

EFFECT OF ETHANOLIC EXTRACT OF TINOSPORA CORDIFOLIA ON OXIDATIVE STRESS INDUCED BY CEREBRAL ISCHEMIA-REPERFUSION IN RATS

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ABSTRACT

Restoration of blood flow to ischemic brain is linked with generation of reactive oxygen species. In Ayurveda, the medicinal properties of *Tinospora cordifolia* have been attributed to its anti-stress, antioxidant, neuroprotective, adaptogenic and nootropic properties. The present study investigates the effect of ethanolic extract of *T. cordifolia* on acute cerebral ischemia-reperfusion in rats. Acute cerebral ischemia-reperfusion (30 min occlusion of bilateral common carotid arteries followed by 45 min reperfusion) in Charles Foster (C.F.) strain rats was produced following standard technique. Effect of *Tinospora cordifolia* on lipid peroxidation, superoxide dismutase (SOD) activity, ascorbic acid, cyclic AMP level and total tissue sulfhydryl (T-SH) group in for brain region in acute cerebral ischemia-reperfusion were evaluated. *T. cordifolia* pre-treatment (100 mg/kg p.o. for 7 days) attenuated the reperfusion induced biochemical alterations. The results suggest protective role of *T. cordifolia* in cerebral ischemia reperfusion injury.

KEYWORDS: Ischemia, Reperfusion injury, Oxidative stress, Neuroprotection, *Tinospora*.**INTRODUCTION**

A number of herbal drugs have been evaluated for their possible role in neurodegenerative disorders and cognitive functions. *Tinospora cordifolia* (TC) or Amrita or Giloya in Hindi and Guduchi in Sanskrit in Ayurveda (the classical Indian system of medicine), has been used for centuries, for a variety of diseases.^[1] *Tinospora cordifolia* (Guduchi) is a large glabrous, deciduous, climbing shrub of Menispermaceae family found throughout tropical India.^[2] It is classed as Medhya Rasayana (Learning and memory enhancer).^[3] Neuroprotective and ameliorative properties are due to their antioxidant and trace element contents.^[4] *Tinospora cordifolia* is known to be a rich source of trace elements (Zinc and Copper) which act as antioxidants and protects cells from the damaging effects of oxygen radicals generated during immune activation.^[5] *Tinospora cordifolia* has been claimed to possess learning and memory enhancing,^[6] antioxidant,^[7,8] and anti-stress activity.^[9] *Tinospora cordifolia* enhanced the cognition in normal and cognition deficits animals.^[10] Mechanism of cognitive enhancement is by immunostimulation and increasing the synthesis of acetylcholine, this supplementation of choline enhances the cognition.^[11] Guduchi may also be attributed to its immunomodulatory properties.^[12]

Chemical constituents' classes are alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds, lignans and polysaccharides.^[13] The polyphenols have the ability to penetrate the blood brain barrier and act as potential neuroprotective agent.

A majority of the present day disease are reported to be due to shift in the balance of pro-oxidant and antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of free radicals caused by excessive oxidative stress or due to their poor scavenging in the body caused by gradual decline in antioxidant defence mechanism.^[14] Oxidative free radicals play an important role in cerebral ischemia as well as reperfusion injury which is a distinct entity from the primary ischemia injury. This study was designed to assess the neuroprotective activity of ethanolic extract of *T. cordifolia* on acute cerebral ischemia-reperfusion.

MATERIALS AND METHODS**Drug and reagents**

1, 1, 3, 3-Tetraethoxypropane (TEP), (Merck, Germany), Thiobarbituric acid (TBA), NADH, nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) (Sigma, USA) were used. All other chemicals and reagents were of the highest analytical grades available.

The raw material of test formulation was procured from Ayurvedic Garden; Banaras Hindu University campus in the month of January. It was authenticated in Department of Dravya Guna, Faculty of Ayurveda, IMS, BHU. The specimen copy has been kept in department for future references. Dried and powdered stem of *T. Cordifolia* was defatted with petroleum ether to remove lipids and fats, and then the residue was extracted with ethanol using soxhlet apparatus. The ethanolic extract was evaporated under reduced pressure at 40°C, using a rotary vapour evaporator. The extract was kept in 4°C for further analysis and experimental studies. The dose selection for the experimental studies was made according to our initial pilot experimental results.

Animals

After approval of Institutional Ethical Committee, the present study was conducted on inbred CF male albino rats weighing 250-300g, obtained from the central animal house of the Institute of Medical Sciences, Banaras Hindu University, Varanasi. They were kept in the departmental animal house in colony cages at an ambient temperature of 25±2°C and 45-55% relative humidity with 10:14 h light: dark cycles. They had free access to standard rodent pellet diet and drinking water. The food was withdrawn 18-24h before the surgical procedure, however, water was allowed ad libitum. Principles of laboratory animal care (NIH Publication No. 86-23, revised 1985) guidelines were followed throughout the experiments.

Experimental Procedure

Surgical Procedure

Surgical technique for induction of cerebral ischemia by bilateral common carotid artery occlusion (BCCAO) was adapted from earlier published method of Iwasaki *et al.*^[15] Rats were anaesthetized by ketamine (100 mg kg⁻¹, i.p). After a midline skin incision in the neck, both common carotid arteries were identified and isolated carefully from accompanying vagosympathetic nerve.

Acute ischemia-reperfusion injury was produced by blocking bilateral common carotid arteries (BCCA) for 30 min (lifting arteries with the help of thread) and reperfusion for 45 min was allowed by releasing the thread. Body temperature was maintained at about 37°C. This protocol was adopted on the basis of earlier reports from our laboratory and elsewhere.^[16,17]

Study Design

The animals were divided into four groups of six animals each. First group served as sham-operated control (underwent all surgical procedure except BCCAO). In second group, ethanolic extract of *T.cordifolia* was administered to sham-operated animals to determine effect of drug per se. Third group of animals underwent 30 min BCCAO and 45 min reperfusion. In the fourth group (treatment) *T.cordifolia* 100 mg/kg/day, p.o. for 7 days, was administered before subjecting animals to ischemia-reperfusion.

Biochemical analysis

At the end of experiments animals were sacrificed by decapitation and frontoparietal part of cerebral cortex from both the hemispheres were separated. After rinsing with ice-cold normal saline the brain tissue were transferred to the appropriate homogenizing medium and analyzed for the biochemical parameters of the oxidant-antioxidant status i.e. thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) activity, tissue total sulfhydryl (T-SH) level, ascorbic acid and cyclic AMP. All the procedures on the brain samples were performed on ice or ice bath and sample were kept at -20°C. For all biochemical parameter studies, frontoparietal part of cerebral cortex of both the hemispheres was analysed.

Lipid peroxidation

Estimation of lipid peroxidation was done by measuring the lipid peroxidation product TBARS (Thio Barbituric Acid Reactive Substances) following the method of Ohkawa *et al.*^[18] TEP was used as an external standard, and the level of lipid peroxidation was expressed as nanomoles TBARS mg⁻¹ of protein.

Superoxide dismutase (SOD)

SOD was estimated by adopting the procedure of Kakkar *et al* and results are expressed in milliunits mg⁻¹ of protein.^[19]

Total tissue sulfhydryl groups (T-SH)

Total T-SH in brain was measured according to the method of Sedlack and Lindsay. The level of T-SH was expressed as moles of SH 100⁻¹ g of wet tissue weight.^[20]

Ascorbic acid

Ascorbic acid levels were determined by the method of Omaye *et al* and the results are expressed in terms of mg/100g wet weight.^[21]

Estimation of brain total protein

The protein content of brain tissue was estimated using the method of Lowry *et al.*^[22]

Cyclic AMP estimation

Cyclic AMP estimation of frontoparietal part of forebrain was done by ELISA using EIATM cyclic AMP kit (Assay Designs Inc., USA). This kit uses a polyclonal antibody to cyclic AMP which binds, in a competitive manner with the cyclic AMP. Results were expressed as nmol of cyclic AMP per g (wet weight) of tissue.

Statistical Analysis

Statistical analysis was performed by applying one-way Analysis of Variance (ANOVA) followed by post hoc Tukey Test for biochemical parameters. A p-value of <0.05 was considered statistically significant.

RESULTS

Acute BCCAO for 30 min followed by 45 min reperfusion induced increase in lipid peroxidation

(TBARS), superoxide dismutase (SOD), activity and fall in T-SH levels. Ethanolic extract of *T.cordifolia* pre-treatment attenuated enhanced TBARS level ($p < 0.01$) and SOD activity ($p < 0.01$) as well as prevented the consumption of T-SH significantly ($p < 0.01$) following cerebral ischemia reperfusion injury. *T.cordifolia* per se had no significant effect on any of these biochemical parameters (Table 1). Ischemia followed by reperfusion increased cyclic AMP level significantly as compared to

that in sham-operated animals ($p < 0.05$). *T.cordifolia* pre-treatment of ischemia reperfused animals led to a significant rise in cyclic AMP level compared to ischemia reperfusion group ($p < 0.01$) (Table 2). Ascorbic acid levels, however, did not show any change after reperfusion injury and/or *T.cordifolia* pre-treatment. Thus total ascorbic acid levels appear unaffected during reperfusion injury (Table-1).

Table 1: Effect of *T.cordifolia* (100 mg/kg p.o. x 7 days) on biochemical parameters of oxidative stress in rat forebrain following cerebral ischemia-reperfusion injury (30 min BCCAO followed by 45 min reperfusion).

Groups	TBARS (nmol/mg protein)	SOD (MU/mg protein)	T-SH ($\times 10^{-5}$ M/mg protein)	Ascorbic Acid(mg/100g wet weight)
Sham-operated control	2.01 \pm 0.51	312.18 \pm 85.00	4.78 \pm 0.45	10.16 \pm 3.01
Per Se	1.95 \pm 0.27	338.01 \pm 79.66	3.91 \pm 0.40	9.33 \pm 1.95
Ischemia-reperfusion	3.95 \pm 0.67	646.80 \pm 115.56	2.46 \pm 0.20	7.80 \pm 2.13
<i>T.cordifolia</i>	2.67 \pm 0.54	405.52 \pm 80.19	3.42 \pm 0.37	7.20 \pm 2.46

All data is expressed as mean \pm SD, n=6 in each group. Sham-operated control and treatment groups are compared with ischemia-reperfusion group. *T.cordifolia* per se is compared with sham-operated control group. Superscript indicates p-value < 0.01 . Statistical analysis was done by one-way ANOVA followed by Tukey test.

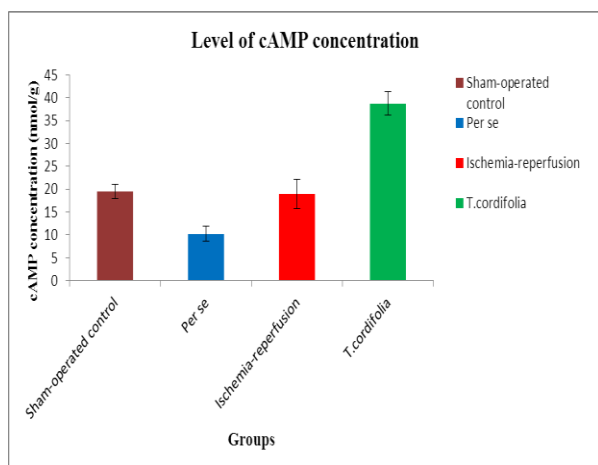


Figure 1: Effect of *T.cordifolia* (100 mg/kg p.o. x 7 days) on level of cyclic AMP in frontoparietal region of rat brain following cerebral ischemia-reperfusion injury (30 min BCCAO followed by 45 min reperfusion). Each bar represents Mean \pm SEM of six rats in each group. * and ** indicates p-value < 0.05 and < 0.01 respectively. Statistical analysis was done by one-way ANOVA followed by Tukey test.

DISCUSSION

The study confirms the previous reports that cerebral post-ischemic reperfusion is associated with generation of free radicals.^[23,24] The analysis of biochemical parameters show that BCCAO for 30 min followed by 45 min reperfusion causes ischemia-reperfusion injury. Increased generation of free radicals initiates lipid peroxidation and this reflected as increased level of TBARS.^[25] Polymorphonuclear leukocytes are known to be involved in cerebral reperfusion injury. Leukocyte accumulation has been noted in brain after cerebral

ischemia.^[26] These activated neutrophils are a source of free radicals, especially superoxide anion.^[27] The increased SOD activity is an indication that brain's antioxidant machinery is activated in response to excessive generation of free radicals.^[28] Enhanced SOD activity catalyzes the conversion of superoxide anion to hydrogen peroxide and molecular oxygen. Hydrogen peroxide, the product of this reaction, is more toxic than the oxygen derived free radicals and requires to be scavenged further by tissue thiols (glutathione redox pathway) and catalase.^[29] A fall in GSH (a non-protein sulfhydryl) during cerebral reperfusion injury is well reported and reduced level of T-SH reflects consumption of tissue thiols.^[30] Sulfhydryl compounds are among the most important endogenous antioxidants. They have role in maintenance of cellular proteins and lipids in their functional states. When these are consumed, the toxic effects of oxidative insult are exacerbated resulting in increased membrane and cell damage.^[31]

The data reveals that *T.cordifolia* could antagonize ischemia-reperfusion injury induces rise in TBARS level. Similarly, *T.cordifolia* reverses ischemia reperfusion induced change in SOD and T-SH. These findings are in agreement with earlier reported antioxidant and neuroprotective properties of *T.cordifolia*. Neuroprotective and ameliorative properties are due to their antioxidant and trace element contents.^[4] *Tinospora cordifolia* has been claimed to possess learning and memory enhancing, antioxidant, and anti-stress activity.^[6,7,8,9] Reperfusion injury did not produce any significant change in ascorbic acid level. Possibly, reperfusion injury increases the ascorbate levels (reduced form of ascorbic acid) without altering the total ascorbic acid levels. This finding receives direct support from an earlier investigation that also suggests lack of change in

total ascorbic acid levels with a decrease in ascorbate levels secondary to cerebral reperfusion injury.^[32]

The study also revealed significant increase in cyclic AMP level in brain (frontoparietal region). Following ischemia-reperfusion injury, cyclic AMP is known to increase in striatum, neocortex and hippocampus and in cerebral cortex.^[33,34,35] The increase in cyclic AMP levels following such injury has been implicated in reversing stroke induced vasospasm in central vessels.^[36] Increased level of cyclic AMP is known to inhibit release of excitatory amino acid like glutamate through modulation of adenosine.^[37] *T.cordifolia* pre-treatment enhanced cyclic AMP concentration in ischemia-reperfused animals. It is rather alluring to hypothesize that part of the therapeutically beneficial effect of *T.cordifolia* might be due to its effect on camp.

Several studies have identified and isolated the active principle of *T.cordifolia* in which lignans, such as 3-(4-hydroxy-3-methoxybenzyl)-4-(4-hydroxy-3-methoxybenzyl) and alkaloids (jatrorrhizine, palmatine, beberine, tembetarine³⁹, choline⁴⁰), has gained attention, as it represents the major constituents responsible for modulation in neurotransmitter levels in the brain of rodents.^[38] Free radical scavenging effect of *T.cordifolia* ethanolic extract could be related to an active constituent.

The data of present studies thus suggested protective role of ethanolic extract of *T.cordifolia* in cerebral ischemia reperfusion induced injury in rats. The present finding, apart from supporting previously reported therapeutic effects of *T. cordifolia* in various animal models of oxidative stress, is suggestive of its efficacy in the treatment of cerebral reperfusion like cerebrovascular insults.

CONCLUSION

Concluding the effects of *T.cordifolia* we demonstrated that *T.cordifolia* prevents the oxidative stress during the cerebral reperfusion injury. In conclusion, the present study provides experimental evidences for *T.cordifolia* as a neuroprotective drug and emphasizes the necessity to appreciate the neuropharmacological effects of *T.cordifolia*.

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