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Neuroprotective activity of *tecoma stans* on cerebral ischemia/reperfusion induced oxidative stress in rats

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ABSTRACT

This study was done to test the neuroprotective effects of Tecoma stans against brain ischemia/reperfusion caused oxidative stress in the rats. The global cerebral ischemia was generated in male albino Wistar rats by occluding the bilateral carotid arteries for 30 min followed by 1 h and 4 h reperfusion. The levels of malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-s-transferase (GST), and hydrogen peroxide (H_2O_2) activity, as well as brain water content, were examined at various periods after reperfusion. Ischemic alterations were preceded by a rise in MDA and hydrogen peroxide concentrations, followed by a reduction in GPx, GR, and GST activity. Ischemia-induced oxidative stress was greatly reduced after treatment with T.stans. In the groups pre-treated with methanolic extract (250 and 500 mg/kg, administered orally in single and double doses/day for 10 days), T.stans administration dramatically reversed and restored to near normal levels in a dose-dependent manner. In the ischemia reperfusion animals, T.stans reversed the cerebral water content. The neurodegenaration also confirmed by the histopathological changes in the cerebral-ischemic animals. The findings from the present investigation reveal that *T.stans* protects neurons from global cerebral-ischemic injury in rat by attenuating oxidative stress.

Keywords: Brain edema, global cerebral ischemia, histopathology, oxidative stress, Tecoma stans.

INTRODUCTION

After myocardial infarction and cancer, stroke is the third greatest cause of mortality, and cerebrovascular illnesses are expected to be the second major cause of death in 2020. In Western nations, this is the major cause of permanent impairment and disability-adjusted loss of independent life-years. According to the Framingham statistics, atherothrombotic and cardioembolic occlusions cause 87 percent of strokes, haemorrhages 14 percent, and other or unknown causes 3

percent.[1] Advanced age, hypertension, prior stroke or transient ischemic attack, diabetes, high cholesterol, cigarette smoking, and atrial fibrillation are all risk factors for stroke. The most important modifiable risk factor for stroke is high blood pressure. [2]

One of the key variables that exacerbates the damage caused by cerebral ischemia is oxidative stress. After cerebral ischemia, many components of reactive oxygen species (ROS) (superoxide, hydroxyl radical, hydrogen peroxide, and peroxynitrite radical) are produced and play an essential role in neuronal death. [3] Superoxide and the hydroxyl radical are both capable of causing lipid peroxidation, which leads to cell membrane damage. [4] After ischemia-reperfusion damage, inducible nitric oxide synthase (iNOS) is increased. As a result, too much nitric oxide (NO) is produced. After cerebral ischemia, the excess NO interacts with superoxide to make peroxynitrite, a strong radical that causes neuronal death. Because of its high rate of oxidative metabolic activity, intense production of reactive oxygen species metabolites, and high content of polyunsaturated fatty acids, as well as its relatively low antioxidant capacity, low repair mechanism activity, and non-replicating nature of its neuronal cells, the brain is particularly vulnerable to oxidative stress injury. [5]

Normal ageing and the pathology of neurodegenerative illnesses including amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease have all been linked to changes in antioxidant status in nerve tissue. Not only has oxidative stress been linked to chronic neuropathologies, but it has also been linked to acute traumas like ischemic stroke. [6] Polyphenolics, which include flavonoids and are present in many herbal extracts, have been demonstrated to be powerful ROS scavengers, antioxidants, and neuroprotectors in vitro. [7]

Tecoma stans (common name yellow bell) also known as yellow trumpet bush belongs o the family bignoniaceae. It is an ornamental plant. It is an erect, branched, sparingly hairy or nearly smooth shrub two to four meters in height. The leaves are opposite, odd-pinnate, Up to 20 centimeters in length with 5 to 7 leaflets. The leaflets are lanceolate to oblong-lanceolate, 6 to 13 centimeters long, pointed at both ends and toothed on the margins. Trumpet shaped flowers are yellow faintly scented and borne in short, dense, terminal clusters. The calys is green. 5 to 7 millimeters long and 5 toothed. Flowering can begin as early as April and continue in to fall. The flowers are followed by 6 inch long, tan pods that are filled with small, papery winged seeds.9

Leaves of *Tecoma stans* contain the alkaloids tecomin and tecostamine are potent hypoglycaemic agent when given intravenously. Anthranilic acid is responsible for the anti diabetic activity. Roots are powerful diuretic and vermifuge10. *Tecoma* is not a toxic because this plant is used in latine America as a remedy for diabetes and moreover for feeding cattle and goats in mexico11. The preliminary phytochemical screening of methanolic extract of flower extract of *Tecoma stans* showed the presence of flavaniods, phenol, alkaloids, tannins, steroids, triterpenes, anthraqunones and saponins etc. [8-12] Present study was undertaken to evaluate the neuroprotective potential of methanolic extract of *T.stans* in bilateral common carotid artery (BCA) occlusion induced global cerebral ischemia model in rats.

MATERIALS AND METHODS

Chemicals and Drugs

Glutathione (oxidized and reduced), nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), and nitroblue tetrazoleum chloride (NBT), were purchased from Sigma Aldrich (St. Louis, MO, USA), SRL, Bombay and other chemicals were AR grade.

Animal

Male Wistar albino rats (250-300 g) were obtained from the National Institute of Mental Health and Neuro Science (NIMHANS), Bangalore. Rats were housed in polypropylene cages in air-conditioned room. Standard rat chow pellets and water was allowed ad libitum.

Plant Material

The areal parts of *Tecoma stans* were collected in the month of May 2015 from Rasipuram (Namakkal District) Tamil Nadu. A herbarium specimen of the plant was deposited in the Department of Pharmacognosy. The plant was identified by Dr.G.V.S.Murthy, Joint Director of the Botanical Survey of India, Southern circle.

Plant Extraction

Fresh stem part of *T.stans* was successively extracted with petroleum ether, chloroform and methanol. Petroleum ether and chloroform extract was discarded. Subsequently, the residue was extracted with methanol (yield: 8.9 g) in a Soxhlet apparatus for 48 h. The methanol solvent was removed under reduced pressure in a rotary vacuum evaporator.

Experimental Protocol for Global Ischemia

The methodology was split into two groups: 1 hour reperfusion models and 4 hour reperfusion models.

Each major group was further broken into six groups, each containing six Wistar male rats that had been given methanolic extract or vehicle for 10 days before to the experiment and were treated as follows:

- Group I: Normal saline (10 ml/kg, orally), no ischemia.
- Group II: Normal saline (10 ml/kg, orally), bilateral carotid artery occlusion (BCAO) for 30 min and followed by 1 h and 4 h reperfusion individually (ischemic control).
- Group III: *T.stans* (250 mg/kg, single dose/day, orally), BCAO for 30 min and followed by 1 h and 4 h reperfusion individually.
- Group IV: *T.stans* (250 mg/kg, double dose/day, orally), BCAO for 30 min and followed by 1 h and 4 h reperfusion individually.
- Group V: *T.stans* (500 mg/kg, single dose/day, orally), BCAO for 30 min and followed by 1 h and 4 h reperfusion individually.
- Group VI: *T.stans* (500 mg/kg, double dose/day, orally), BCAO for 30 min and followed by 1 h and 4 h reperfusion individually.

Induction of Global Cerebral Ischemia and Reperfusion (I/R)

Bilateral carotid artery blockage was performed on a group of animals. Thiopentone sodium (40 mg/kg, i.p.) was used to anaesthetize rats. The animals were placed on their backs, and a midline ventral incision in the neck was made. The animal's trachea was opened, and the right and left common carotid arteries were discovered. Both carotid arteries were exposed, with particular care taken to separate and preserve the vagus nerve fibres. For 30 minutes, ischemia was produced by passing a cotton thread below each carotid artery and tying a surgical knot on both arteries. The thread was removed after 30 minutes of global cerebral ischemia to allow blood to reflow via the carotid arteries (reperfusion) for 1 hour and 4 hours, respectively. The body temperature of rats was maintained at 37 0.5°C using a heated surgical platform during the surgery. Sham control animals had the identical surgical procedures as the experimental animals, with the exception that the BCA was not blocked. The mice were evaluated for their neuroprotective activity once the reperfusion time was completed, and then euthanized. The brains were dissected for biochemical parameter measurement, brain weight determination, histopathology investigation, and assessment of cerebral infract size.

Preparation of Post-Mitochondrial Supernatant

After creating BCAO, the animals were promptly decapitated and killed. Their brains were removed, rinsed in pre-chilled 0.9 percent saline, and frozen for 5 minutes at 20°C. The brain was weighed and homogenised in cold sodium phosphate buffer (0.1 M, pH 7.4) using a REMI tissue homogenizer after being blotted on filter paper. Post-mitochondrial supernatant (PMS) was collected from 10% (w/v) brain tissue and kept at -10°C for future tests after centrifugation at 10,000 g for 20 minutes at 4°C.

BIOCHEMICAL ANALYSIS

Malondialdehyde

A sample of 0.2 ml PMS (10 percent w/v), 0.2 ml 8.1 percent sodium dodecyl sulphate, 1.5 ml 20 percent acetic acid solution, and 1.5 ml 0.8 percent aqueous solution of TBA were mixed to a sample of 0.2 ml PMS (10 percent w/v), 0.2 ml 8.1 percent sodium dodecyl sulphate, 1.5 ml 20 percent acetic Using distilled water, the mixture was produced up to 5 mL and then cooked in an oil bath at 95°C for 60 minutes with a glass ball as a condenser. After chilling with tap water, 5 ml of the n-butanol/pyridine (15:1 v/v) combination was added and forcefully shaken. The organic layer was collected after centrifugation at 4000 g for 10 minutes, and its absorbance was measured at 532 nm. MDA levels in tissue were calculated using a standard graph of known MDA values and represented as nmol/g tissue.

Glutathione peroxidase activity

The reaction mixture consisted of 0.4 ml of phosphate buffer (0.4 M, pH 7.0), 0.2 ml of EDTA (0.8 mM), 0.1 ml of sodium azide (10 mM), 0.1 ml of reduced glutathione (4 mM), 0.1 ml of H 2 O 2 (30 mM), and 0.2 ml of PMS (10%, w/v). The mixture was incubated at 37°C for 10 min. Stored the tubes at room temperature and added 0.5 ml of 10% TCA and centrifuged at 200 g for 10 min, supernatant 0.1 ml of DTNB (0.04% in 1% trisodium citrate solution) solution was added. The optical density was read at 420 against blank (i.e. without homogenate).The enzyme activity was calculated as nM of glutathione oxidized/min/mg protein by using molar extinction coefficient 6.22×10 3 M−1 cm−1.

Glutathione reductase activity

The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml NADPH (0.1 mM), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), and 0.1 ml PMS (10%, w/v) in a total volume of 2.0 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nM NADPH oxidized/min/mg protein by using molar extinction coefficient of 6.22×10−3 M−1 cm−1 .

Glutathione-S-transferase activity

The reaction mixture consisted of 1.425 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM), and 0.3 ml PMS (10%, w/v) in a total volume of 2.0 ml. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nM CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6×10 3 M−1 cm−1.

Hydrogen peroxide

Hydrogen peroxide was estimated by added 0.2 ml of PMS to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0), and 1.0 ml of 1M of potassium iodide. The absorbance of the reaction mixture was measured at 390 nm. The rate of H 2 O 2 production was calculated using a standard graph of H 2 O 2 and expressed as µM/g tissue.

Protein concentration

Protein concentration in all PMS 10% (w/v) samples was determined by the method of Lowry et al. [13] by using span diagnostic kit.

Brain weight and water content

In another study involving the same protocol and treatment, brains from twelve groups of animals were removed and immediately weighed in pre-weighed glass vials and the wet weights recorded. [14] The percentage water content of each brain was calculated.

Histopathology study

Similar protocol was placed for studying the histopathology of brain. Twelve groups of six animals each and were given similar treatment and induced the BCAO for 30 min and followed by 1 h and 4 h reperfusion individually. Coronal brain sections from control and experimental groups of global ischemia were removed, rinsed with cold normal saline and were fixed with a mixture of formaldehyde (40%), glacial acetic acid and methanol (1:1:8, v/v). Brain slices cut into 4-5 mm thickness and embedded in paraffin blocks. Brain sections of 4-6 µm thickness were cut and stained with hematoxylin and eosin.

Statistical analysis

The results were expressed as mean ± S.E.M. Statistical difference between mean were determined by one-way analysis of variance (ANOVA), followed by Dunnett t-test. The diagrammatic representation of the data was performed by using; Microcal™ Origin® Version 6.0 (Origin 6.0 AddOn, Data analysis and Technical graphics) software was used for all statistical calculations. Differences were considered significant at $P<0.05$.

RESULTS

Effect of *T.stans* on Biochemical Analysis

The biochemical results are showed in [Figure 1-5]. The results showed that the cerebral ischemia and reperfusion significantly decreased antioxidative activities (GPx, GR, and GST) and increased the level of lipid peroxidation (malondialdehyde content, an index of lipid peroxidation) and hydrogen peroxide (H 2 O 2) in the injured brain tissue of rats as compared with the sham control group. However, the pretreatment of rats with *T.stans* (250, 500 mg/kg, single and double dose/day) was markedly increased GPx, GR, and GST activity. In contrast, MDA and H 2 O 2 content in the injured brain tissue of rats decreased significantly ($P<0.01$) in *T.stans* extract treated group compared to ischemic control group. However, the accumulation of MDA and H 2 O 2 content was significantly lower in cerebral ischemia in extract treated animals. Interestingly, the double dose/day treated group (250 and 500 mg/kg) had significantly alters enzyme activities than the single dose/day treated group with methanolic extract of *T.stans* in cerebral ischemia-subjected rats.

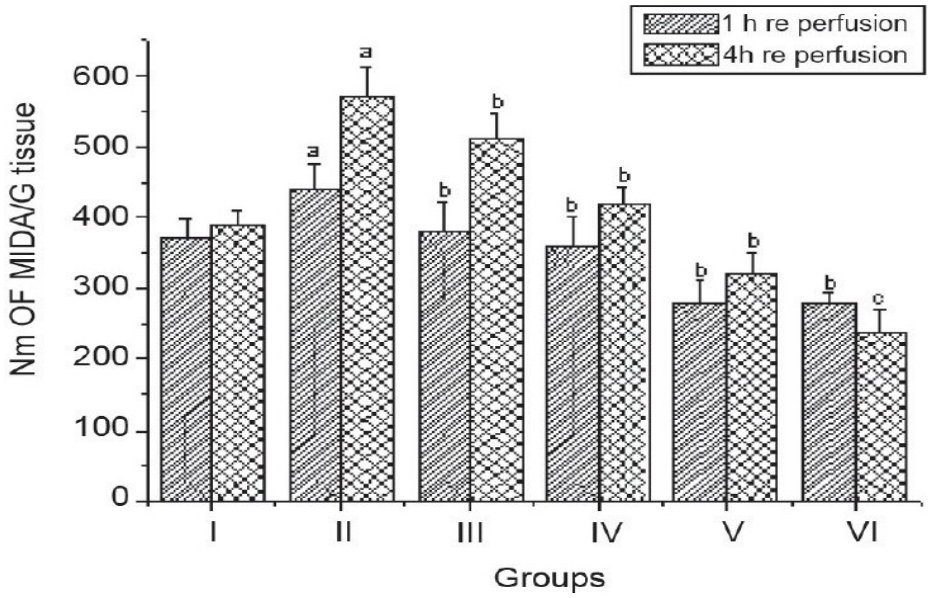

Figure 1: Effect of *T.stans* on MDA in rats subjected to global cerebral ischemia followed by reperfusion. I: Sham control, no occlusion, II: ischemic control (normal saline, 10 ml/ kg, p.o.), III: *T.stans* (250 mg/kg, single dose/day, p.o.) + ischemia, IV: *T.stans* (250 mg/kg, double dose/day, p.o.) + ischemia, V: *T.stans* (500 mg/kg, single dose/day, p.o.) + ischemia, VI: *T.stans* (500 mg/kg, double dose/day, p.o.) + ischemia. Values are expressed as mean ± S.E.M., (n = 6). a = *P*<0.01 vs Sham control, b = *P*<0.01 vs ischemic control, by one-way analysis of variance (ANOVA), followed by Dunnett t-test.

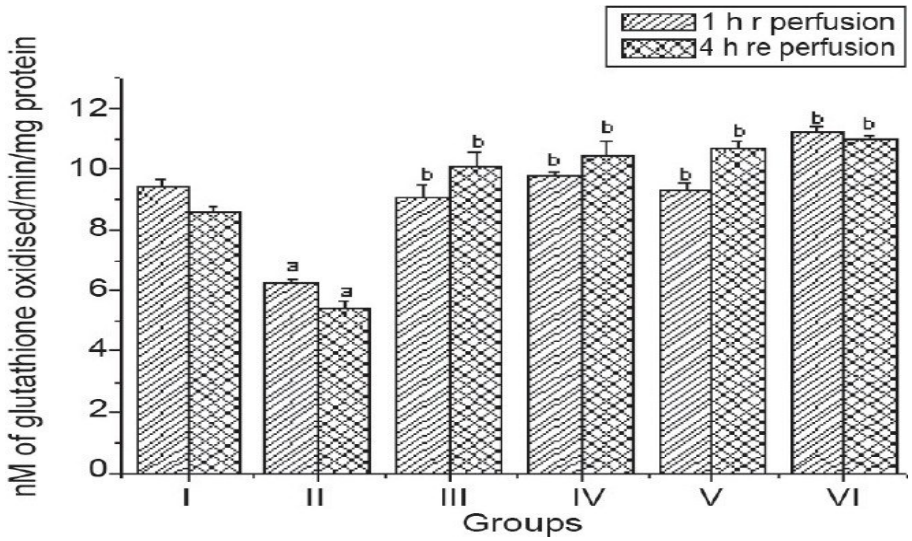

Figure 2: Glutathione peroxidase (GPx) (a = P<0.01 and b = P<0.001 vs Sham control, c = P<0.01 and d = P<0.001 vs ischemic control (see the legends in Fig. 1)

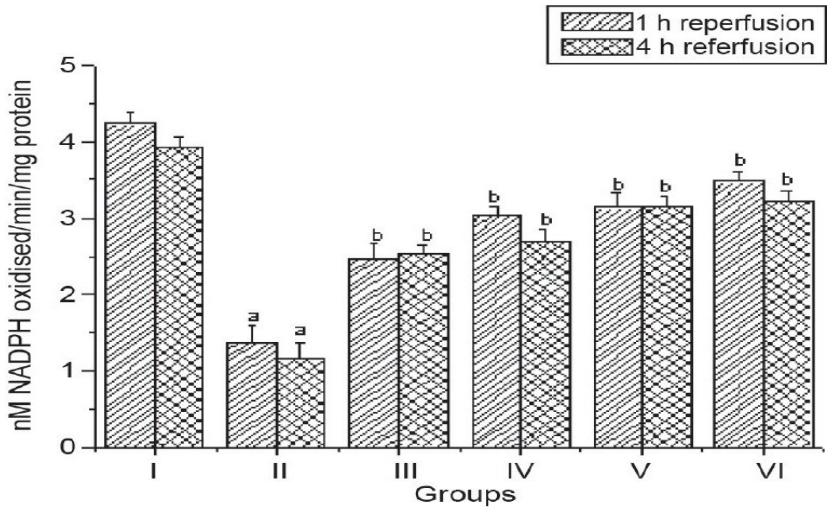

Figure 3: Glutathione reductase (GR) (a = P<0.001 vs Sham control, b = P<0.01 vs ischemic control (see the legends in Fig. 1)

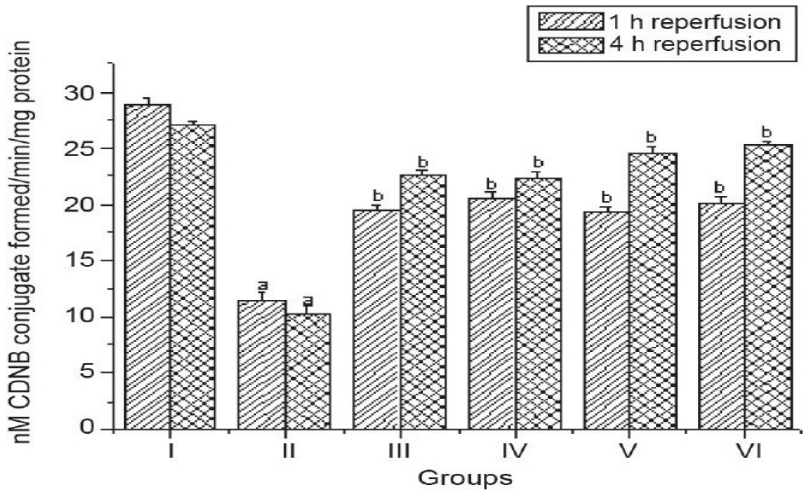

Figure 4: Glutathione-S-transferase (GST) (a = P<0.001 vs Sham control, b = P<0.01 vs ischemic control (see the legends in Fig. 1)

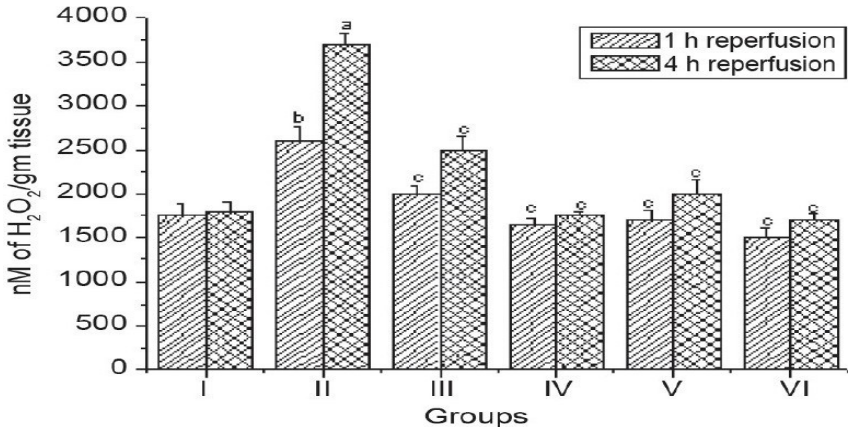

Figure 5: Hydrogen peroxide (H2O2) (a = *P*<0.001 vs Sham control, b = *P*<0.01 vs Sham control, c = *P*<0.01 vs ischemic control (see the legends in Fig. 1)

Effect of *T.stans* on Brain Weight and Water Content

In the ischemic/reperfusion control group, the amount of cerebral water (edoema) was considerably higher. Pretreatment with T.stans (250, 500 mg/kg) resulted in a substantial (P0.01) drop in water content, with a more than two-fold decrease in treated rats compared to the ischemia control group. However, in the treated groups, there was a considerable reduction in brain weight. [Figure 6].

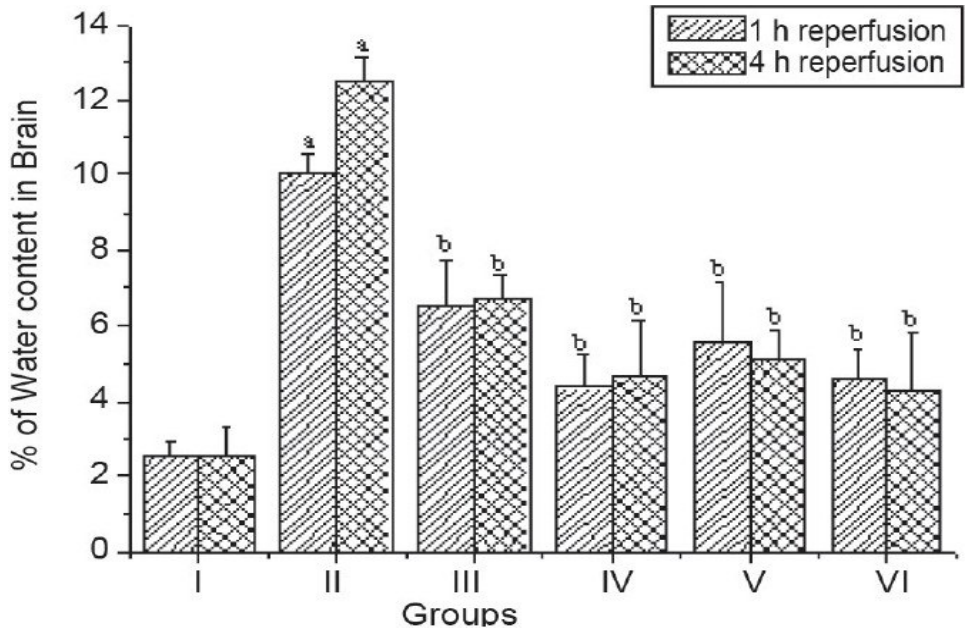

Figure 6: Effect of methanolic extract of *T.stans* on water content in brain in rats subjected to global cerebral ischemia followed by reperfusion. a = *P*<0.001 vs Sham control, b = *P*<0.01 vs ischemic control (see the legends in Fig 1).

Effect of *T.stans* on Histopathology

The histopathology of the brain of ischemia-reperfusion and extract treated groups are showed in [Figure 7] and [Figure 8]. From the histopathological study, it was observed that section of brain tissue showing swollen neurons, dilated blood vessels with neuronal loss occurred in brain regions of I/R rats induced by BCAO for 30 min followed by 1 h and 4 h reperfusion in ischemic control group [Figure 7]b and [Figure 8]b. While no apparent morphological changes in sham control and brain section showing normal structure [Figure 7]a and [Figure 8]a. *T.stans* (250 and 500 mg/kg) treated group of 1 h reperfusion brain section showed significantly prevented the neuron loss by compared with ischemic control group and in the other hand no significant difference between the doses of 250 mg/kg of *T.stans* on 4 h reperfusion ischemic treated groups.

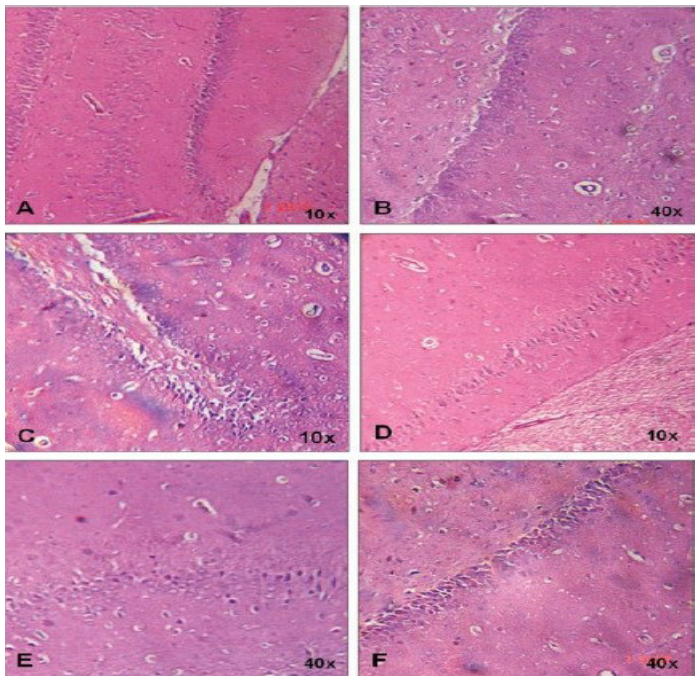

Figure 7: Histopathological photographs of coronal sections of brain after 30 min of occlusion and 1 h of reperfusion in bilateral common carotid arteries occluded rats. (A) Sham control, no ischemia (normal saline, 10 ml/kg, p.o.); (B) ischemic control (normal saline, 10 ml/kg, p.o.); (C) *T.stans* (250 mg/kg, single dose/day, p.o.) + ischemia; (D) *T.stans* (250 mg/kg, double dose/day, p.o.) + ischemia; (E) *T.stans* (500 mg/kg, single doses/day, p.o.) + ischemia; (F) *T.stans* (500 mg/kg, double doses/day, p.o.) + ischemia.

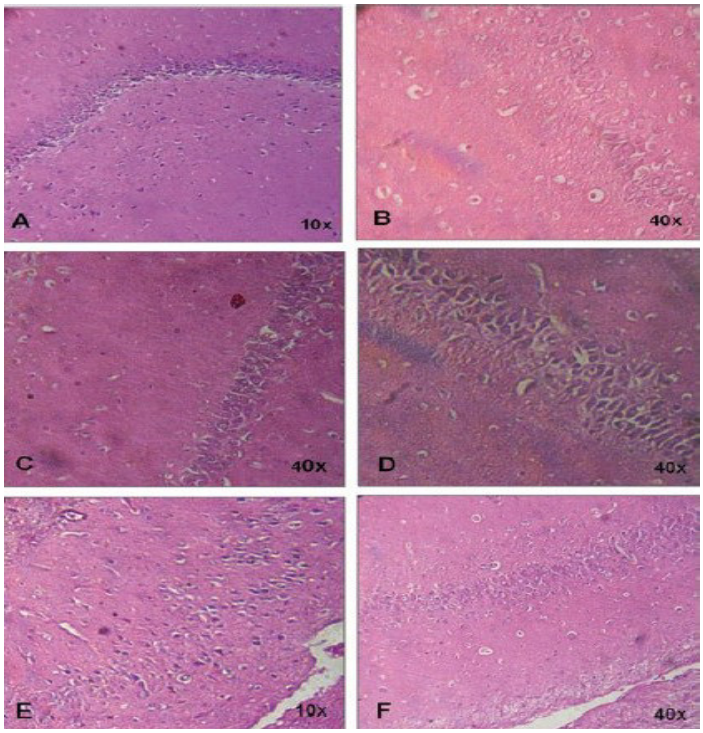

Figure 8: Histopathological photographs of coronal sections of brain after 30 min of occlusion and 4 h of reperfusion in bilateral common carotid arteries occluded rats (see the legends in Fig. 7).

DISCUSSION

T.stans was shown to have therapeutic potential in a worldwide model of ischemia for the investigation of stroke in rats in the current investigation. Because the extent of damage induced may differ greatly, we used two alternative reperfusion (1 h and 4 h reperfusion) models. Furthermore, our goal was to assess the brain damage produced by both reperfusion models, as well as the preventive effect of T.stans methanolic extract pretreatment.

It was observed that *T.stans* attenuated the impaired neurological deficit and sensory motor functions of the ischemic rats. The activity of *T.stans* appears to restore the altered antioxidant enzymes as well as decreased the production of MDA in brain regions induced by BCA occlusion. There was a considerable evidence supported the role of ROS in the pathogenesis of I/R induced oxidative stress in brain. [15] [16] The endogenous antioxidant enzyme activity of the brain impaired by I/R is particularly important and measurement of those antioxidant enzymes after reperfusion can assess the vulnerability of the particular areas of the brain. [17] Therefore, the potent radical scavenging properties or lipid proxidation inhibiting ability of polyphenolic natural compounds protecting the neurons from oxidative stress may provide useful therapeutic agent for the treatment of neurodegenerative diseases such as I/R induced oxidative stress. Peroxidation of lipid bi-layer was reported after cerebral IR injury.[18] So that lipids are most susceptible macromolecules to oxidative stress. From results of our work reveals that the production of MDA was significantly elevated in ischemic brain regions of the rats after 1 h and 4 h of reperfusion period. The results demonstrated that pretreatment with *T.stans* had markedly reduced the MDA level and inhibits the neuronal injuries from propagating chain reaction of lipid peroxidation.

The presence of several antioxidant enzymes in the brain, such as GPx, GR, and GST, protects these tissues against oxidative damage caused by free radical production. [19] Another essential enzyme involved in hydrogen peroxide scavenging is GPx. In numerous investigations, GPx dysfunction has been linked to a decrease of protective action exerted by these enzymes, which has been shown as an increase in infarction. [20] Antioxidant treatment should prevent the loss of GPx activity by effectively scavenging the excess ROS. GPx are involved in the detoxification of H 2 O 2 , both at high and low concentrations. In this study, reduction in the GPx activity was significantly prevented by *T.stans* administration.

Glutathione reductase, a key enzyme for maintaining intracellular reduced glutathione concentrations, also serves as a free radical scavenger.[21]Glutathione reductase deficiency might both cause and reflect oxidative stress. After ischemia damage, the amount of brain glutathione reductase was dramatically lowered. [22] It was also considerably lower in the I/R control group compared to the sham control group in our research. This depletion was linked to an increase in lipid peroxidation in the brain. A variety of neurodegenerative illnesses have been linked to glutathione system dysfunction. [23] Therefore, further extends the support to our idea for observing the glutathione levels in the present study. Glutathione-S-transferase catalyses the detoxification of oxidized metabolites of catecholamines (o-quinone) and may serve as antioxidant, preventing degenerative processes. [24]

Accumulation of hydrogen peroxide was reported to impair the mitochondrial function. H 2 O 2 is a longer-lasting reactive species electrically neutral and is able to pass through cell membranes. Hydrogen peroxide is reported to be more stable than superoxide anion, hydroxyl free radical, and other ROS. [25] Therefore, hydrogen peroxide may persist for longer time after reperfusion to produce neuronal injury. Level of H 2 O 2 in I/R rats brain were markedly reversed and restored to near normal levels in the groups' pre-treated with *T.stans*. Due to it increases in endogenous antioxidant defense enzymes and may prevent the neuronal injury produce by hydrogen peroxide.

Brain edema has also been studied extensively in ischemia to assess the impact of brain damage. [14],[26] Cerebral edema occurs as a result of ionic imbalance (and hence the osmotic pressure) across the cellular membrane. A simple pathophysiological mechanism is that energy failure results in neuron depolarization, which causes activation of glutamate receptors, which in turn alters ionic gradients of Na + , Ca ++ , Cl - and K + . As glutamate increases in the extracellular space, peri-infarct depolarization occurs. Then, as water shifts occur, cells swell with resulting cerebral edema. *T.stans* also reversed effects on brain water content in the ischemia reperfusion animals as compared to the ischemic control group. These results are pointing to its potential therapeutic value in cerebrovascular diseases including stroke. The observed results leading to neurodegeneration is also confirmed by the histopathological differences between treatment and ischemic control groups. There was reversal of the brain damage observed in extract administered twice a day and it was prevented the neuron loss by approximately 40%. It is suggested that pretreatment of *T.stans* could exert a neuroprotective effect on the brain subjected to I/R.

CONCLUSION

Methanolic extract of *T.stans* could reduce neuronal loss of the ischemic brain tissue. *T.stans* showed antioxidant activity in reperfusion induced oxidative stress.

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