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Research Article

NEUROPROTECTIVE EVALUATION OF *LAGENARIA VULGARIS* EXTRACT HYPOXIC NEUROTOXICITY INDUCED RATS

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ABSTRACT

Objective: The present study was aimed to evaluate the neuroprotective effects of *Lagenaria vulgaris* seed oil (LVSO) in hypoxic neurotoxicity induced neurodegeneration in mice.

Methods: Hypoxia was induced by providing sodium nitrite (5 ml/kg) in drinking water to mice for 14 days and mice were evaluated for elevated plus maze test. On 15th day, mice were sacrificed and brain was isolated for estimation of non-enzymatic antioxidants (glutathione [GSH]), enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), total protein (TP) in the brain tissue, and histopathological study.

Results: Treatment with LVSO significantly prevented raise in levels of oxidant enzymes. CAT, GSH, SOD, and TP content have significantly restored in LVSO treated groups whereas toxic group has shown a significant decrease in levels compare to control group. LVSO (0.5 mg/kg) has shown maximum protection. The observed results leading to neurodegeneration is also confirmed by the histopathological differences between treatment and hypoxic mice

Conclusion: LVSO ameliorated antihypoxic effects induced by sodium nitrite and this effect may be mediated by antioxidant mechanism, thereby enhancing neuroprotection.

Keywords: Neuroprotective, Antioxidants, Hypoxia, Seed oil.

INTRODUCTION

Oxidative stress resulting due to the imbalances between the altered production of reactive oxygen species and their removal has been generally recognized as a cause of various diseases. In the central nervous system, a variety of neurodegenerative disorders including Alzheimer's disease and Parkinson's disease and aging process have a common pathology involving oxidative stress [1,2].

The highest degree of oxidative damage occurs in brain, heart, and skeletal muscles as these organs are composed primarily of post mitotic cells. All the cells in our body are equipped with antioxidative enzymes such as superoxide dismutase (SOD), glutathione (GSH) peroxidase, GSH reductase, and reduced GSH. They are generated for protecting the oxidative attack [3-5].

Pharmaceutical approach for novel prevention and treatment strategies of neurodegeneration involves the use of neuroprotective agents in order to delay or stop neuronal cell death or to strengthen cellular defense system. However, there is still lack of effective therapies for such encounters [6]. A number of natural compounds are being used as a brain tonic to help restore debilitated conditions. Since plants produce a significant amount of antioxidants, they represent a potential source of compounds with antioxidant activity. Antioxidants are becoming popular in combating oxidative stress related diseases.

Lagenaria vulgaris (LV), commonly known as bottle-gourd (in English), belongs to the Cucurbitaceae family [7]. The plant is widely available throughout India.

A wide range of chemical compounds including sterols, terpenoids, flavonoids, and saponins have been isolated from the species [8]. Further, antihepatotoxic, analgesic and anti-inflammatory, hypolipidemic, antihyperglycemic, immunomodulatory, and antioxidant activities of its fruit extract have been evaluated [9-13]. LV exhibited stronger *in vitro* free radical scavenging activity and suggests that LV seed extract might play an important role in the oxidation-related deterioration of brain activities.

Hence, the present study was designed to evaluate the protective effect of seed extract of LV in hypoxic neurotoxic-induced mice.

METHODS

Plant material and preparation of extracts

The fruits of LV were obtained from a local market of Kolhapur, Maharashtra (India), which were taxonomically identified and authenticated by Botanical Survey of India, Pune. A voucher specimen was submitted at institute's herbarium department for future reference. The fruit were dried under shade and seeds were removed, dried, and used for further studies. The seeds of LV were coarsely powdered and extracted using n-hexane as a solvent. The extract was concentrated to dryness to obtain oil (yield 58%).

Preliminary phytochemical study

The preliminary phytochemical screening was performed for the presence of different constituents using standard methods [14].

Experimental animals

Adult albino mice weighing around 20-30 g were used in the present study. Animals were housed in polypropylene cages in groups of 5-6 animals per cage under laboratory conditions (alternating light and dark cycle of 12 hrs each). Animals were acclimatized to laboratory conditions before the test. The experimental protocols were approved by the Institutional Animal Ethics Committee and conducted according CPCSEA, New Delhi, India

Acute oral toxicity studies

The acute toxicity was performed according to the OECD 423 guidelines. The extract at the dose of 5, 50, 300, and 2000 mg/kg body weight,

was administered to the rats and they were subsequently observed closely for the first 4 hrs for any untoward symptoms such as tremors, convulsions, exophthalmos, salivation, diarrhea, and lethargy followed by observation for a further 14 days. At the end of the experimental period, the animals were observed for any changes in behavioral pattern and mortality. No toxicity was found in all the four doses, so the extract was declared safe up to 2000 mg/kg.

Induction of hypoxia

Hypoxia was induced by administration of sodium nitrite drinking water (sodium nitrite 30 mg/kg dissolved in normal water) by gavages (5 ml/kg dosing volume) for 14 days except the control group, which was provided with normal water [15,16].

Experimental groups

Group 1: Control mice received normal saline for 14 days.

Group 2: Mice orally received sodium nitrite in drinking water (sodium nitrite 30 mg/kg dissolved in normal water) by gavages (5 ml/kg dosing volume) (hypoxic group).

Group 3: Mice orally received the sodium nitrite in normal saline and administered orally with LV seed oil (LVSO) (0.25 g/kg body weight) for 14 days weeks. The LVSO was administered 60 minutes before sodium nitrite administration.

Group 4: Mice orally received the sodium nitrite in normal saline and administered orally with LVSO (0.25 g/kg body weight) for 14 days weeks. The LVSO was administered 60 minutes before sodium nitrite administration.

During the drug treatment, mice were observed for the behavioral changes for 50 minutes daily. On 14th day after sodium nitrate administration, mice were evaluated for elevated plus maze test. On 15th day, elevated plus maze test was performed and mice were sacrificed and brain were isolated for estimation of GSH, catalase (CAT), lipid peroxidation (LPO), SOD, total protein (TP), and histopathological study.

Parameters monitored

Measurement of body weight change

Animal body weight was noted on the first day and last day of the experimentation. Percentage change in body weight was calculated in comparison to the initial body weight on the first day of the experimentation.

Elevated plus maze test for spatial memory

Memory dysfunction is evaluated using elevated plus maze [17], which consists of two opposite open arms (50 cm × 10 cm), crossed with two closed arms of same dimensions with 40 cm high wall. The arms are connected with central square (10 cm × 10 cm). Acquisition of memory was assessed on day 14th after initiating hypoxic treatment. Mice were placed individually at one end of an open arm facing away from the central square. The time taken by animal to move from open arm and enter into one of the closed arm was recorded as initial transfer latency (TL). Mice were allowed to explore the maze for 30 seconds after recording TL and returned to its home cage. Retention TL was noted again on the 15th day.

Estimation of antioxidant enzyme levels in brain tissue

Preparation of tissue homogenate

The whole brain dissected out, blotted dry, and immediately weighed. The brain regions cerebral cortex, cerebellum, hippocampus, and striatum were subsequently dissected from the intact brain carefully on ice plate (4±2°C). A 10% brain homogenate was prepared with ice-cold phosphate buffered saline (0.1 M, pH 7.4) using Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 rpm at −4°C for 15 minutes and the pellet discarded. The supernatant obtained was used for the quantification of antioxidant levels like GSH, CAT, LPO, SOD, TP levels [18].

CAT

In brief, the incubation mixture contained 0.1 ml of diluted homogenate, 1.0 ml of phosphate buffer and 0.4 ml of distilled water to which 0.5 ml of H_2O_2 solution was added to initiate the reaction, while the H_2O_2 solution was left out in control tubes. After incubating for 1 minutes at 37°C, the reaction was stopped by addition of 2 ml of potassium dichromate acetic acid reagent. The samples were kept in boiling water bath for 15 minutes, finally cooled and the absorbance measured at 570 nm against control. The CAT content was calculated by using molar extinction coefficient = 58.03×10^{-3}/M/cm and the values are expressed as nmoles/mg protein [19].

LPO

Briefly, the reaction mixture contained 0.1 ml of brain regions homogenate (1 mg protein), 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid (0.8% w/v), and 0.2 ml sodium dodecyl sulfate. Following these additions, tubes were mixed and heated at 95°C for 1-hr on a water bath and cooled under tap water before mixing 1 ml of distilled water and 5 ml mixture of n-butanol and pyridine (15:1). The mixture was centrifuged at 2200 g for 10 minutes. The amount of malondialdehyde (MDA)/thiobarbituric acid reactive substances formed was measured by the absorbance of upper organic layer at a wavelength of 532 nm. The results are expressed as nmol MDA/mg protein. The absorbance of the clear pink color supernatant was measured at 532 nm against appropriate blank. The amount of LPO was determined by using molar extinction coefficient 1.56×10^5/M/cm and the results were expressed as nmoles MDA/g of protein [20].

Reduced GSH

The assay is based on the principle of Ellman's reaction. The sulfhydryl group of GSH reacts with 5, 5'- dithiobis-2-nitrobenzoic acid (DTNB) and produces a yellow colored TNB. Measurement of the absorbance of TNB at 412 nm provides an accurate estimation of GSH in a sample. Briefly, 0.5 ml of homogenate is mixed with 0.1 ml of 25% trichloroacetic acid to precipitate proteins and centrifuged at 4000 rpm for 5 minutes. Then 0.3 ml of the supernatant was mixed with 0.5 ml of 0.1 M phosphate buffer (pH 7.4) and 0.2 ml of 10 mM DTNB. This mixture was incubated for 10 minutes and the absorbance was measured at 412 nm against appropriate blanks. The GSH content was calculated by using extension coefficient 13.6×10^3/M/cm. The values are expressed as nmoles/mg protein [21].

SOD

The assay mixture contained 0.1 ml of sample, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of phenazine methosulfate (186 µm), 0.3 ml of nitro blue tetrazolium (300 µm), and 0.2 ml of NADH (750 µm). Reaction was started by addition of NADH. After incubation at 300°C for 90 seconds, the reaction was stopped by the addition of 1 ml of glacial acetic acid. The mixture was allowed to stand for 10 minutes. The color intensity of the chromogen was measured at 560 nm against blank and concentration of SOD was expressed as units/minutes/mg of protein [22,23].

TP

The TP of brain tissue was determined by Biuret method [24,25].

Histopathological study

A section of the brain was fixed with 10% formalin and embedded in paraffin wax and cut into sections of 5 µm thickness. The sections were stained with hemotoxylin and eosin dye for histopathological observations. Depending on the model, either hippocampal or striatal neurons were observed for morphological changes [26].

Statistical evaluation

The data were expressed as mean ± standard error mean. Statistical comparisons were performed by one-way ANOVA followed by Dunnett test t-test using Graph Pad Prism version 5.0. *p<0.5, **p<0.01, ***p<0.001 will be considered as significant compared to toxic control.

RESULTS

Phytochemical analysis

The phytochemical study of seed oil showed the presence of fats and oil, amino acids, phenolic compounds, and flavonoids.

Acute toxicity studies

The mice treated with the extract of LV seed extract up to 2000 mg/kg, p.o., exhibited normal behavior. The extract did not produce any toxic effects or mortality up to the dose level of 2000 mg/kg body weight in mice. Hence, the extract was considered as safe for pharmacological screening.

Effect of LVSO on body weight change in hypoxic induced neurotoxicity in mice

Administration of sodium nitrite (30 mg/kg) resulted in a change in body weight when compared to normal mice. In hypoxic neurotoxic mice, the body weight was significantly (p<0.001) decreased than initial body weight. Treatment with LVSO (0.25 and 0.5 g/kg p.o.) markedly prevented the hypoxic neurotoxicity induced decrease in body weight (Fig. 1).

Elevated plus maze paradigm

In the present experiment, mean initial TL on day 14th day was relatively stable in all the animals within the group except hypoxic mice which showed significant (p<0.01) increase TL compared to normal control animals. Normal control animals entered closed arm quickly and mean TL was shorter when compared to its own TL. However, hypoxic mice performed poorly and showed an increased mean TL compared to its own TL. This indicates there is cognitive dysfunction in hypoxic neurotoxic mice. Pretreatment with LVSO (0.25 and 0.5 mg/kg p.o.) to hypoxic neurotoxic minimum inhibitory concentrations showed significant (p<0.01) improvement in memory performance when compared to hypoxic mice (Fig. 2).

Antioxidant parameters

The result indicated an increase in the levels of MDA and hydroperoxides in hypoxic neurotoxic mice group compared to control group. Treatment with LVSO significantly restored the levels. CAT, GSH, SOD, and TP content have significantly increased in LVSO treated groups whereas toxic group has shown a significant decrease in levels compare to control group. LVSO (0.5 mg/kg) has shown maximum protection (Fig. 3). Induction of hypoxia significantly (p<0.001) increased the acetylcholinesterase activity and brain glutamate level when compared with the control group.

Histopathological study

The results of histopathological study showed that there are no morphological changes of the brain in the normal animals. The CA3 region shows intact pyramidal cells in tight clusters. Hippocampus was found to be normal. In hypoxic induced neurotoxicity in mice, the CA3 region shows decreased number of intact pyramidal cells and most of the pyramidal cells show degenerative changes. Treatment with CVSO effectively reduced the degenerative changes in pyramidal cells. There was very few of the pyramidal cells showing degenerative changes, thereby indicating CVSO prevented hippocampal damage (Fig. 4).

DISCUSSION

The present study revealed the neuroprotective effects of n-hexane extract of LV on sodium nitrite induced hypoxia deficits in mice. During hypoxia, changes will occur in the diffusion parameters of the extracellular concentration of energy-related metabolites and glutamate in rat cortex. Hypoxia in brain cortex has shown a decrease in space volume and in toxicity within few minutes followed by cardiac arrest. In the LVSO treated groups 0.25 mg/kg and 0.5 mg/kg, there was a significant reduction in enzyme levels when compared with the

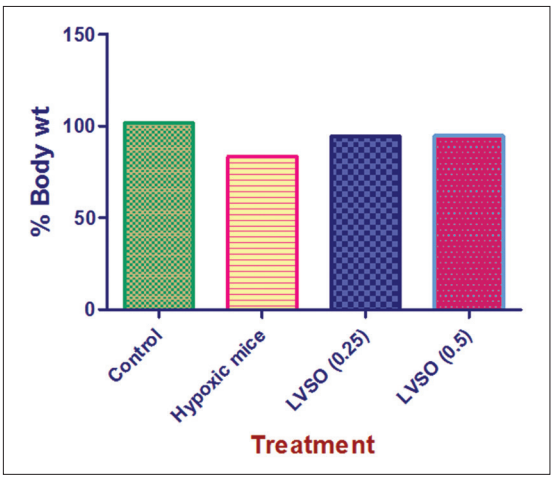

Fig. 1: Effect of *Lagenaria vulgaris* seed oil on body weight change in hypoxic neurotoxicity in mice

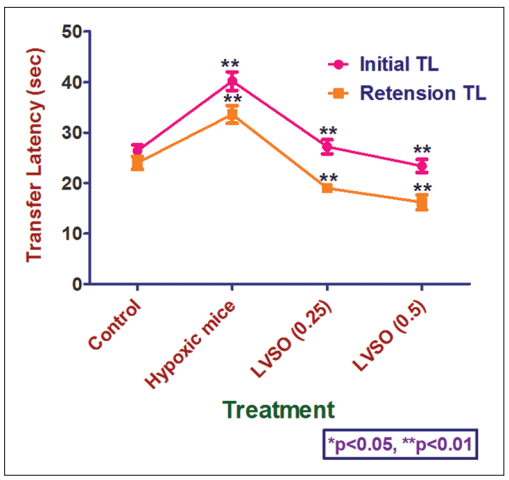

Fig. 2: Effect of *Lagenaria vulgaris* seed oil on transfer latency in hypoxic neurotoxicity in mice

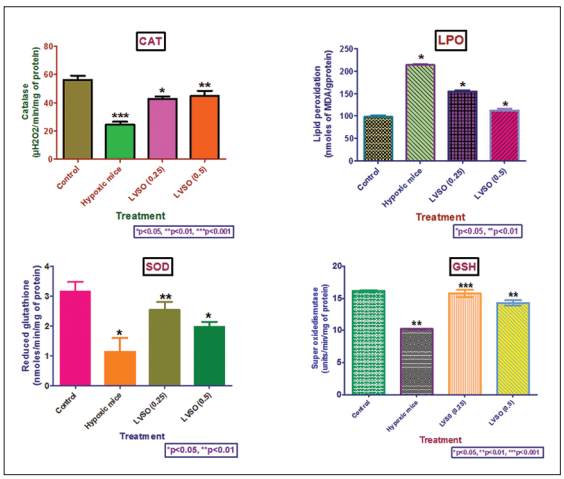

Fig. 3: Effect of *Lagenaria vulgaris* seed oil on levels of catalase, lipid peroxidation, glutathione, and superoxide dismutase in hypoxic neurotoxicity in mice

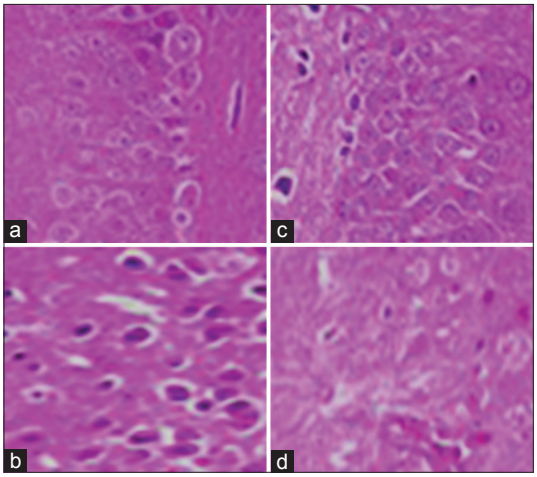

Fig. 4: Histopathological observations of brain in hypoxic neurotoxicity in mice, (a) Control group, (b) hypoxic group, (c) Lagenaria vulgaris seed oil (LVSO) (0.25 g/kg), (d) LVSO (0.5 g/kg)

hypoxia group. The involvement of oxidative damage and mitochondrial dysfunction are implicated in hypoxic toxicity along with possible involvement of excitotoxicity and generation of free radicals [27]. In the present study, hypoxic neurodegeneration, produced oxidative stress increased levels of LPO (as evident by increased MDA levels), and depleted levels of an endogenous antioxidant enzyme (CAT, reduced GSH, SOD levels). These results support the oxidative stress based theory of neurotoxicity caused [28]. Treatment with the LVSO has produced a more significant reversal of CAT, GSH, and SOD suggesting the possible involvement of antioxidant action of LVSO in preventing hypoxic neurotoxicity. The observed results leading to neurodegeneration is also confirmed by the histopathological differences between treatment and hypoxic mice. There was a reversal of the brain damage observed in LVSO treated animals and it prevented the neuron loss.

CONCLUSION

Our findings show that long-term intake of LVSO improves antihypoxic effects induced by sodium nitrite, and this effect may be mediated by antioxidant mechanism thereby enhancing neuroprotection. The findings support further investigation of the potential of LV as neuroprotective agents.

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