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Cerebroprotective effect of *Glycyrrhiza glabra* Linn. root extract on hypoxic rats

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Abstract

The present study was carried out to evaluate the cerebroprotective effect of the aqueous extract of the roots of *Glycyrrhiza glabra* Linn. (250 and 500 mg/kg) in hypoxic rats. Hypoxia was induced by providing sodium nitrite drinking water to rats for 14 days. Extract at the tested doses promoted the locomotor activity and spatial behavior significantly, which was impaired in hypoxic rats. The extract administration restored the decreased levels of brain enzymes such as glutamate and dopamine and decreased acetylcholinesterase (AChE) activity significantly. Levels of anti-oxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase were reduced due to hypoxia and were restored to near normalcy by administration of ethanol extract of *G. glabra*. Increased lipid peroxidation in hypoxic rats was also restored significantly by extract treatment. Thus, this study suggests that ethanol extract of *G. glabra* possess a cerebroprotective effect in hypoxic rats, which may be mediated by its anti-oxidant effects.

Introduction

Oxidative stress is implicated as one of the primary factors that contribute to the development of neurodegenerative diseases like, Alzheimer's, Parkinsonism and neurological conditions like epileptic seizures, stroke, brain damage, neurotrauma, hypoxia etc (Srinivasan, 2002). The highest degree of oxidative damage usually occurs in organs like brain, heart and skeletal muscles, since these organs are composed primarily of post mitotic cells. the central nervous system shows increased susceptibility to oxidative stress because of its high oxygen consumption rate (20% of the total oxygen inhaled by the body) that accounts for the increase in generation of oxygen free radicals and reactive oxygen substances like superoxide radical (O_2), single oxygen (O_2), H_2O_2 and hydroxyl radical (OH) (Srinivasan, 2002).

All the cells and tissues of our body are also equipped with anti oxidative enzymes like super oxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GRD) and substances like reduced glutathione (GSH). They dispose the free radicals as and when they are generated there by protecting the cells and tissues from the oxidative attack. Normally the balance is maintained between the oxidative attack of the free radicals and the anti-oxidative defense system prevailing in the cells and tissues of our body (Srinivasan, 2002).

Glycyrrhiza glabra Linn. of the family Leguminosae, is a genus of perennial herbs and under shrubs distributed in the subtropical and warm temperate regions of the world, chiefly in the Mediterranean countries and China. The dried, peeled or unpeeled underground stems and roots constitute the drug known in the trade as liquorice (Anonymous, 1985).

The roots and rhizomes possess demulcent, anti-inflammatory, anti stress, anti depressive and expectorant property and useful in the treatment of peptic ulcer. Carbenoxolone, one of the oleandane derivatives from prepared from *G. glabra* possess considerable mineralo-

corticoid activity (Khare, 2007). The roots and rhizomes of *G. glabra* has been studied with respect to spatial learning and passive avoidance (Ravichandra et al., 2007); anxiolytic activity (Ambawade et al., 2001); memory enhancement (Dinesh et al., 2004); preliminary free radical scavenging (Toshio et al., 2003); cerebral ischemia (Zhan and Yang, 2006) and anti-oxidant capacity towards LDL oxidation (Vaya et al., 1998).

Accordingly, the present study was designed to explore the potential of *G. glabra* roots to protect brain from hypoxic damage induced by sodium nitrite.

Materials and Methods

Plant material

Roots of *G. glabra* were purchased from an herbal drug store in Chennai, Tamil Nadu, India during August 2007. The material was identified by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai. A voucher specimen was deposited in our laboratory for future reference.

Preparation of extract

The roots were shade dried and pulverized in a grinder to a coarse powder. Weighed quantity (1 kg) was passed through sieve number 40 and subjected to soxhlet extraction using distilled water. The extract was dried and weighed. A brownish black waxy residue with 21.1% w/w yield was obtained. This aqueous extract of *G. glabra* was used for animal administration.

Experimental animals

Inbred colony strains of Wister rats of either sex weighing 150-250 g procured from the animal house of C. L. Baid Metha College of Pharmacy were used for the study. The animals were maintained in polypropylene cages of standard dimensions at a temperature of 28 ± 1°C and standard 12:12 hours day/night rhythm. The animals were fed with standard rodent pellet diet (Hindustan Lever Ltd.) and water *ad libitum*. Prior to the experiment, the animals were acclimatized to the laboratory conditions. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) constituted under CPCSEA. (IAEC Ref No: IAEC/XIII/06/CLBMCP/2007-08 dated 24-07-2007).

Preliminary phytochemical study

Preliminary phytochemical screening was performed as per procedure of Kokate et al. (2004).

Acute toxicity studies

Albino mice weighing 22-25 g selected by random sampling technique were used in the study. Acute oral toxicity was performed as per OECD-423 guidelines (Ecobichon, 1997). The animals were fasted overnight, provided only water after which aqueous extract was administered to the groups orally at the dose level of 5 mg/kg body weight by gastric intubation and the groups were observed for 14 days. If mortality was observed in 2 or 3 animals among 6 animals then the dose administered was assigned as a toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2,000 mg/kg body weight. The animals were observed for toxic symptoms such as behavioral changes, locomotion, convulsions and mortality for 72 hours.

Induction of hypoxia

Hypoxia was induced by administration of sodium nitrite water (30 mg/kg body weight of sodium nitrite dissolved in normal water) by gavage (5 mL/kg dosing volume) for 14 days except the control group, which was provided with normal water (Hirneth and Classen, 1984)

Experimental design

The animals were divided into 4 groups of 6 rats each as follows: Group I served as control and received normal saline. Group II served as hypoxic rats and received sodium nitrite water for 14 days. Group III animals received extract (250 mg/kg; p.o) suspended in 1% gum acacia and sodium nitrite water for 14 days. Group IV animals received extract (500 mg/kg; p.o) suspended in 1% gum acacia and sodium nitrite water for 14 days.

Locomotor activity

The locomotor activity was monitored by using actophotometer. The cognitive effect was measured by placing the animals in the actophotometer and the readings were recorded at 0, 30, 60, 90, 120 and 150 min for 10 min. The locomotor activity was expressed in terms of total photo beam interruption counts/min/animal (Turner, 1965).

Water maze task

The Morris water maze was performed using a circular water tank of standard dimensions (Morris, 1984). After several trails, the test was conducted on the 14 day of sodium nitrite water drink. The time required to escape on to the platform was recorded.

Estimation of dopamine and metabolic enzymes

On the day of experiment rats were sacrificed, whole brain was dissected out and separated the sub cortical region (including the striatum). Weighed quantity of tissue and was homogenized in 0.1 mL hydrochloric acid-butanol, (0.9 mL of 37% hydrochloric acid in one liter *n*-butanol for spectroscopy) for 1 min in a cool environment. The sample was then centrifuged for 10 min at 2,000 rpm. 0.08 mL of supernatant phase was removed and added to an Eppendorf reagent tube containing 0.2 mL of heptane (for spectroscopy) and 0.025 mL 0.1 M hydrochloric acid. After 10 min of vigorous shaking, the tube was centrifuged under same conditions to separate two phases. Upper organic phase

was discarded and the aqueous phase (0.02 mL) was used for dopamine estimation (Schlumpf et al., 1974).

Estimation of dopamine- To this phase 0.005 mL of 0.4 M hydrochloric acid and 0.01 mL EDTA/sodium acetate buffer (pH 6.9) were added, followed by 0.01 mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0.01 mL Na_2SO_3 in 5 M NaOH (0.5 g Na_2SO_3 in 2 mL H_2O + 18 mL 5 M NaOH). Acetic acid (0.01 mL, 10 M) was added 1.5 min later. The solution was then heated to 100°C for 6 min. when the sample again reached room temperature, excitation and emission spectra were read in the microcuvette. The readings were taken at 330-375 nm (Schlumpf et al., 1974).

Estimation of acetylcholinesterase (AChE)- 20 mg of brain tissue per mL of phosphate buffer (0.1 M; pH 8) was homogenized in a potter-elvehjem homogenizer. A 0.4 mL aliquot of brain homogenate was added to a cuvette containing 2.6 mL of 0.1 M phosphate buffer. 100 µL of dithiobisnitro benzoic acid reagent was added to the cuvette and the absorbance was measured at 412 nm. 20 µL of acetylcholine chloride was added. A change in the absorbance was recorded and the change in absorbance/min was calculated. It was expressed in µ mol/min/g tissue (Ellman et al., 1961).

Brain glutamate estimation- Weighed quantity of the brain portion was homogenized with 2 parts by weight of perchloric acid and centrifuged for 10 min at 3,000 rpm. 3 mL of supernatant fluid was adjusted to pH 9.0 with 1 mL phosphate solution. It was allowed to stand for 10 min in an ice bath and then filtered through fluted filter paper. Absorbance was measured at 340 nm. Similarly a blank reading at 340 nm was measured. The level of glutamate was expressed as µmol/g tissue (Bernt and Bergmeyer, 1965).

Estimation of anti-oxidant enzymes- 100 mg of the brain tissue was weighed and homogenate was prepared in 10 mL tris hydrochloric acid buffer (0.5 M; pH 7.4) at 4°C. The homogenate was centrifuged and the supernatant was used for the assay of cyto-protective enzymes namely catalase (Aebi, 1983); glutathione peroxidase (Lawrence and Burk, 1976); superoxide dismutase (Marklund and Marklund, 1974); glutathione reductase (Dobler and Anderson, 1981) and lipid peroxidation (Luck, 1965).

Results

The preliminary phytochemical screening carried out on aqueous extract of *G. glabra* revealed the presence of phytoconstituents such as alkaloids, carbohydrates, saponins, triterpenoids, flavanoids and flavones.

The extract did not produce any toxic symptoms of mortality up to the dose level of 2,000 mg/kg body weight in rats, and hence the drugs were considered safe for further pharmacological screening. According to the OECD-423 guidelines for acute oral toxicity, the LD_{50} dose of 2000 mg/kg and above is categorized as unclassified.

There was an increase in escape latency in negative control group in the water maze task when compared with the control group ($p<0.01$). The groups treated with 250 mg/kg and 500 mg/kg aqueous extract showed the significant decrease in the escape latency ($p<0.05$ and $p<0.01$) respectively. The hypoxia induced group (negative control) indicated decrease in locomotor activity ($p<0.01$) in comparison with the control group (Table I). The extract treated groups exhibited significant ($p<0.05$ and $p<0.01$) increase in locomotor activity respectively for 250 mg/kg and 500 mg/kg of aqueous extract when compared with the negative control group (Table II). Induction of hypoxia significantly ($p<0.01$) increased the AChE activity and brain glutamate level when compared with control group. In the aqueous extract-treated groups, (250 and 500 mg/kg) there was a significant ($p<0.01$) reduction in enzyme levels when compared with the hypoxic

Table I

Effect of *G. glabra* on water maze task

Group	Escape latency (sec)
I	29.0 ± 4.1
II	57.5 ± 8.2[b]
III	37.7 ± 2.5[a]
IV	30.6 ± 3.3[b]

Values are mean ± SEM; n = 6; [a]p<0.05, [b]p<0.01

Table II

Effect of *G. glabra* on locomotor activity

Group	Time					
	0 min	30 min	60 min	90 min	120 min	150 min
I	375 ± 30.1	405 ± 36.4	401 ± 33.9	416 ± 20.3	425 ± 15.2	417 ± 9.5
II	201 ± 15.5[b]	224 ± 17.5[b]	231 ± 16.3[b]	239 ± 20.2[b]	245 ± 18.5[b]	257 ± 20.2[b]
III	260 ± 14.7[a]	274 ± 10.6[a]	291 ± 11.9[ns]	320 ± 16.4[a]	325 ± 16.5[a]	350 ± 18.0[a]
IV	340 ± 27.4[b]	390 ± 11.1[b]	395 ± 28.1[b]	410 ± 21.0[b]	427 ± 17.9[b]	429 ± 10.5[b]

Values are mean ± SEM; n = 6; [a]p<0.05, [b]p<0.01, ns = not significant

Table III

Effect of *G. glabra* on brain enzymes

Group	AChE (µmol/ min/mg protein sec)	Glutamate (µmol/ g tissue)	Dopamine (pg/mg tissue)
I	16.3 ± 0.1	73.8 ± 1.3	642.3 ± 14.6
II	19.3 ± 0.1^{a**}	85.3 ± 1.5^{a**}	521.5 ± 10.6^{a}
III	18.0 ± 0.1^{b*}	78.7 ± 1.3^{b**}	580.6 ± 11.9^{b*}
IV	16.0 ± 0.1^{b}	74.5 ± 1.4^{b**}	592.5 ± 12.2^{b**}

Values are mean ± SEM; n = 6; *p<0.05, **p<0.01; ^{a}Group I vs. Group II; ^{b}Group II vs. Groups III and IV

group. The dopamine levels in negative control group was significantly decreased (p<0.01) when compared with the control group. The levels of dopamine in 250 and 500 mg/kg of aqueous extract treatment, significantly increased the reduced dopamine level (p<0.05 and p<0.01) respectively (Table III).

The levels of cytoprotective (anti-oxidant) enzymes such as super oxide dismutase, glutathione peroxidase, glutathione reductase and catalase were reduced (p<0.01) due to induction of hypoxia in Group II, with an increase in lipid peroxidation (p<0.01). Administration of aqueous extract increased the levels of the enzymes significantly (p<0.05 and p<0.01) for 250 and 500 mg/kg respectively. Lipid peroxidation was significantly decreased (p<0.01) by the administration of aqueous extract 250 and 500 mg/kg (Table IV).

Discussion

The present study has revealed the neuroprotective effect of aqueous extract of *G. glabra* on sodium nitrite induced hypoxia deficits in rats. *G. glabra* is a medicinal plant with anti-oxidant properties. Previous studies show that oral administration for seven days protect against cerebral ischemia damage, cerebral energy metabolism, brain Na^+K^+ATPase activity, malondialdehyde content, total adenine nucleotides in dependent manner. Na^+K^+ATPase responsible for establishing the electrochemical gradient of Na^+ and K^+ ions across the cell membrane in central nervous system (Tao et al., 2001).

During hypoxia, changes will occur in the diffusion parameters of the extracellular space and extracellular concentration of energy–related metabolites and glutamate in rat cortex. Hypoxia in rat cortex has shown decrease in extracellular space volume and increase in tortuosity with in few minutes following cardiac arrest. Glucose levels were critical in regulating redox state during hypoxia; the cellular redox state was determined by increasing the reactive oxygen species. Supply of glucose increases cellular redox state and lowers the reactive oxygen species and cell death (Holingan and Jain, 2006).

Exposure to hypoxia alters the dendritic carbonization of hippocampal neurons and impairs the spatial learning and memory. Hypoxia will affect dendric morphology of the CA1 neurons. Exposure to more days result in significant reduction in branching points, intersections and dendric length in most of the segments, significantly elevated levels of calcium and protein synthesis may lead to delayed neuronal death in CA1 region after hypoxia. The CA3 cells have higher level of metabolic activity than the CA1 cells and this may make them more vulnerable to hypoxia (Titus et al., 2007)

Glutamate is a major excitatory neurotransmitter in brain. Down regulation of glutamate transporter expression and uptake, activity was observed during hypoxia. GABA levels were highly correlated with endogenous glutamate levels during hypoxia, it increases GABA levels when glutamate level raises above the normal level when glutamate level decreases GABA level decreases automatically inhibitors of glutamate decarboxylase and GABA transaminase suggested that increased synthesis and decreased catabolism may both contribute to increase in GABA levels during hypoxia (Ales et al., 2006). ATP depletion effects on the release and redistribution of glutamate and aspartate in rat hippocampal slices, glutamate is released during ATP depletion by reversal of co-transporters.

Table IV

Effect of *G. glabra* on anti-oxidant enzymes

Parameter	Group I	Group II	Group III	Group IV
Superoxide dismutase (U/mg)	8.1 ± 0.3	5.6 ± 0.1^{a**}	6.9 ± 0.4^{b*}	8.04 ± 0.3^{b**}
Catalase (µmol/mg)	2.3 ± 0.03	1.6 ± 0.1^{a**}	1.9 ± 0.05^{b*}	2.2 ± 0.1^{b**}
Glutathione peroxidase (U/mg)	34.1 ± 1.2	26.2 ± 1.0^{a**}	31.0 ± 0.7^{b*}	34.8 ± 1.4^{b**}
Glutathione reductase (U/mg)	31.7 ± 0.4	24.7 ± 0.4^{a**}	27.9 ± 0.3^{b**}	32.1 ± 0.6^{b**}
Lipid peroxidation (nmol TABRS/mg)	2.6 ± 0.04	4.4 ± 0.05^{a**}	3.8 ± 0.1^{b**}	2.2 ± 0.1^{b**}

Values are mean ± SEM; n = 6; *p<0.05, **p<0.01; ^{a}Group I vs. Group II; ^{b}Group II vs. Groups III and IV

The treatment with aqueous extract ameliorated cognitive deficits in sodium nitrite drinked rats. In water-maze test consumption of aqueous extract decreased the escape latency almost to normal levels in dose-dependent manner; it is possible that neuroprotection plays a role in favorable effect of *G. glabra* on sodium nitrite induced cognitive effects.

The AChE activity has been shown to be increased with in and around hypoxic brain. The calcium influx followed by oxidative stress is involved in the increase in activity of AChE induced by sodium nitrite, decreasing cell membrane order and ultimately leading to the exposure of more active sites of the enzyme. Increasing in AChE activity and ROS production indicates that it can be possible to ameliorate cholinergic function by inhibiting sodium nitrite, which induces increase in AChE activity.

The AChE activity in the brain was increased in rats treated with sodium nitrite when compared with the normal; in addition, the sodium nitrite induced increase in AChE was attenuated by aqueous extract treatment.

Conclusion

This study suggests that markedly aqueous extract improves antihypoxic effects induced by sodium nitrite and this effect may be mediated by its anti-oxidant properties.

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