopamine estimation using HPLC, Complex I activity and Glutathione in rats.

Sr.No	Group	Rotarod-Retention Time (Sec)	Dopamine Concentration (ng/mg of protein)	Complex I activity (nmol/min/mg protein)	Glutathione reductase (nmol/mg of protein)
1	Sham control	156.0 ± 1.468	4.654±0.125	65.67 ± 0.532	0.993 ± 0.008
2	6-OHDA control	54.50 ± 0.567***	0.649±0.547***	50.83 ± 1.078***	0.543 ± 0.032***
3	Levodopa	154.5 ± 3.006###	3.574±0.452###	80.0 ± 1.423###	0.743 ± 0.004###
4	EUR	150.33 ± 2.342###	2.523±0.001###	69.18 ± 0.426###	0.767 ± 0.007###

Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with Sham control group; ###P<0.001 when compared 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

When compared with sham control group the retention time in rotarod was significantly reduced (P<0.001) for 6-OHDA. It showed the lesion induced bradykinesia in 6-OHDA groups. In the same time, EUR and levodopa treatment significantly increased (P<0.001) retention time when compared with 6-OHDA group.

When compared with sham control animals, 6-OHDA control showed a significant (P<0.001) reduction in dopamine concentration, but levodopa showed higher degree of dopamine levels. When compare with 6-OHDA control, EUR group significantly (P<0.001) retained the dopamine level.

When compared with sham control group the mitochondrial activity was significantly reduced (P<0.001) for 6-OHDA.While the activity significantly (P<0.001) improved in EUR and levodopa treated animals when compared to 6-OHDA treated groups.

When compared with sham control animals the amount of glutathione reductase was significantly (P<0.001) reduced for the treatment with 6-OHDA control, levodopa, EUR. But when compared with 6-OHDA control, EUR and levodopa showed significant (P<0.001) retention of glutathione reductase.

4. Discussion and Conclusion
The efficacy of *Uncaria rhynchophylla* in 6-OHDA induced PD has not been well established. In our study, we have demonstrated the anti-Parkinson's activity and antioxidant effect of plant extract.

Rotarod experiment demonstrated the impairment in the locomotor function and coordination in Parkinson's rats. Lack of motor coordination and maintenance of normal limb posture has been reported in PD condition [14]. The treatment with EUR in rats increased the fall off time when compare to 6-OHDA control rats. This could be the effect of test drugs in mid brain dopaminergic neurons exert a dopamine agonism. The evaluation revealed the efficacy of EUR increasing muscle co-ordination and thus could co-relate with possible action on CNS.

In our study conditions EUR reversed the catalepsy induced by 6-OHDA induction. The pyramidal dopamine facilitatory actions may be the possible action of this test drug.

The EUR retained the equal levels of dopamine as that of levodopa treated groups. The test drug remarkably improved the complex-I activity, where as levodopa treatment could not. This suggested the protective role of EUR in mitochondrial function of PD brain.

The turnover of dopamine in nigral cells plays a major role in controlling motor function. In our study, we reported that EUR caused a pronounced increase in dopamine levels in mid brain regions of 6-OHDA rats and it could a result of protection of dopaminergic neurons by these drugs. The beneficial roles of EUR in retaining dopamine levels demonstrated the protection of nigral neuron by test drugs.

In our study, we have estimated GSH to demonstrate whether the drug treatment could elevate or suppress GSH level intracellularly in mid brain region. With respect to this objective, our finding showed that EUR treatment could maintain the normal range of GSH in brain tissue. This given us a knowledge of possible role of GSH in protecting the mitochondrial activities and reduce in-vivo oxidative stress in neuron. In the further investigation, the reduced level of complex-I activity and relevant reduction in anti-oxidant enzymes in untreated groups demonstrated the possible role of EUR in neuroprotection in PD. Because chronic, generalized mitochondrial deficits have been found in sporadic PD [15, 16] and the increasing GSH could protect the nigral neurons.

In view of the above facts we are concluding that aqueous extract of *Uncaria rhynchophylla* plant showed significant anti-Parkinson's activity. The estimated parameters were closely relevant to clinical Parkinsonism and the drug treatment protected the diseased brain of rat. And we appreciate further detailed molecular studies with this drug in anti-Parkinson's pharmacology and toxicology.

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