Synopsis of the Thesis

EVALUATION OF ANTIPARKINSON'S ACTIVITY OF *Uncaria rhynchophylla*, *Mentha aquatica* and *Banisteriopsis caapi* - PLANTS WITH MONOAMINE OXIDASE-B (MAO-B) INHIBITION POTENTIAL

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DOCTOR OF PHILOSOPHY

By

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MARCH 2015

CERTIFICATE

This is to certify that the work embodied in the synopsis entitled "**Evaluation of antiparkinson's activity of *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* - plants with monoamine oxidase-B (MAO-B) inhibition potential**" submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai, was carried out by **Mr. Biswajit Pal** (Ref.No.AC-I(2)/29596/2011) in the Department of Pharmaceutics, Padmavathi College of Pharmacy and Research Institute, Dharmapuri, for the award of Doctor of Philosophy (Pharmacy) under my direct supervision and guidance.

This work is original and has not been submitted in part or full for any other degree/diploma or academic award of any other university.

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CERTIFICATE

This is to certify that **Mr. Biswajit Pal** (Ref.No.AC-I(2)/29596/2011) carried out research work on "**Evaluation of antiparkinson's activity of *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* - plants with monoamine oxidase-B (MAO-B) inhibition potential**" for the degree of Doctor of Philosophy (Pharmacy) in The Tamilnadu Dr.M.G.R. Medical University, Chennai, within the requisite period under the regulation enforce and the synopsis of the thesis is a bonafide record of the work done under our supervision and guidance. This work is original and has not been formed on the basis of the candidate for any degree/diploma, fellowship or other similar title. We state that the entire synopsis of the thesis represents the independent work of **Mr. Biswajit Pal** and all the experimental techniques employed in the work were actually undertaken by the candidate himself under our guidance.

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INTRODUCTION

Parkinson's disease [1, 2, 3]

Parkinson's disease (PD) is a neurodegenerative disorder caused by the progressive loss of mesencephalic dopaminergic neurons in the substantia nigra innervating the striatum. It was first described by neurologist James Parkinson in 1817 that he called ''Shaking Palsy'', or ''paralysis agitans''. The causes are unknown although risk factors in the genetic and toxic domain are being discovered. An important pathophysiological feature in PD is the loss of part of the dopaminergic neurons in the substantia nigra (SN) resulting in a specific dysorganisation of the complicated basal ganglia (BG) circuits. The relay functions at the level of the striatum e.g., are out of balance leading to disturbed subcorticocortical interactions. Parkinson's disease (PD) is the second most common neurodegenerative disease, primarily affecting people of ages over 55 years (approximately 1.5% to 2.0%), although young adults and even children can also be affected. Research on the pathogenesis of PD has rapidly advanced due to the development of animal models. Through the use of these models, the striatal dopamine deficiency could be associated with the motor symptoms of PD, and levodopa (dihydroxyphenylalanine or L-dopa) was first applied to compensate striatal dopamine losses. L-Dopa treatment still remains the standard of PD therapies. Unfortunately, long-time use of L-dopa results in dyskinesia (involuntary movements). Moreover, the specific etiology of PD is still unknown. Thus, the development of animal models is essential for better understanding pathogenesis and progression of PD and testing therapeutic agents for the treatment of PD patients.

MAO-B, MAO-B inhibitors and PD[4]

MAO exists as two isoforms with different substrate selectivities and different distributions in brain and between species. In man, dopamine is largely metabolised by MAO-B although it can also be a substrate for MAO-A. However, dopaminergic neurones in the striatum contain relatively little MAO-B but the A-isoform is present. Rather MAO-B is found extensively in glial cells and localised to the outer mitochondrial wall. Under normal physiological conditions, dopamine released into the synapse by impulse flow is rapidly 'inactivated' by the high affinity reuptake process that constitutes the dopamine transporter. In PD, the number of presynaptic terminals in the striatum is extensively depleted and now MAO-B in surrounding glial cells becomes a major focus for dopamine metabolism. This provides a targeted and disease specific mechanism through which dopamine degradation can be inhibited

by the use of selective MAO-B inhibitors. Selegiline and rasagiline are selective MAO-B inhibitors that irreversible inhibit the enzyme by the covalent binding of the propargylamine moiety to the active site in the mitochondrial membrane. The long lasting inhibition of MAO-B is held responsible for the symptomatic improvement in motor symptoms occurring in monotherapy in early PD and as adjunct therapy to L-dopa and/or dopamine agonist treatment in midand late-stage illness. However, it has been implied that both drugs may also alter the rate of progression of PD through the DATATOP study and subsequent investigations of selegiline and the TEMPO and ADAGIO studies of rasagiline. This raises the question of how such effects might be mediated. Initially, the inhibition of the metabolism of dopamine by MAO-B was thought to lower oxidative stress by preventing the formation of toxic oxygen free radical species. But subsequently the emphasis has evolved to support an action of both selegiline and rasagiline in preventing apoptotic processes leading to cell death through effects at the level of mitochondria. Recent interest has centred on rasagiline's actions based on the finding of improved clinical scores with early drug use in the ADAGIO study and the preclinical evidence to support such an effect will now be explored in greater detail.

In the search of natural drugs to treat PD, we found that Kong *et al.* isolated three protoberberine alkaloids like jatrorrhizine, berberine and palmatine from the methanol extract of *Coptis chinensis* rhizome and evaluated their MAO inhibitory action[5].

The piperine alkaloids from piper nigrum was found to be potent MAO-B inhibitors[6].
Han et al. isolated some flavonoids such acacetin, apigenin, diosmetin, eriodictyol and luteolin from *Chrysanthemum indicum* and screened for their MAO-B inhibition. Their research revealed that the flavonoids acacetin and diosmetin showed good inhibitory action towards rat liver mitochondrial monoamine oxidase MAO-B[7].

Uncaria rhynchophylla[8] 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, three from Africa and the Mediterranean and two from the neotropics. The total alkaloid content in *Uncaria rhynchophyl* is about 0.2 %, in which rhynchophylline (Rhy) is 28 %-50 %, isorhynchophylline (Isorhy) is 15 %. 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

enzymes. 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 herb Cat's Claw has been used in Peru and Europe since the early 1990s as a treatment for cancer and AIDS, as well as for other diseases that target the immune system.

Mentha aquatica[9] is a perennial plant in the genus Mentha and family Lamiaceae. It is also known as Water Mint and distributed throughout Europe except for the extreme north, and also northwest Africa and southwest Asia. The chemical components include (S)-naringenin, oxygenated monoterpenes (+)-pulegone and (+)-menthofuran, viridiflorol. The volatile oil of *Mentha aquatica* main constituents are: menthofuran (51.27%), limonene (12.06%), izomenthone (8.11%), β – cis – ocimene (7.92%), ledol (3.01%). The phytochemical (S)-naringenin showed a potent in vitro inhibitory activity against human brain monoamine oxidase (MAO)-B enzyme. The leaves are anodyne, antiseptic, antispasmodic, astringent, carminative, cholagogue, diaphoretic, emetic, refrigerant, stimulant, stomachic, tonic and vasodilator. A tea made from the leaves has traditionally been used in the treatment of fevers, headaches, digestive disorders and various minor ailments.

Banisteriopsis caapi[10] also known as Ayahuasca, Caapi or Yage, is a South American jungle vine of the family Malpighiaceae. It contains harmine, harmaline, and tetrahydroharmine, all of which are both beta-carboline harmala alkaloids and MAO-B inhibitors. The stems contain 0.11-0.83% beta-carbolines, with harmine and tetrahydroharmine as the major components. It is used to prepare Ayahuasca, a decoction that has a long history of entheogenic uses as a medicine and "plant teacher" among the indigenous peoplesof the Amazon Rainforest.

In PD conditions, the neurons are highly vulnerable to oxidative stress, which are induced by dopamine metabolism in CNS. The approach to limit or control dopamine metabolism is one of the approach to retain the loss of dopaminergic neurons and also to reduce the toxic dopamine metabolite accumulation in CNS. This was our hypothesized concept before we start the practical aspect of this thesis. In this regard, we surveyed the three plants viz. *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi* and understood that, this plats may offer a protective or palliative role in the treatment of Parkinson’s disease through this preclinical study.

OBJECTIVE

In the present study, we would like to evaluate the possible Anti-Parkinson's activity of *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi* plants which are showing monoamine oxidase-B (MAO-B) inhibition and anti-oxidant activity and to find out the possible actions of these plants for alleviating or preventing the neurodegeneration and mitochondrial dysfunction for the treatment of Parkinson's disease.

Monoamine oxidase (MAO), a flavin-containing enzyme, is widely distributed in both the central and peripheral nervous systems[20] and plays a central role in the control of substrate availability and activity. MAO catalyzes the oxidation of a variety of amine-containing neurotransmitters to yield the corresponding aldehyde, hydrogen peroxide (H_2O_2), and ammonia[17]. MAO exists in two forms, MAO-A and MAO-B, which are distinguished on the basis of different pharmacological and biochemical characteristics. 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[18] and is especially elevated in certain neurodegenerative diseases[19]. 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.

The major hallmarks of the Parkinsonism such as deposition of ubiquiten, Alpha amyloid plaques, neuroinflammation, mitochondrial dysfunction, nigral iron deposition etc., will be studied and the therapeutic benefit of plants having monoamine oxidase B (MAO-B) inhibitors will be validated in various experimental models of Parkinson disease. The pathological interventions during the disease development and after the occurrence of disease will be explored during the study periods. The ultimate aim of our research will be identifying a potential therapeutic herbal drug for the treatment of Parkinson's disease without unacceptable clinical troubles.

AIM OF THE RESEARCH

- To investigate whether monoamine oxidase-B (MAO-B) inhibition potential plants [*Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi*] protects brain against neurodegenaration in 6-OHDA rat models, and to analyse the murine preclinical therapeutic efficacy of test drugs in attaining postural stability after completion of treatment.
- To explore the beneficial effects of *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi* in reducing neurodegenaration by controlling iron induced neurotoxicity, retaining dopamine concentrations and lowered oxidative stress in experimental PD.
- To study the effectiveness of *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* for neuroprotection in PD model through controlling MAO-B associated pathways of metabolism.

PLAN OF WORK

The work consists of following stages

Stage I:

- Literature survey

Stage II:

- Collection and authentication of the medicinal plant
- Extraction of plants by using continuous hot extraction method
- Standardization and Quantification active constituents by HPTLC method

Stage III:

Carried out the pharmacological screening by using extract of *Uncaria rhynchophylla* (EUR), *Mentha aquatica* (EMA), *Banisteriopsis caapi* (EBC) and mixture of all three extract (1:1:1 ratio) (EEE).

- Acute toxicity study of plant extract
- Selection of suitable doses of extracts
- Grouping of animals and induction of Parkinsonism.
- Estimation rat brain monoamine oxidase
- Lesion verification: quantification of circling behavior
- Anti-Parkinson's activity screening
- Apomorphine-induced circling behavior
- Rotarod (Grip strength) Grip strength
- Catalepsy test (Fore limb placing test)
- HPLC measurement of dopamine and metabolites
- Localization of iron in substantia nigra
- Anti oxidant study
- Isolation of mitochondrial fractions and Complex I activity assay
- Estimation of Calcium ions in mitochondria

Stage IV

- Documentation of results
- Evaluation of statistical significance of the results by using a computer aided program and reporting of research finding.

MATERIALS AND METHODS

Animals

Healthy, adult Wistar rats of both sexes (180-220g) were obtained from the Central animal house facility from Padmavathi College of Pharmacy, Dharmapuri, Tamilnadu. The animals were kept in a well ventilated room and the animals had exposed to 12 hrs day and night cycle with a temperature between 20 ± 3^0C. The animals were housed in large spacious, hygienic polypropylene cages during the course of the experimental period. The animals were fed with water and rat feed *adlibitum*. All experiments were performed after obtaining prior approval from CPCSEA and IAEC. The animals were housed in suitable environmental conditions.

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

Collection and authentication of plant material

Uncaria rhynchophylla, Mentha aquatica and *Banisteriopsis caapi* were collected from local vender from Coimbatore district, Tamilnadu, India. The collected plants were authentified by Dr. S Rajan, Field Botanist, Survey of Medicinal Plants & Collection Unit, Central Council for Research in Homoeopathy, Dept. of AYUSH, The Nilgiris, Tamilnadu.

Extraction of plants[8,9,10]

The extract of *Uncaria rhynchophylla* and *Banisteriopsis caapi* plants were obtained by continuous hot extraction method and *Mentha aquatica* extract was obtained by using hydro-alcoholic solvent. Freeze dryer was used for drying the extract. Percentage yield of the extract was found to be 12.8% w/w for *Uncaria rhynchophylla,* 15.3%w/w for *Mentha aquatica* and 21.2% w/w for *Banisteriopsis caapi.*

Standardization and Quantification active constituents by HPTLC method[21,22, 23]

HPTLC standardization method was carried out for all the three plants extracts to determine the active constituent quality of extracted materials. Catechin, naringenin and harmine were used as a marker constituents for *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi* respectively.

Acute toxicity study[11]

The acute toxicity study of EUR, EMA, EBC and EEE extracts were performed using up and down procedure at a dose level of 5000 mg/kg body weight orally in rats, as per OECD 425 guidelines on female rats and observed for mortality for 24 h.

Based on acute toxicity study suitable dose was selected for EUR, EMA, EBC and EEE.

Grouping of animals

Animals were divided into seven groups of either sex rats in each group.

Group I : Sham Control

Group II : 6-OHDA Control

Group III : 6-OHDA +L-DOPA (Standard)

Group IV : 6-OHDA+ EUR

Group V : 6-OHDA+ EMA

Group VI : 6-OHDA+ EBC

Group VII : 6-OHDA+ EEE

Induction of Parkinsonism by 6-OHDA[14, 15]

On the zero day desipramine (Sigma, St. Louis, MO) (25 mg/kg, IP) was administered 30 min before surgery, to protect noradrenaline containing terminals from the effects of 6-OHDA (Sigma). All animals were anaesthetised with ketamine (100mg/kg ip), xylaxine (15mg/Kg im) and were fixed in a stereotaxic apparatus (USA). After the skull was exposed, a burr hole was drilled for the accommodation of needle. 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.

Lesion verification: quantification of circling behavior[25,26,27]

After 48 hours of surgery, the animals were tested for circling behaviour. Circling behaviour was induced by subcutaneous injection of 0.5 mg/kg apomorphine. The animals were observed for 10 minutes period for counting circling behaviour. During observation period the animals were not disturbed. Only animals showing at least 7 turns/ min in both tests were included in this study.

Treatment

EUR, EMA, EBC and EEE were given orally after 48hr of induction for 60days. L-DOPA was given orally as standard drug[12,13]. Treatment drugs were suspended in 0.3%cmc solution and control animals were treated with 0.3% CMC in saline.

Estimation rat brain monoamine oxidase[24]

The quantification of MAO-B from rat brain homogenate was carried our as per the earlier procedure. The herbal extract treated animals which showed more inhibition of MAO-B compared with 6-OHDA control group were included for further investigation of below parameters.

Parameters evaluated

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

1. Pharmacological evaluation

1.1. Apomorphine-induced circling behavior[25,26,27]

At the end of the treatment period the animals were tested for circling behavior. Circling behavior was induced by 0.25 mg/kg apomorphine (s.c.) respectively. The animals were observed for 10 minutes period for counting circling behavior. During observational period the animals were not disturbed. The numbers of full and counter clock wise turns were observed for ten minutes among different groups. The total numbers of circle for ten minutes were recorded.

1.2. Rotarod (Grip strength)[28,29]

The main symptom of the Parkinsonism disease is muscle rigidity. The loss of muscle grip is an indication of muscle rigidity. This effect can be easily studied in animals by using rotarod apparatus. Rotarod has been used to evaluate muscle grip strength by testing the ability of rats to remain on revolving rod. The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. First rotarod apparatus was turned on then selected 20 rpm as an appropriate speed. Each rat was given five trials before the actual reading was taken. The animal was placed individually one by one on the rotating rod. The 'fall of time' was noted when animal falls from the rotating rod and then the fall off time of animals were compared in treated group.

1.3. Catalepsy test (Fore limb placing test)[30,31]

For the catalepsy test, rats were removed from their home cages and their forelimbs were placed onto a stable bar (about the diameter of a standard pencil) which was elevated 8 cm above the testing surface. As soon as the experimenter released the rat from this imposed stance, a timer was started, and the time taken for the rat to return both forelimbs back to the tabletop was recorded.

2. Biochemical evaluation

2.1. HPLC measurement of dopamine and metabolites[32,33]

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. 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.

2.2. Localization of iron in substantia nigra[34,35]

(Perl's diaminobenzidene (Perl's-DAB) method)

Brain tissues were stained for ferric ion using the perl's-DAB method. The isolated and partially frozen brains were cut horizontally to get 30-40 µm sections on a vibratome and then mounted on a glass slide. The sections were immersed in 2% potassium ferrocyanide and 2% hydrochloric acid for thirty minutes at room temperature and then rinsed with deionized water for five minutes. The perl's reaction was intensified by placing the tissue in 0.5% diaminobenzidine (DAB) in cold phosphate buffer (pH 7.4) for fifteen minutes. Next, 2ml of 1% hydrogen peroxide was added for every 200 ml of DAB solution. The sections remained in the solution for twenty five minutes. Following DAB treatment the sections were rinsed in deionized water for fifteen minutes. Counter stained the sections by using thionine solution. The slides of the section were made using a motic microscope (model Motic images plus 2.0), under the magnification of 40x equipped with camera.

3. Anti oxidant studies

3.1. Lipid peroxidation assay[36]

Lipid peroxidation in rat brain homogenate was carried out essentially as described earlier. Rat forebrain (stored at -80ºC for less than 8 days) was homogenized in 20 mM Tris-HCl, pH 7.4 (10 ml) at 4ºC using a Polytron homogenizer. The homogenate was centrifuged at 1000 g for 10 min at 4°C, and the supernatant collected. Then acetic acid 1.5 ml (20%; pH 3.5), 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml of sodium dodecyl sulphate (8.1%) were added to 0.1 ml of supernatant and heated at 100 ºC for 60 min. Mixture was cooled and 5 ml of *n*-butanol-pyridine (15:1) mixture, 1 ml of distilled water was added and vortexed vigorously. After

centrifugation at 1200×*g* for 10 min, the organic layer was separated and absorbance was measured at 532 nm using Elisa plate reader. Malonyldialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen–thiobarbituric acid reactive substance.

3.2. Estimation of catalase (CAT)[37]

Catalase measurement was carried out by the ability of CAT to oxidize hydrogen peroxide (H_2O_2). 2.25 ml of potassium phosphate buffer (65 mM, pH 7.8) and 100 µl of the brain homogenate were incubated at 25 ºC for 30 min. A 650 µl H_2O_2 (7.5 mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240 nm for 2–3 min and the results were expressed as CAT µmol/min mg of protein.

3.3. Estimation of Superoxide dismutase assay (SOD)[38]

SOD activity was analyzed by the method described earlier. Assay mixture contained 0.1 ml of supernatant, 1.2 ml of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 ml of phenazine methosulphate (186 µm), 0.3 ml of nitro blue tetrazolium (300 µM), 0.2 ml of NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30 ºC for 90 s, the reaction was stopped by addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 ml of *n*-butanol. Colour intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm and concentration of SOD was expressed as U/mg of protein.

3.4. Analysis of GSH/ Glutathion[39]

GSH was measured enzymatically by the method described by Owen. The striata were homogenized in ice-cold perchloric acid (0.2 M) containing 0.01% EDTA. The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min. 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 (ΔA) of test solution with the (ΔA) of standard GSH.

4. Molecular pharmacology

4.1. Isolation of mitochondrial fractions and Complex I activity assay[40]

Brain tissue was homogenized in a Dounce tissue grinder (Wheaton, Millville, NJ, USA) in mitochondrial isolation buffer and suspensions were centrifuged at 800 g, 4°C, for 10 min. The supernatant fluids were centrifuged at 13,000 g, 4°C, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at 13,000 g, 4°C, for 10 min to obtain the crude mitochondrial fraction. NADH: ubiquinone oxidoreducase (Complex I) activity was measured in the SN.

4.2. Estimation of mitochondrial calcium[41]

The crude mitochondrial fraction was collected from brain tissue homogenate and added to ice-cold PBS solution (25ml) and homogenized with an Omni 5000 homogenizer over ice for 5 min. The homogenized was centrifuged (3000 rpm-5min) and the supernatant was separated and stored at -80 ^{0}C before AAS analysis.

The concentration of calcium present in the supernatant was determined by atomic absorption spectroscopy. The standards of different Ca concentrations (i.e., 1, 1.5, 2 and 2.5 µg/ml) were prepared from stock standard. The standards and samples were read against the blank solution. The absorbance of samples, standards and blank were noted. The concentration of calcium in the brain was calculated by reading from the standard curve.

OBSERVATIONS

Standardization and Quantification of active constituents by HPTLC method

HPTLC standardization of EUR, EMA and EBC was performed using catechin, naringenin and harmine as standards. From the chromatogram the presence of catechin, naringenin and harmine in the sample was confirmed and the amount of these constituents present in the sample was calculated and confirmed its normal quantity as per earlier references.

Effect of EUR, EMA, EBC and EEE on monoamine oxidase-B levels in rats

The treatment with EUR, EMA, EBC and EEE in 6-OHDA lesioned rats showed a significant MAO-B inhibition. In the untreated group, the MAO-B activity was not altered during the entire course of experimental periods.

Effect of EUR, EMA, EBC and EEE on Quantification of circling behaviour in rats

When compared with 6-OHDA control the number of Ipsilateral turns in 10 minutes was significantly ($P<0.0001$) reduced for the EUR, EMA, EBC and EEE. The levodopa treated group also showed significant reduction in rotation. The results suggested that, treatment with levodopa as well as EUR, EMA, EBC and EEE significantly reduced the unilateral lesion in the experimental animals.

Effect of EUR, EMA, EBC and EEE on rotarod performance in rats

When compared with sham control group the retention time was significantly reduced for 6-OHDA. It showed the lesion induced bradykinesia in 6-OHDA groups. In the same time, EUR, EMA, EBC, EEE and levodopa treatment significantly increased retention time. When compared with 6-OHDA group, EUR, EMA, EBC and EEE showed significant improvement in bradykinesia as same to levodopa treated group.

Effect of EUR, EMA, EBC and EEE on Catalepsy test (Fore limb placing test)

When compared with sham control group, EUR, EMA, EBC and EEE showed a significant reduction in the catalepsy score as same to that of levodopa. When compared with 6-OHDA control, group 4 to 5 showed less cataleptic score and it indicated increase in dopaminergic activity among that groups.

Effect of EUR, EMA, EBC and EEE on dopamine estimation using HPLC in rats

Dopamine concentration in striatal region was measured by HPLC using UV detector. When compared with sham control animals, 6-OHDA control showed more significant reduction in dopamine concentration, but levodopa showed higher degree of dopamine levels. When

compare with 6-OHDA control, EUR, EMA, EBC and EEE significantly retained the dopamine level.

Effect of EUR, EMA, EBC and EEE on localization of iron in substantia nigra (Perl's DAB iron asymmetry) in rats

The mean Perl's DAB substantia nigra iron asymmetry ratio for the treatment and sham control groups were compared with population mean of 1.00 using one way ANOVA. The sham control animals showed an iron asymmetry ratio of (1.02 ± 0.03). The 6-OHDA animals showed an iron asymmetry ratio of (1.9 ± 0.02). The levodopa treated animals showed an iron asymmetry ratio of (1.73 ± 0.02). The test drugs treatment significantly ($p<0.001$) reduced iron asymmetry ratio.

Effect of EUR, EMA, EBC and EEE on lipid peroxidation level in rats

The lipid peroxidation level in brain tissues were analyzed. When compared with sham control animals the lipid peroxidation was significantly ($P<0.001$) increased for 6-OHDA operated group. When compared with 6-OHDA operated control the level of lipid peroxidation was significantly ($P<0.001$) reduced for the L-DOPA as well as EUR, EMA, EBC and EEE treated groups.

Effect of EUR, EMA, EBC and EEE on catalase levels in rats

When compared with sham control animals the 6-OHDA operated group showed a significant ($P<0.001$) reduction in the level of catalase activity. While compared with 6-OHDA operated groups, the catalase activity was significantly ($P<0.001$) increased for L-DOPA, EUR, EMA, EBC and EEE treated groups.

Effect of EUR, EMA, EBC and EEE on superoxide dimutase levels in rats

The superoxide dismutase was measured and statistically compared. When compared with sham control animals, it showed a significant ($P<0.001$) reduction for 6-OHDA operated group. But when compared with 6-OHDA operated control, the L-DOPA EUR, EMA, EBC and EEE significantly ($P<0.001$) retained the level of SOD.

Effect of EUR, EMA, EBC and EEE on glutathione level in rats

When compared with sham control animals the amount of glutathione reductase was significantly reduced for the treatment with 6-OHDA control, levodopa, EUR, EMA, EBC and EEE. But when compared with 6-OHDA control, levodopa, EUR, EMA, EBC and EEE showed

significant retention of glutathione reductase. The treatment with standard drug, EUR, EMA, EBC and EEE showed retention of glutathione.

Effect of EUR, EMA, EBC and EEE on complex I activity in rats

The complex I activity was estimated from mitochondrial fractions isolated from brain tissue homogenate. When compared with sham control animals the mitochondrial activity was significantly reduced for 6-OHDA, levodopa, EUR, EMA, EBC and EEE. When compared with 6-OHDA group, complex I activity was equally significant with the value of ($P<0.001$), for levodopa, EUR, EMA, EBC and EEE.

Effect of EUR, EMA, EBC and EEE on rat brain mitochondrial calcium level by AAS

Brain mitochondrial calcium concentration in striatal region was measured. When compared with sham control animals, 6-OHDA control showed more significant increase in calcium concentration, but levodopa showed lower degree of calcium levels. When compare with 6-OHDA control, EUR, EMA, EBC and EEE were showed a significant decrease in calcium concentration.

INFERENCES

The efficacy of *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi* in 6-OHDA induced PD has not been well established. In our study, we have demonstrated the anti-Parkinson's activity of not only the above mentioned three plant extract but also evaluated the combined effects of all three extracts.

In this study, first we have demonstrated the Apomorphne induced circling behavior. Apomorphne is a mixed (D_1 and D_2) dopamine receptor agonist that does not share transport or metabolic pathways with L-DOPA and presumably acts by direct stimulation of dopamine receptor[26].

In our study, the circling controversial to the lesion side following the administration of L-DOPA or dopamine agonist result from stimulation of dopamine receptor rendered supersensitive by partial denervation. The lesioned rats showed a greater level of circling behavior and other treatment groups might be replenishing dopamine or already protected dopaminergic nurons in mid brain (SNpc). Further it could presumably suggest the confirmation of nigral lesion in all the treatment groups. Anyhow, the significant levels in comparing the degree of lesioning is not mandatory in our study, because post treatment lesion verification.

Rotarod experiment demonstrated the impairment in the motor function and coordination in Parkinson's rats. Parkinson's rats showed lower fall off time from the rotating rod when compare to control suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. Lack of motor coordination and maintenance of normal limb posture has been reported in PD condition[16]. The treatment with EUR, EMA, EBC and EEE 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, EMA, EBC and EEE increasing muscle co-ordination and thus could co-relate with possible action on CNS.

In our study conditions, EUR, EMA, EBC and EEE reversed the catalepsy induced by 6-OHDA induction. The pyramidal dopamine facilitatory actions may be the possible action of those test drugs.

The turnover of dopamine in nigral cells plays a major role in controlling motor function. In our study, we reported that EUR, EMA, EBC and EEE 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 duration of treatment was for 60 days and it might be a longer duration for rats. The beneficial roles of these drugs in retaining dopamine levels demonstrated the protection of nigral neuron by test drugs. L-DOPA treatment hiked the dopamine levels and it is easily demonstrated by its high concentration after treatment for 60 days. In the test drugs group it was slight decreasing in numerical values of dopamine and it insisted us to further investigate nigral pathology. With this light we assumed that some other factor may contribute for the protection of nigral cells apart from retaining the dopamine levels.

Oxidative stress and oxidative damage to critical biomolecule based on the important process mediating cell death in PD[42]. The studies showed an increase in lipid peroxidation for 6-OHDA control group and L-DOPA treated groups but not for EUR, EMA, EBC and EEE treated groups and the activities of superoxide dismutase and catalase were also mreduced in 6-OHDA group but increased in all other treatment groups. Reduction in GSH might impair H_2O_2 clearance and promote OH radical's formation and produces oxidative stress. The all antioxidant defense mechanisms were related and disturbance on one might damage the balance in all. The depletion in glutathione reductase content and enhancement of lipid peroxidation leads to the degeneration of nigrostriatal neurons and consequently leads to reduction in the content of catecholamine.

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, EMA, EBC and EEE 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, EMA, EBC and EEE in neuroprotection in PD. Because chronic, generalized mitochondrial deficits have been found in sporadic PD[43,44] and the increasing GSH could protect the nigral neurons.

The unilateral nigral lesion produced pronounced levels of Iron accumulation. The brain iron asymmetry data clearly demonstrate the degeneration of transferrin and subsequent deposition of iron in nigral cells. The 6-OHDA increased iron deposition was significantly lowered by EUR, EMA, EBC and EEE. This pharmacological action explained overall protection of test drugs in retarding iron deposition. However the EUR, EMA, EBC and EEE

could reduce iron deposition, and increase mitochondrial activity is a remarkable question. To answer for this question, we have assessed the levels of intracellular calcium in all treatment groups. From that result, it was clear that the EUR, EMA, EBC and EEE significantly reduced intracellular calcium levels and consequent reduction in iron deposition and retain the mitochondrial activity.

So these issues addressed the role of calcium in other factors viz, iron deposition and mitochondrial impairment. This fact further answered with the levodopa treated groups, where iron deposition was more and mitochondrial complex-I activity was improved when compared with the test drug treated groups. This supports the earlier reports of L-DOPA treatment and which leads to iron degeneration and impairs complex-I activity[45].

The Monoamine oxidase system is highly vulnerable to dopamine metabolism. The inhibition of MAO-B caused by these extracts are one of the possible mode of action, in sense the dopamine induced toxicities might be reduced by this enzyme inhibition. The mechanism of action can be correlated with the improvement in mitochondrial functions and low levels of iron degeneration in mid brain regions. Further we identified the possible anti-Parkinson's activity of the test extracts by measuring anti-oxidant enzymes and we found a valid result of anti-oxidant property offered by test drugs. These findings suggested a possible MAO-B associated anti-oxidant mechanism through reduced or controlled production of amido-quinones and para-quinones. These research finding are much promising information to consider the test drugs as anti-Parkinson's agent.

SUMMARY AND CONCLUSIONS

In view of the above facts we are concluding that *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi-* plants showed significant anti-Parkinson's activity, and earlier these plants were ethanopharmacologically proven for its anti oxidant, anti ulcer, antiseptic, antispasmodic, anti diabetic, immunostimulent, anti cancer and CNS activities. The evaluation of anti Parkinson's activities of these plants might be leading to a new drug molecule or herbal moiety which can ameliorate the anti-Parkinson's drug toxicities or can be an anti Parkinson's drug in future. The anti-Parkinson's activity of herbal extracts was performed. The extracts showed significant anti-Parkinson's activity in 6-OHDA lesioned rat models. 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 these drugs in anti-Parkinson's pharmacology and toxicology. From these findings we suggest that, these drug molecules can be a future drug of choice for the treatment of clinical Parkinsonism.

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