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Research Article

Methanolic Extract of *Mucuna pruriens* Seed Acts for Neuroprotection and Antioxidant Defense in A Fish Model

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ABSTRACT
The anti-oxidative and neuroprotective effects of *Mucuna pruriens* seeds and L-Dopa were evaluated in Nile tilapia. Fish were divided into five groups (n=30, 2 replicates). One group was fed control diet, one with diet containing methanolic *M. pruriens* seed extract (0.2 g/kg feed) and one with diet containing L-Dopa (0.034gm/kg) for 30 days. The 4th and 5th group of fish were fed control diet but injected with *Mucuna* extract (0.015gm/mL) and L-Dopa (0.0008gm/mL), respectively, once daily for three days from 27th to 30th day of the feeding period. After 30 days, levels of different enzymatic and non-enzymatic antioxidants, acetylcholinesterase (AChE), monoamine oxidase (MAO), nitric oxide (NO), H_2O_2 protection, hydroxyl radical scavenging, alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT) activities were measured in brain tissue and serum. There was significant ($P<0.05$) increase in AChE, H_2O_2 protection, MAO, superoxide dismutase, glutathione S-transferase, glutathione and glutathione reductase activities in all four treatment groups compared to those in control fish. Glutathione peroxidase, ALP and GPT levels decreased significantly ($P<0.05$) in all treatment groups than those in control.

Keywords: *Mucuna pruriens*, L-Dopa, Neuroprotection, Antioxidant, Methanol extract, Nile tilapia

INTRODUCTION

Neurodegenerative diseases are often considered to be chronic and progressive disorders, with variable symptoms and outcomes. Clinical signs of such disorders are usually related to the topography of the lesions, which involve neuronal degeneration and loss of function[1]. Neurodegeneration can be found in many different levels of neuronal circuitry ranging from molecular to systemic[2]. Intense efforts have been made to comprehend the complex pathogenesis and design effective therapeutics for neurodegenerative diseases. Many neural diseases are associated with deficiencies of neurotransmitters such as dopamine (DA)[3]. Neuropsychiatric disorders such as attention deficit hyperactivity disorder (ADHD), Tourette Syndrome (TS), schizophrenia, psychosis, depression and neurodegenerative diseases like Parkinson's disease (PD), Huntington disease (HD), multiple sclerosis (MS) have been related with depletion of DA and disturbances of DA transmission[4]. Oxidative damage due to formation of free radicals in neurons has also been associated with many neural disorders. Besides, alterations of glutathione (GSH) metabolism in brain have been implicated in oxidative stress and neurodegenerative diseases as well[5].

Fish are often exposed to different xenobiotics such as pesticides, heavy metals and hydrocarbons in the aquatic environment. These harmful substances may have a negative impact on general fish health, increase susceptibility to different neurological diseases in fish and thereby reduce production to affect aquaculture economics[6,7]. Most of these pollutants may directly enhance ROS formation whereas others act indirectly by binding with cellular thiols and reducing antioxidant potential[8].

L-Dopa, the precursor of dopamine has been reported to transport across the blood brain barrier and provide symptomatic benefit to the patients suffering from neurodegenerative disorders. However, chronic exposure to L-Dopa has been found to show detrimental effects on health, promote oxidative DNA damage and increase the levels of oxidized glutathione[9]. Besides, different synthetic chemotherapeutics used for treatment of ROS-related ailments in fish are reported to have residual effect on human after the consumption of such fish[10]. Thus, considerable efforts have been made for development of safe, natural alternatives to synthetic immunostimulating agents in aquaculture from plant materials[11]. Different plant materials have been reported to provide symptomatic relief and help to control neurodegenerative disorders in different animals including fish. Methanolic extract of *Stachytarpheta indica* have been found to show neuroprotective activity on rotenone induced Parkinson's condition in Zebra fish[12].

The seed powder of the leguminous plant, *Mucuna pruriens* has long been used in traditional Ayurvedic

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Indian medicine for treatment of different neurodegenerative diseases including Parkinson's. The plant was reported to contain L-Dopa and other phenolic compounds, and thus might be used as a potent neuroprotecting agent[13]. It was documented to have anti-depressant potency, libido enhancing effect, antispasmodic, antipyretic and anti-inflammatory activity as well[14]. However, no observations have been made related to its *in vivo* effect on the neuroprotective function and anti-oxidant properties in any fish species. The Nile tilapia (*Oreochromis niloticus*), often being cultured in sewage-fed aquaculture system is exposed to many xenobiotic and neurodegenerative agents and thus may act as suitable fish model for studying the neuroprotective and antioxidative efficacy of *M. pruriens*. Considering these aspects, the objective of the present study was to investigate the potential effect of *M. pruriens* as a possible therapeutic source for neuroprotection and antioxidant activities in Nile tilapia. Besides, the efficacy of this plant material on anti-oxidant and redox status in fish brain and specific brain enzymes controlling neuronal activity was compared with those of L-Dopa treatment to understand the suitability of this natural product in lieu of a synthetic chemotherapeutics. Moreover, the plant extract and L-Dopa were administered either through diet or by intraperitoneal injection to determine functional difference in their neuroprotecting efficacy, if any, with respect to different modes of administration.

MATERIALS AND METHODS

Collection of fish

Nile tilapia, *Oreochromis niloticus* (Linnaeus) (mean weight 58.38±3.04 gm and mean length 15.68±0.63 cm) was collected from the Fish Hatchery of West Bengal Government, oxygen packed and transported to the laboratory. In the laboratory, the fish was kept in 45 l aerated aquaria maintaining the physiological parameters such as water temperature (T=27±2ºC), pH (7.2-7.5) and dissolved oxygen (5-5.5 mg/l) at appropriate values.

Plant extract preparation

M. pruriens seeds were procured from the local plant market, washed in sterile distilled water, air-dried in shade and powdered. These powdered plant materials (250 gm) were extracted with 500 ml methanol in a percolator for 48 h at room temperature, evaporated to dryness under pressure at 45ºC using a rotary evaporator and stored under nitrogen at -20ºC in amber glass bottle until it was used.

Determination of plant extract yield

The yield of evaporated dried extract based on dry weight basis was calculated from the following equation:

Yield (%) = $(W_1 \times 100) / W_2$

Where W_1 was the weight of extract after evaporation of the solvent and W_2 was the dry weight of the fresh plant sample.

Determination of L-Dopa content of M. pruriens

Total amount of L-Dopa in the plant extract was measured by a spectrophotometric assay following the standard protocol[15].

Experimental design

After 10 days of acclimatization period fish were randomly distributed in 5 treatment categories (n=30, 2 replicates) as follows:

- Fish fed diets containing methanol extract of *M. pruriens* at concentration of 0.2 gm/kg food for 30 days.
- Fish fed diets containing L-Dopa at concentration of 0.0634gm/Kg food for 30 days.
- Fish given intraperitoneal injection with methanol extract of *M. pruriens* at concentration of 0.015gm/mL with normal physiological saline for 3 consecutive days.
- Fish given intraperitoneal injection with L-Dopa at concentration of 0.0008gm/mL with normal physiological saline for 3 consecutive days.
- Control fish fed diets without plant extract and L-Dopa and without any injection.

The doses of L-Dopa were selected based on the percentage content of L-Dopa in methanol extract of *Mucuna*. The plant extract and L-Dopa was dissolved in ethanol and sprayed over commercial fish feed containing 30% protein (500 ml ethanol/Kg feed). Control feed was sprayed with ethanol only without the addition of the plant extract and L-Dopa. Fish from treatment categories 3 and 4 were fed control diet for 30 days and injection was given for the last 3 consecutive days of culture. After 30 days of treatment, brain tissue and serum samples were collected from each fish from control and all the treatment groups. All samples were stored in -20ºC for biochemical analysis.

Measurement of enzymatic and non-enzymatic antioxidants in brain tissue

Brain tissues were homogenized with Tris buffered saline (10 mM Tris–HCl, 0.1 mM EDTA-2Na, 10 mM sucrose, 0.8% NaCl, pH 7.4), centrifuged and supernatant was used to measure levels of different enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd), glutathione peroxidase (GPx), glutathione S-transferase (GST), malondialdehyde (MDA) and glutathione (GSH) according to standard methods[16]. In brief, SOD activity was measured following a spectrophotometric method based on assessment of $O_2.^-$-mediated nitro blue tetrazolium reduction by an aerobic mixture of NADH and PMS. For measurement of CAT activity, absorbance was monitored at 240 nm up to 90 s at 15 s intervals. The assay was validated by treating the tissue homogenates with sodium azide, a known inhibitor of CAT activity[17]. For GPx activity, absorbance was measured at 492 nm against blank (100 µl extra OPD solution instead of sample). The activity of GRd was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm, in a reaction mixture containing 950 µl of 0.15 mM NADPH, 0.5 mM glutathione, and 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5) and 50 µl extract. The GST activity was spectrophotometrically assessed using glutathione (GSH, 2.4 mM/l) and 1-chloro-2, 4-dinitrobenzen (CDNB, 1 mM/l) as substrate. The level of MDA equivalents was measured in brain by

thiobarbituric acid reactive substances (TBARS) assay with minor modifications. Quantity of the non-enzymatic antioxidative agent GSH in each tissue extract was measured following the method described by Ellman[18].The level of GSH in each sample was calculated by extrapolating the data from the standard graph prepared using GSH. Protein concentrations in the supernatants were determined using Bradford's procedure.

Measurement of activity of brain enzymes

Acetylcholinesterase (AChE) activity in brain homogenates was determined with an AChE kit according to standard method[18]. The activity of 1 U of AChE was defined as the number of hydrolyzed micromoles of acetylthiocholine iodide per minute per microgram of protein. The activity of monoamine oxidase (MAO) was determined using a detection kit, which assessed the production of benzyl aldehyde from the reaction of MAO and its specific substrate, aniline hydrochloride[19]. NO content in brain homogenates was expressed as micromoles per milligram of hypothalmic protein (µmol/mg protein)[20]. NO activity was defined as the amount that increased the time dependence absorbance by 0.01 at 37°C at 401 nm.

Hydroxyl (OH) radical scavenging activity

Deoxyribose assay was used to determine the hydroxyl radical scavenging activity[21]. The reaction mixture containing $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (1 mM) and 2-deoxy-D-ribose (2.8 mM) were mixed with extract of *Mucuna* at various concentrations (10-250 µg) to prepare a final volume of 1 ml with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 hr at 37ºC. The mixture was heated at 95ºC in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm. The negative control without any plant extracts was considered 100% deoxyribose oxidation. The % hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive control.

Hydrogen peroxide (H_2O_2) Protection Assay

H_2O_2 in the tissue samples was measured using a Hydrogen Peroxide Assay Kit (ab102500) using a highly sensitive, simple, direct and HTS-ready colorimetric and fluorometric assay for measuring H_2O_2 in biological samples. In the presence of Horse Radish Peroxidase (HRP), the OxiRed Probe reacts with H_2O_2 to produce product with color (570 nm) and red-fluorescent (Ex/Em=535/587 nm). The detection limit can be as low as 2 pmol per assay (or 40 nM concentration) of H_2O_2 in the sensitive fluorometric assay.

Measurement of serum biochemical parameters

Blood samples were collected and serum were isolated for analysis of biochemical parameters such as alkaline phosphatase activity (ALP), glutamic oxaloacetic transaminase activity (GOT) and glutamic pyruvate transaminase activity (GPT) for different treatment groups using standard protocols[22].

Statistical analysis

All data were expressed as Mean±SD and analyzed by one-way ANOVA. Where F values indicated significance, the means were compared by a post hoc multiple range test taking $P<0.05$ as the threshold.

RESULTS

The yield for methanolic extract of *Mucuna pruriens* seed was 7.80% and the concentration of L-dopa in *Mucuna* extract was 31.7%. All the L-Dopa and *Mucuna* treated groups showed significant variations ($P<0.05$) for several neuroprotective parameters, antioxidant enzymes and serum biochemical enzymes compared to that of control (Table 1, 2, 3). There was significant ($P<0.05$) increase in AChE activity, H_2O_2 protection activity, MAO, SOD, GST, GSH and GRd in all four treatment groups compared to those in control fish (Table 1, 2). GPX, ALP and GPT levels decreased significantly ($P<0.05$) in all treatment groups than those in control (Table 2, 3). No significant difference ($P>0.05$) was observed between the control fish and fish injected with *Mucuna* extract for GOT activity (Table 3).

Intraperitoneal injection with L-Dopa and *Mucuna* extract showed better neuroprotective and antioxidant activity compared to dietary administration for the same (Table 1, 2). The level of H_2O_2 protection assay, AChE activity, SOD, CAT, GRd, GST and GSH levels showed a significant increase ($P<0.05$) for injection with *Mucuna* extract and L-Dopa than those for dietary treatment categories (Table 1, 2). Fish injected with *Mucuna* extract showed the highest level of AChE activity, H_2O_2 protection activity, CAT, GRd and GSH activity, which was significantly higher ($P<0.05$) compared to all other treatment categories (Table 1, 2). Intraperitoneal injection with L-Dopa resulted in the highest activity level for GST, MAO and NO (Table 1, 2). However, fish fed diets containing L-Dopa and *Mucuna* extract showed significantly lower level ($P<0.05$) of MDA compared to fish injected with L-Dopa and *Mucuna* extract, as well as control fish (Table 2). Fish fed diet fortified with *Mucuna* extract showed significant decrease ($P<0.05$) in ALP, GOT and GPT levels compared to those in fish injected with *Mucuna* extract, and similar results were obtained for fish fed L-Dopa fortified diet and injected with L-Dopa (Table 3).

DISCUSSION

Oxidative stress is a multistep pathway ending in imbalance between pro-oxidant and antioxidant defense mechanism and tissue damage[23]. In aquatic environment, fish are often exposed to different pollutants that may cause oxidative stress leading to neurodegeneration[24,25]. The allelochemical L-Dopa is reported to possess differential functional effect on neurons at different concentrations[26]. Thus, efforts should be made to find suitable alternatives to L-Dopa for treatment of neurodegenative diseases. Present study indicated the efficacy of methanol extract from an Indian herb, *Mucuna pruriens* for improvement of brain enzyme synthesis and reduction of reactive oxygen species in a fish model,

Table 1: Effect of *Mucuna pruriens* and L-Dopa treatments on neurological parameters of Nile tilapia.

Neurological Parameters (U/mg protein)	Control	*Mucuna* Injection	L-Dopa Injection	*Mucuna* Feeding	L-Dopa Feeding
OH radical scavenging activity	53.85±2.90^{a}	65.00±3.68^{b}	60.90±1.98^{ab}	56.75±1.34^{ab}	57.00±1.27^{a}
AChE activity	2.400±0.14^{a}	6.27±0.10^{e}	5.55±0.21^{d}	4.86±0.07^{c}	3.81±0.14^{b}
H_2O_2 protection assay	6.76±0.07^{a}	13.50±0.42^{d}	11.20±0.28^{c}	9.60±0.14^{b}	8.76±0.21^{b}
MAO	10.71±0.28^{a}	16.30±0.84^{c}	19.20±0.42^{d}	13.45±0.35^{b}	14.50±0.42^{bc}
NO	0.91±0.03^{a}	2.35±0.21^{bc}	3.00±0.28^{c}	1.55±0.21^{ab}	2.05±0.21^{b}

Notes: OH- Hydroxyl, AChE- Acetylcholinesterase, H_2O_2- Hydrogen peroxide, MAO- Monoamine oxidase, NO- Nitric oxide. Data are Mean±SD (n=6). Different alphabets within same row indicate significant difference ($P<0.05$) in mean values.

Table 2: Effect of *Mucuna pruriens* seed methanol extract and L-Dopa treatments on antioxidant parameters of Nile tilapia.

Antioxidant Parameters (U/mg protein)	Control	*Mucuna* Injection	L-Dopa Injection	*Mucuna* Feeding	L-Dopa Feeding
SOD	41.35±0.64^{a}	53.20±0.42^{c}	51.65±0.35^{c}	45.55±0.50^{b}	44.80±0.14 ^{b}
CAT	10.40±0.28^{a}	16.55±0.35^{d}	15.15±0.21^{c}	11.10±0.14^{a}	12.25±0.35^{b}
GPx	13.30±0.28^{b}	12.60±0.14^{b}	11.35±0.21^{a}	12.85±0.07^{b}	12.70±0.28^{b}
GRd	11.95±0.07^{a}	16.10±0.14^{d}	15.05±0.07^{c}	13.65±0.21^{b}	13.50±0.14^{b}
GST	10.30±0.14^{a}	14.10±0.14^{d}	14.20±0.14^{d}	12.85±0.07^{c}	11.70±0.14^{b}
GSH	12.45±0.07^{a}	19.60±0.14^{e}	18.05±0.07^{d}	15.75±0.07^{c}	14.70±0.28^{b}
MDA	13.60±0.28^{c}	12.47±0.47^{bc}	12.00±0.28^{b}	9.65±0.21^{a}	10.00±0.14^{a}

Notes: SOD - Superoxide dismutase, CAT- Catalase, GPx- Glutathione peroxidase, GRd- Glutathione reductase, GST- Glutathione S-transferase, GSH- Glutathione, MDA – Malondialdehyde. Data are Mean±SD (n=6). Different alphabets within same row indicate significant difference ($P<0.05$) in mean values.

tilapia.

Different antioxidant enzymes act as a defense system against oxidative stress and provide the system a balance. The SOD-CAT system provides the first line of defense against oxygen and hydroxyl radical toxicity[27]. Decrease in the activity of SOD and CAT has been reported to be the most likely primary response against toxicant stress and indicative of failure to neutralize the impact of increased ROS generation[28]. Inhibition of SOD and CAT activities in catfish tissues was reported after long term exposure to toxicant[29]. Reduced glutathione (GSH) acts as the internal non-enzymatic anti-oxidant and most potent free radical scavenger in the cellular level[30]. GRd plays an important role in cellular antioxidant protection and adjustment processes of metabolic pathways, thereby maintains the reduced form of glutathione within threshold level and sustains GSH: GSSG ratio[31]. GPx catalyzes the reduction of H_2O_2 and levels of lipid peroxidation[32]. Together all these enzymatic and non-enzymatic anti-oxidants constitute an important line of defense against oxidative stress. Depletion of antioxidants, or the excessive accumulation of ROS, or both, may lead to tissue damage. MDA, a lipid peroxidation indicator, is generally used to evaluate the levels of oxidative stress[33]. NO acts as an important mediator in cellular signaling mechanism, neurotransmission, cell protection and regulatory machinery in various cells and their endocrinal and physiological status[34]. AChE is involved in synaptic transmission at cholinergic synapses by controlling the action of acetylcholine, whereas MAO plays an important role in the metabolism of different neurotransmitters[35]. Therefore, increase in levels of NO, AChE and MAO in L-Dopa and *Mucuna* extract treated fish groups compared to those in control fish might be indicative of the neuroprotective function of L-Dopa and *Mucuna*.

According to some previous studies, dopaminergic neurons are prone to ROS toxicity and leads to degeneration of the dopaminergic neurons during dopamine metabolism[36]. Overall, ROS can attack cellular components, denaturing and inhibiting proteins, causing damage to DNA and membranes[25]. In this context, some studies have reported contradictory effects of dopamine with respect to the formation of ROS, suggesting a possible antioxidant activity of dopamine[37]. However, the present study showed significant increase in OH^- radical scavenging H_2O_2 protection activities, increase in enzymatic and non-enzymatic antioxidant defense systems, and in agreement with a decreased ROS levels, reduction of lipid peroxidation, after both *Mucuna* extract and L-Dopa treatment. The result of *Mucuna* extract treatment suggested that the plant might have the potential to be used as an effective antioxidant and neuroprotective agent in tilapia. Similar antioxidant and

Table 3: Effect of *Mucuna pruriens* and L-Dopa treatments on serum biochemical parameters of Nile tilapia.

Serum Biochemical Parameters (U/mg protein)	Control	*Mucuna* Injection	L-Dopa Injection	*Mucuna* Feeding	L-Dopa Feeding
ALP	0.28 ± 0.01^{d}	0.24 ± 0.01^{c}	0.20 ± 0.01^{b}	0.12 ± 0.01^{a}	0.13 ± 0.01^{a}
GOT	22.20 ± 0.28^{d}	23.40 ± 0.56^{d}	22.05 ± 0.07^{c}	18.30 ± 0.42^{b}	16.25 ± 0.35^{a}
GPT	1.87 ± 0.28^{e}	1.81 ± 0.50^{d}	1.73 ± 0.21^{c}	1.70 ± 0.07^{b}	1.63 ± 0.07^{a}

Notes: ALP- Alkaline phosphatase, GOT- Glutamic oxaloacetic transaminase, GPT- Glutamic pyruvate transaminase. Data are Mean±SD (n=6). Different alphabets within same row indicate significant difference ($P<0.05$) in mean values.

neuroprotective effects of methanol extract of the plant were observed in carp and catfish as well[24,25].

Enzymes such as ALP, GOT and GPT are normally localized within the cells of the liver, heart, gill, kidney, muscles and other organs. The enzymes are of major importance in assessing and monitoring liver cytolysis and their presence in the serum might give information on organ dysfunction[38]. Injection with either L-Dopa or *Mucuna* extract have resulted in significant increase in the levels of these enzymes with respect to feeding treatment with corresponding material (Table 3). This might be due to probable tissue damage during injection treatment of the fish. Feeding treatment showed lower levels of these enzymes in the serum compared to those in control group, indicating better state of general health for fish fed diets fortified with *Mucuna* extract and L-Dopa. A high level of ALP, GOT and GPT was observed in *Clarias gariepinus* from a polluted aquatic environment[39]. Tilapia exposed to glycophosphate herbicide has been reported to show significant increase in serum concentration of ALP, GOT and GPT[40]. Considering this, dietary administration of the plant extract and L-Dopa might be postulated to be better application method rather than invasive intraperitoneal injection.

CONCLUSION

Reactive oxygen species plays a crucial role in various neurodegenerative diseases. Antioxidant capacity of plant materials is often exploited as a potential mean to reduce such stress related neural disorders. *Mucuna* extract treatment exhibited prominent antioxidant and neuroprotective activity and strongly inhibited lipid peroxidation in fish. The functional efficacy of the plant extract was found comparable to that of a potent neuroprotective compound, L-Dopa. The potential use of this plant material through non-invasive dietary administration might be useful in therapy of free radical pathologies. However, further study will be required to corroborate this observation with other aquatic organisms and higher vertebrates, and for determination of the extent of pharmacological activity of *M. pruriens*.

DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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