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RESEARCH ARTICLE

Neuroprotective and antioxidant potential of terpenoid fraction from *Hygrophila auriculata* against transient global cerebral ischemia in rats

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Abstract

Context: The plant *Hygrophila auriculata* (K. Schum) Heine. (Acanthaceae) is widely used in the Indian System of Medicine as "Rasayana" for treating brain and liver diseases.

Objectives: The present study evaluated the *in vivo* antioxidant and neuroprotective effect of a terpenoid rich fraction (TF) from *Hygrophila auriculata* in a rat model of transient global cerebral ischemia (tGCI).

Materials and methods: Male Wistar rats were grouped as sham control, tGCI control, vitamin E (500 mg/kg) and TF (100 & 200 mg/kg) treated groups. Following 7 days of drug administration, animals were subjected to tGCI by permanent occlusion of both vertebral and transient occlusion of carotid arteries for 10 min followed by reperfusion. The neuroprotective effect was assessed by tGCI induced neurological, sensory motor deficit in rats. Brain antioxidants such as superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were investigated. Further, a histopathological examination was done in CA1 hippocampus.

Results: tGCI induction resulted in an increase in beam balance score (5.1), number of entries in open field (131) and a decrease in time spent in rotorod (47 s). In contrast, TF treatment resulted in a significant decrease in ($p < 0.01$) beam balance score (2.9), number of entries (67) and increased time spent in rotorod (63.25 s). There was also a significant ($p < 0.001$) decrease in brain SOD and GSH with an increase in MDA. TF treatment resulted in restoration of antioxidants and protection of hippocampal CA1 neurons against tGCI insult.

Conclusion: It is concluded that TF from *Hygrophila auriculata* shows neuroprotective potential against tGCI induced oxidative stress.

Keywords: Oxidative stress, hippocampal CA1 neuron, motor coordination, beam balances test

Introduction

Global cerebral ischemia is a clinical outcome occurring as a consequence of conditions such as cardiac arrest (CA), coronary artery bypass graft (CABG) and reversible severe hypotension. Ischemic damage to brain cells caused by clinical stroke is reported to involve a complex cascade of events that involves a number of phenomena including excitotoxicity (Sheng & John, 2002), inflammation (Liu & Hong, 2003), cytokines (Block et al., 2000) released by activated microglia and invading neutrophils, and oxidative damage (Sugawara & Chan, 2003) caused by free radicals. The induction of transient global cerebral ischemia (tGCI) by permanent vertebral occlusion and temporary carotid ligation (four-vessel occlusion) is a widely accepted rodent model which mimics the above pathogenesis. Many experimental stroke studies demonstrated that oxidative stress to neurons caused by excess production of free radicals on reperfusion to be an important step in which impairment in neurobehavior and ischemic insult to special subcortical neurons in the brain may appear later

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(Paganelli et al., 2006; Trushina & McMurray, 2007). The pyramidal neurons of the hippocampus are the cells that are most vulnerable to ischemic injury. Neuronal death in the CA1 region after cerebral ischemia has been shown to occur in a delayed fashion (Candelario-Jalil et al., 2001). Epidemiological studies suggest that the consumption of high antioxidant containing fruits, vegetables and medicinal herbs is associated with lower risk of stroke which is in part attributed to antioxidant properties (Joshipura et al., 1999; Tero et al., 2000). Most notably the terpenoids from *Panax ginseng* L. (Araliaceae) and *Ginkgo biloba* L. (Ginkgoaceae) (ginkgolides A and B) have been shown to be effective in numerous pre-clinical models of stroke (Clark et al., 2001), including transient and permanent occlusion of the middle cerebral artery (MCA) in rat and glucose oxygen deprivation induced stroke in *in vitro*. *Hygrophila auriculata* is a terpenoid rich herb widely used in the Indian traditional System of Medicine (ISM), Ayurveda, for the treatment of various ailments such as pain (Shanmugasundaram & Venkataraman, 2005), liver cirrhosis (Singh & Handa, 1995), and haematinic (Gomes et al., 2001). The crude alcohol extract of the plant has been shown to be hepatoprotective and increase endogenous antioxidants in hepatocytes (Hewawasam et al., 2003; Shanmugasundaram & Venkataraman, 2006). The Indian system of medicine, Ayurveda, describes *Hygrophila auriculata* as a brain tonic (Nadkarni, 1996) and the decoction of this crude plant material has been in use for stroke patients near Paddapai village, Kancheepuram District for many years. Our earlier work on the terpenoid fraction from *Hygrophila auriculata* on free radical scavenging of total antioxidant capacity assay using rat brain homogenate has been published (Vijaykumar et al., 2006; Hussain et al., 2009). However, the *in vivo* neuroprotective and antioxidant effect of terpenoids fraction from *Hygrophila auriculata* has not yet been investigated. So, the present study evaluated the antioxidant effect of terpenoids from *Hygrophila auriculata* in transient global cerebral ischemia (tGCI) in rodents.

Materials and methods

Animals

Inbred Wistar male rats weighing around 250–300 g were used. Animals were procured from the central animal house, School of Pharmaceutical Sciences, Vels University. Animals were housed in polypropylene cages at an ambient temperature of 25 ± 2°C and 45–55% relative humidity with 12 h light/dark cycle. They had free access to pellet chow (Brook Bond, Lipton India) and water *ad libitum*. The experimentations on animals were approved by the Institutional Animal Ethical Committee (IAEC) under the regulation of CPCSEA, New Delhi. The approval No. is (CPCSEA/ 12.12.2000/ PH-07-08).

Plant material and extraction

The fresh plant was collected in the month of August from the field area of Paddapai, Kancheepuram Dist, Tamilnadu, India. The plant specimen was authenticated by field botanist Dr. P. Jayaraman, Plant Anatomy Research Center, Chennai, Tamilnadu. A voucher specimen no PARC 55/01/2007 has been deposited at the department herbarium.

Extraction, fractionation, and purification

The powdered plant material (5 kg) was macerated with 75% v/v of ethanol for 48 h. The ethanol extract was concentrated under reduced pressure. The resulting ethanol extract was concentrated with a rotary vacuum evaporator. The residue obtained was successively washed with diethyl ether, chloroform, and acetone, finally dissolved in methanol and filtered. The filtrate was poured into excess diethyl ether (Et_2O) resulting in a light brown mass precipitate. The precipitate was separated by filtration and purified by repeating the above process of dissolving in methanol (MeOH) and precipitating with diethyl ether (Et_2O). The qualitative phytochemical test and thin layer chromatography indicated that the purified filtrate tested positive for only terpenoids and preliminary TLC, and the Rf values were in agreement with an earlier publication on *Hygrophila auriculata* terpenoids (Minocha & Tiwari, 1981). The fraction is named the terpenoid rich fraction (TF).

HPTLC analysis of terpenoids

A pre-coated TLC plate of silica gel 60 F254 (E. Merck, India) of 0.2 mm thickness was used. Ten microliters of terpenoid fraction was spotted in the form of a band using a CAMAG Linomat V Automatic Spotter (CAMAG, Switzerland). The TLC pattern of the terpenoid fraction was developed using *n*-hexane: ethyl acetate (7:3) as a solvent system. Then the plates were scanned in a CAMAG TLC Scanner-III and the peaks were recorded at a wavelength of 366 nm. The TLC and HPTLC finger printing studies on the terpenoid fraction showed the presence of various terpenoids with their respective Rf values. The observed Rf values from TLC were in concordance with HPTLC finger prints.

Acute toxicity study and gross behavior

An acute toxicity study was performed according to Organisation for Economic Co-operation and Development guidelines. Two groups of control rats (n = 3 in each group) were given TF separately 2000 mg/kg p.o. After oral ingestion, the animals were observed continuously for 2 h under the following profiles like alertness, restlessness, irritability, fearfulness spontaneous activity, reactivity, touch response, pain response, defecation and urination. After periods of 24 and 72 h, animals were observed for signs of lethality or for death.

Surgical procedure

Transient global cerebral ischemia is induced by the four vessel occlusion (4 VO) method. Briefly, on the first day, rats were anesthetized with ketamine and xylazine (80 + 5 mg/kg, i.p). Both vertebral arteries were

occluded permanently with 5/0 nylon filament through the alar foramen of the first cervical vertebra. The rats were allowed to recover overnight. Twenty-four hours later, both the common carotid arteries were exposed through ventral midline cervical incision and carefully isolated from surrounding adventia and vagus nerve, and occluded for 10 min with micro aneurysm clips to induce transient global cerebral ischemia (tGCI). Reperfusion of the blood flow was ensured visually. Rectal temperature was maintained at 37 ± 0.5°C during the surgical procedures to avoid hypothermia. Sham operated animals were treated similarly to the ischemic group but the common carotid and vertebral arteries were not occluded (Buchan et al., 1991).

Drug administration

Rats were randomly assigned to the following groups: Group 1 (n = 12) Sham operated rats treated with vehicle 0.1% carboxy methyl cellulose (CMC). Group 2 (n = 12) tGCI operated rats treated with vehicle (0.1% CMC). Group 3 (n = 12) tGCI operated rats treated with vitamin E 500 mg/kg. Group 4 (n = 12) tGCI operated rats treated with terpenoids fraction (TRP) 100 mg/kg. Group 5 (n = 12) operated rats treated with terpenoids fraction (TRP) 200 mg/kg. Vitamin E and test drugs were prepared with 0.1% CMC as a suspension and administered to rats by oral route in a volume of 5 mL/kg body weight. All drugs and vehicle are pretreated once a day for 7 days and tGCI was performed on 7th and 8th day. The neurobehavior was performed 24 h after tGCI that is on 9th day.

Mortality rate

With a total of 60 rats, 48 were subjected to tGCI surgery and 12 were treated as sham control. Four animals in tGCI, 1 animal in tGCI + vitamin E, 2 animals from TGCI + TF 100 mg/kg, and 1 animal from tGCI + TF 200 mg/kg, were killed on the 9th day. The total mortality rate was 8 out of 60 animals. The mortality rate was 13%.

Assessment of sensory motor and neurobehavior function

Beam balance test

The beam balance was used to evaluate gross vestibulomotor function. It requires a rat to balance on an elevated 120 cm long beam (2.3 cm in diameter, 50 cm above the floor). The rat was perpendicularly positioned on the center of the beam and maintained for a maximum of 60 s. The rats performance is assessed with a score, which ranked from 0 to 6 [0 = balances with steady posture; 1 = grasps side of beam; 2 = hugs the beam and one limb falls down from the beam; 3 = hugs the beam and two limbs fall down from the beam, or spins on beam; 4 = attempts to balance on the beam but falls off (>40 s); 5 = attempts to balance on the beam but falls off (>20 s); 6 = falls off: no attempt to balance or hang on to the beam (<20 s)] (Goldstein & Davis, 1990).

Rota rod test

In the rotarod experiment, all the animals were given one day testing before induction of tGCI. Animals were kept in a rotating rotarod and the speed was maintained at 10 rpm for 20 s and then increased up to 25 rpm. The motor coordination was evaluated by the ability of the rat to hold the rotating rotor. The time for which the rat holds the rotor was calculated and the result was expressed in seconds. The duration was 5 min (Nathalie et al., 2004).

Open field test

An open field test is used to detect motor and exploratory behavior. The open field is made of black wood and consisted of a floor (96 × 96 cm) with 50 cm walls. The floor of the box is painted with white lines (6 mm) to form 16 equal squares. Animals were placed in a random corner of the open-field and behavior monitored for 10 min during which the number of square entries (in periphery and center) was recorded for the initial and final 5 min testing period. Since habituation is dependent on baseline levels of performance within each animal (considered here as the first 5 min of exploration), habituation profiles for individual animals during the single 10 min open field exposure were calculated (Prut & Belzung, 2003).

Preparation of brain tissue for estimation of oxidative stress marker

Animals (n = 6) from each group were sacrificed by deep carbon dioxide euthanasia. The brains of the animals were removed after immediately neurobehavioral testing. They were weighed and various brain regions of cortex and hippocampus were dissected out and weighed and homogenized with 10 times (w/v) ice cold phosphate buffer saline (50 mM pH 7.8) in a Teflon glass homogenizer. The homogenate was centrifuged at 1000 rpm at 4°C for 3 min and divided in to two portions in which one portion of supernatant was used for measurement of lipid peroxidation (LPO). The remaining supernatant was again centrifuged at 12,000 rpm at 4°C for 15 min and used for the measurement of antioxidant enzymes (Lowry et al., 1951).

Measurement of lipid peroxidation

Estimation of various brain regions of LPO was carried out by measuring the LPO end product malondialdehyde (MDA) (Ohkawa et al., 1979). Brain homogenate (0.1 mL) was treated with 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL thiobarbituric acid and 0.2 mL sodium dodecyl sulphate. The mixture was then heated at 100°C for 60 min. The mixture was cooled with tap water and 5 mL of n-butanol-pyridine (15:1% v/v) and 1 mL of distilled water was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm. The concentration of MDA formed was expressed as mM/mg of protein.

Estimation of superoxide dismutase

Superoxide dismutase SOD activity in ischemic and drug treated brain regions was measured (Saggu et al., 1989). A mixture of 2.80 mL of sodium carbonate (0.05 mM) buffer (pH 10.2), 100 µL of EDTA (1.0 mM) and 20 µL of brain homogenate or sucrose (blank) was incubated at 30°C for 45 min. Thereafter, the reaction was imitated by adding 100 µL of adrenaline solution (9.0 mM). The change in absorbance was recorded at 480 nm for 8–12 min. The results were expressed as SOD µmoles/min/mg of protein.

Estimation of brain glutathione

Glutathione (GSH) was measured according to the method of Ellman (1959). An equal quantity of brain homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 mL of this supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 5′5-dithiobis (2-nitrobenzoic acid) and 0.4 mL of double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as nM/mg of protein.

Histopathological examination

Following the completion of the behavior testing, rats ($n = 3$) were immediately euthanized and transcardially perfused with buffered formalin. Brains were removed, and kept in the same fixative for 3 days. Serial coronal sections (10 µm) through the dorsal hippocampus (between 1.5 and 2.0 mm posterior to the bregma) were obtained using a cryostat and stained with hematoxylin and eosin. Analysis of neuronal density was performed in hippocampal sectors CA1 using the images taken at 400× magnification. The number of intact neurons in the hippocampal CA1 subfield per mm of the pyramidal cell layer was counted by a blinded investigator according to the method of Buchan et al. (1991). The neuronal density for a given animal represents the average of both the right and left neuronal cell densities. Neuronal density values were expressed as mean ± SEM.

Statistical analysis

Results are reported as mean ± SEM. Statistical analysis was performed using one way analysis of variance (ANOVA). If the overall p value was found statistically significant ($p < 0.05$), further comparisons among groups were made according to *post hoc* Dunn's test (for parametric data), Mann–Whitney test (for non-parametric data). All statistical analyses were performed using Graph Pad Prism statistical version 3 software package (Graph Pad® Inc., USA).

Results

Effect of TF in acute toxicity and gross behavior in rats

The rats treated with the TF fraction exhibited normal behavior up to 2000 mg/kg orally. All animals were alert with normal grooming, touch response, pain response and there was no sign of passivity, stereotypy, and vocalization. There was no abnormal change in motor activity, secretary signs or their body weight and water intake during drug administration.

Effect of TF in sensory motor and neurobehavior

A significant increase (H = 31; $p < 0.001$) in beam balance score and impaired motor function was noted in vehicle-treated tGCI group when compared to vehicle-treated sham group (Figure 1). Vitamin E and TF treatment of (H = 20; $p < 0.05$) (TF_{100} = H = 21; $p < 0.05$) (TF_{200} = H = 25; $p < 0.01$) treated tGCI rats showed a significant decrease in the beam balance score assumed in Mann-Whitney test. Figure 2 represents the effect of vitamin E and TF treatments on the rotarod test in tGCI rats. The sham group stayed for a longer time in the rotarod as compared to vehicle-treated tGCI group. The tGCI group stayed for a shorter time which was found to be significant vs sham treated = df (4, 59) = 5.4 ($p < 0.001$). Vitamin E = df (4, 59) = 3.5 ($p < 0.001$), TF_{100} = df (4, 59) = 3.3 ($p < 0.001$) and TF_{200} = df (4, 59) = 3.6 ($p < 0.001$) treatments resulted in improved motor performance in the rotarod test when compared with the tGCI animal group.

In the open field, tGCI rats displayed obvious behavioral alterations shown in Figure 3 (A-C). tGCI rats showed hyperactive behavior as indicated by an increased number of entries at 5 min df (4, 55) = 15.98 ($p < 0.001$) and at 10 min df (4, 55) = 24.95 ($p < 0.001$) than the vehicle-treated sham control group. Administration of TF 100 df (4, 55) = 4.647, $p < 0.001$ at 5 min) df (4, 55) = 11.67, $p < 0.001$ at 10 min) and TF 200 mg/kg dose df (4, 55) = 11.61, $p < 0.001$ at 5 min) df (4, 55) = 15.94, $p < 0.001$ at 10 min) dependently decreased the total number of entries in the open field compared with the vehicle-treated tGCI rats. The percentage and total number of central square entries were significantly decreased ($p < 0.001$) in vehicle-treated tGCI rats compared with the sham control group. TF pre-administration to tGCI rats showed a significant increase ($p < 0.001$) in % of central square entries in a dose-dependent manner compared with the tGCI animals. In total number of central square entries, TF 10 and 20 showed significance at the level $p < 0.05$.

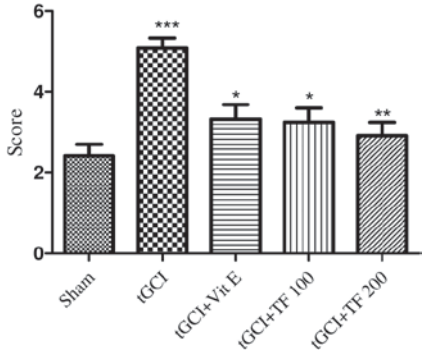

Figure 1. Beam balance score of tGCI rats treated with vitamin E and TF. Values are represented as mean ± SEM, where ***$p < 0.001$ Sham vs tGCI, **$p < 0.01$ vs TF 200 mg/kg vs tGCI, *$p < 0.05$ vitamin E, TF 100 mg/kg vs tGCI.

Effect of TF and vitamin E in brain MDA levels

The level of LPO marker MDA levels in different brain regions following ischemia/reperfusion induced brain injury is represented in Figure 4. MDA level was found to be significantly elevated in cortex df (4, 9) = 12.87, ($p < 0.001$) and hippocampus (4, 9) = 9.7, ($p < 0.001$) of tGCI animals as compared to the sham control group. Dose-dependent and significant reduction in MDA levels were observed in the cortex (4, 9) = 6.87, ($p < 0.001$) and hippocampus (4, 9) = 4.6, ($p < 0.001$) regions of the tGCI rats treated with TF 100 mg/kg extract. In addition, TF 200 mg/kg extract significantly lowered the MDA level in the cortex (4, 9) = 6.87, ($p < 0.001$) and hippocampus (4, 9) = 4.6, ($p < 0.001$) of the tGCI rats. Vitamin E administration showed significant ($p < 0.001$) reduction in MDA formed in the cortex and hippocampus compared to the vehicle treated TF rats.

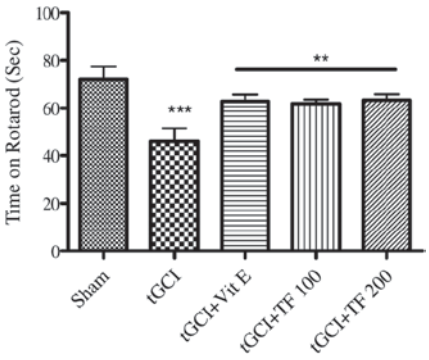

Figure 2. Motor coordination behavior of tGCI rats treated with vitamin E and TF in rotarod test. Values are represented as mean ± SEM, where ***$p < 0.001$ tGCI vs sham, **$p < 0.01$ vitamin E, TF vs tGCI group.

Effect of TF and vitamin E on brain SOD level

The effect of oral administration of TF and vitamin E on SOD levels in various brain regions is represented in Figure 5. Depletion of SOD protein was observed in brain cortex df (4, 9) = 13.11 ($p < 0.001$) and hippocampus df (4, 9) = 9.57 ($p < 0.001$) regions of the ischemic animal brain as compared to the sham treated animal group. Administration of TF (100 & 200 mg/kg) significantly elevated SOD levels in the cortex df (4, 9) = 7.3; 8.4 ($p < 0.001$) and hippocampus df (4, 9) = 4.4; 7.1 ($p < 0.001$) of the ischemic animal groups compared with the vehicle-treated tGCI group. The antioxidant effect of TF to elevate the SOD level was found to be dose-dependent.

Effect of TF and vitamin E in reduced GSH level

Figure 6 represents the effect of tGCI on changes in GSH levels in various brain regions. It was observed that a significant depletion of GSH level was noted in cortex df (4, 9) = 14.22 ($p < 0.001$) and hippocampus df (4, 9) = 13.30 ($p < 0.001$) of tGCI as compared to the sham treated group. Pre-administration of TF at doses of 100 and 200 mg/kg significantly elevated brain GSH level in the cortex df (4, 9) = 9.26, 10.26 ($p < 0.001$) and hippocampus df (4, 9) = 10.54, 13.09 ($p < 0.001$) region of tGCI animals as compared to the sham treated control.

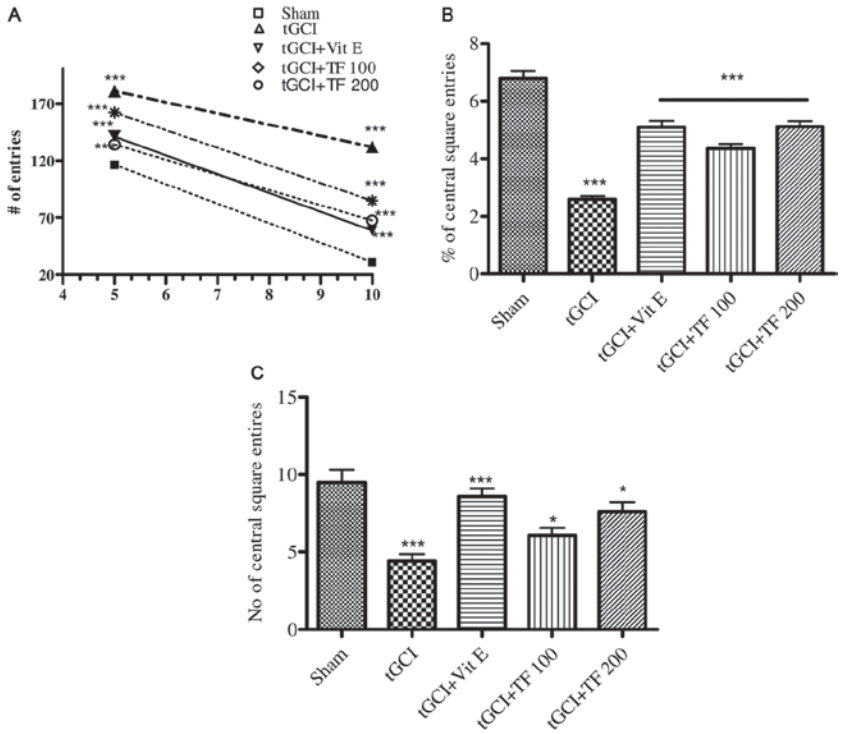

Figure 3. (A) Open field exploratory behavior test (number of entries) of tGCI animals treated with vitamin E and TF. Values are represented as mean ± SEM, ***$p < 0.001$ sham vs tGCI, and vitamin E, TF vs tGCI group. (B) Open field exploratory behavior test (% central square entries) of tGCI animals treated with vitamin E and TF. Values are represented as mean ± SEM, ***$p < 0.001$ sham vs tGCI, and TF vs tGCI group. (C) Open field exploratory behavior test (number of central square entries) of tGCI animals treated with vitamin E and TF. Values are represented as mean ± SEM, ***$p < 0.001$ sham vs tGCI, vitamin E, *$p < 0.05$ TF vs tGCI group.

Histopathological examination

Histopathological examination of CA1 regions of tGCI and test drug treated tGCI were performed. Hippocampus CA1 regions of sham, tGCI and test drug treated tGCI results are shown in Figure 7 (A–E). It was observed that tGCI animals showed a significant decrease in neuronal density ($p < 0.001$) with edema indicating neuronal damage as compared to the sham operated group which shows regular arrangement of neuronal cells (Figure 8). Terpenoid fraction at the dose of 200 mg/kg only showed a significant effect as observed by an increase in neuronal density in the hippocampal region as compared to the vehicle-treated sham tGCI group. The effect was statistically significant ($p < 0.05$). However, vitamin E and TF 100 mg/kg showed an increase in neuronal density in the CA1 field of the tGCI rat brain but it was not found to be statistically significant (Buchan et al., 1991).

Discussion

The present investigation showed the neuroprotective effect of crude terpenoids from *Hygrophila auriculata* against tGCI induced oxidative stress. The crude extract has been used for the management of bedridden plegic patients at Padappai villagers for many years in the past. The pentacyclic triterpene leupeol was isolated from the leaves of *Hygrophila auriculata* and the percentage content of lupeol has been standardized in *Hygrophila auriculata* (Sunita & Abishek, 2008; Daniel, 2008). In this work we have demonstrated that TF administrated prophylactically improves the impaired neurological deficit and sensory motor function. In addition, treatment with TF markedly protects the hippocampus CA1 region from cell loss during 10 min of tGCI followed by reperfusion. This clearly suggests the neuroprotective role of terpenoids against ischemic insult. The plant *Hygrophila auriculata* is one of the widely used herbs in Ayurveda as "Rasayana" drugs for the treatment of CNS related diseases. There is strong preclinical and clinical evidence support that the production of ROS such as superoxide radicals hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) are increased after transient cerebral ischemia and reperfusion injury (Chan, 1996; Vijayakumar et al., 2006). Since the rate of oxidative metabolic activity and the contents of polyunsaturated fatty acid are high and antioxidant enzyme activity is low in the brain, neurons are more vulnerable to ischemic events. ROS that escape the local defense mechanism may cause oxidative damage to membrane lipids, proteins and DNA are important underlying factors in tGCI induced alteration in the CNS (Candelario-Jalil et al., 2001). In the present study, we showed that ROS spurt in brain homogenates which is increased 24 h after tGCI as indicated by increased lipid per oxidation in hippocampus and cortex.

Our results indicate that SOD and GSH activities in rat brain are reduced following transient ischemia, and subsequent reperfusion periods. It has been reported the decrease in antioxidant enzyme activities during ischemia and reperfusion is due to the attack of sulphydril (-SH) groups of enzymes by oxygen free radicals and interaction of enzymes with peroxidation products which affect the active site of the enzyme (Mishra et al., 1990; Sinha et al., 2001). In the present study, the reduction in the SOD activity was prevented by TF administration. The increased SOD level may be due to the increased expression of SOD gene product in brain by TF. It has been reported that the crude ethanol extract of *Hygrophila auriculata* upregulates SOD gene expression in rat kidney against CCl_4 induced liver cirrhosis (Shanmugasundaram & Venkataraman, 2005). TF treatment also reversed the reduction in GSH

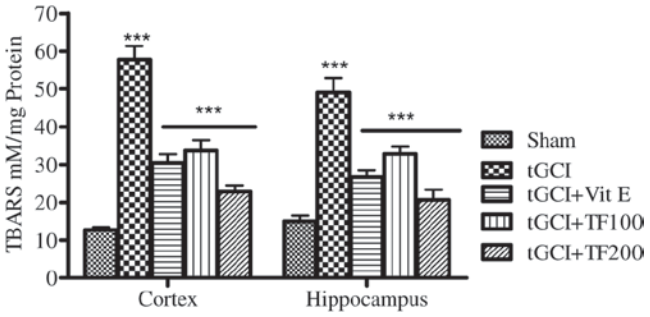

Figure 4. Effect of vitamin E and TF on brain MDA levels following tGCI injury. Values are represented as mean ± SEM, ***$p < 0.001$ tGCI vs sham and vitamin E, TF vs tGCI.

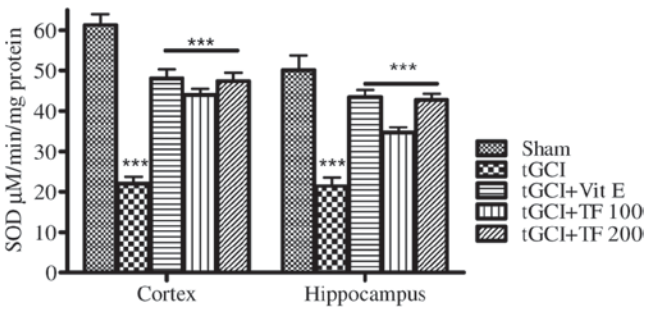

Figure 5. Effect of vitamin E and TF on brain superoxide dismutase (SOD) levels following transient tGCI cerebral ischemia/reperfusion injury. Values are represented as mean ± SEM, ***$p < 0.001$ tGCI vs sham and vitamin E, TF vs tGCI.

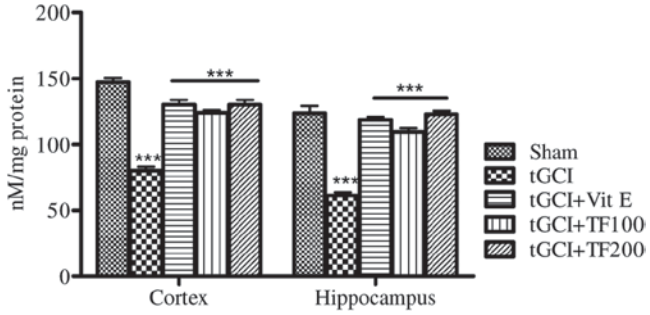

Figure 6. Effect of vitamin E and TF on brain glutathione (GSH) levels following transient tGCI cerebral ischemia/reperfusion injury. Values are represented as mean ± SEM, ***$p < 0.001$ tGCI vs sham and vitamin E, TF vs tGCI.

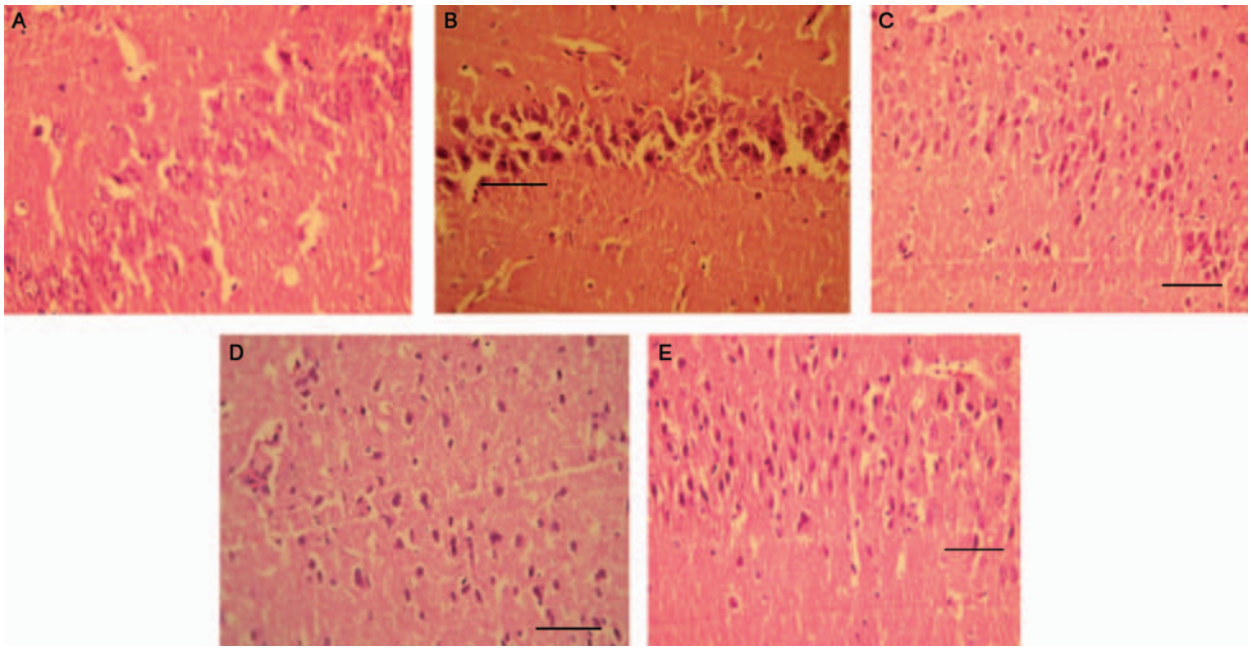

Figure 7. Showing histopathological sections of CA1 hippocampal neuron. (A) sham control, (B) tGCI control, (C) tGCI treated with vitamin E, (D) TF (100 mg/kg) treated tGCI group, and (E) TF (200 mg/kg) tGCI treated. Magnification 100× (H&E Staining).

activity in the cortex and hippocampus of tGCI group. Our results are in agreement with the earlier report of Vijay Kumar et al. (2006), where they showed that the chronic treatment of rats with crude extract of HA increased the activity of GSH in liver homogenate. In brain, GSH is considered as intra- and intercellular signaling molecules which includes synthesis and release of glutamate and GABA (Weber, 1999) regulation of NMDA and non-NMDA glutamate receptors activity (Oja et al., 2000). It has been shown that GSH and its related enzyme deficiency leads to various neurological impairment and disorders (Cooper & Meister, 1992). The results of MDA and glutathione are consistent with neurological and histological recovery. This suggest that the antioxidant property of TF acted as a possible mechanism in protecting neurons, possibly by increasing the endogenous antioxidant capacity of the brain to combat oxidative stress induced by transient ischemia and reperfusion.

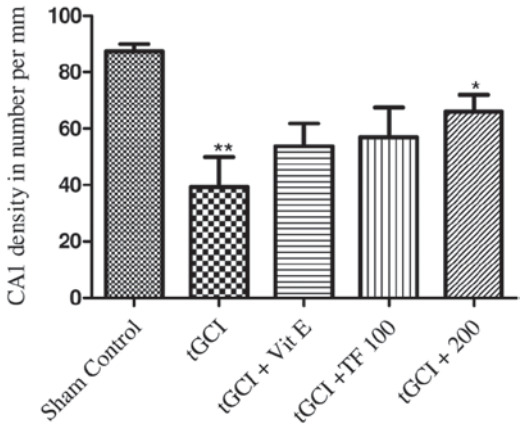

Figure 8. Effect of vitamin E and TF on hippocampal CA1 neurons following cerebral ischemia/reperfusion injury. Values are represented as mean ± SEM, $^{**}p < 0.01$ tGCI vs sham. $^{*}p < 0.05$ with tGCI vs TF.

The neuroprotective effect of terpenoids from curcuma oil was reported in a filament model of focal stroke (Rathore et al., 2008) and the antioxidant effect of terpenoids from *Asparagus racemosus* Willd root has been established in rat model of global cerebral ischemia (Nandagopal et al., 2011) recently. Medicinal herbs like *Ginkgo biloba* L., green tea extract and flavonoids found in many herbal extracts have been shown to decrease oxidative stress in animal models of cerebral ISH/RF injury (Calapai et al., 2000; Hong et al., 2001). Therefore, pharmacological agents capable of scavenging free radicals and inhibiting LPO and thereby protecting neurons from oxidative stress may provide useful therapeutic agents for the prevention or treatment of neurodegenerative disorders. Earlier studies with vitamin E have clearly shown that vitamin E treatment prevents reactive oxygen spurt (ROS) and carbonyl protein depletion in hippocampal neurons caused by cerebral ischemia and reperfusion in normal gerbils and stroke prone spontaneously hypertensive rats but does not significantly affect CA1 architecture (Tagami et al., 1999). In the present investigation, data clearly demonstrate the neuroprotective effect of the terpenoid fraction from *H. auriculata* in protecting CA1 neuron architecture is due to antioxidant properties. In addition to antioxidant properties, biolobalide from *Ginkgo biloba* and asiatic acid from *Centella asiatica* were reported to inhibit GABAergic mediated CA1 neuron excitability in

rat hippocampal slices (Keiko et al., 1999; Nasir et al., 2010), and show neurotrophic (Li et al., 2003) and anti-apoptotic properties (Stephan et al., 2000).

Further, the tGCI animals exhibited a score of more than five in beam balance task and impaired motor function as assessed in the rotarod experiment which clearly indicates the altered sensory and motor behavior induced by tGCI. Previous experiments have confirmed the positive correlation between impaired sensory and motor deficit in rodents being directly proportional to magnitude of the ischemic insult, reactive oxygen production and neuronal damage in cortical and subcortical areas (Liang et al., 2008). Impaired beam balances and altered motor function of the rats following tGCI may be correlated with cortical neuron damage. Rats with tGCI have shown improvement with TF administration which may be due to attenuation of neuronal death in hippocampal neurons and protecting the brain against oxidative stress by enhancing antioxidant systems. In the open field test, tGCI rats showed hyperactive behavior as indicated by an increased number of entries, and decreases in percentage and number of central squares crossed. It has been shown by many investigators that hyperactive locomotion in the open field is due to damage of hippocampal neuron (Kiyotaka et al., 2003). The hyperactive behavior of tGCI rats was attenuated by TF administration suggesting a neuroprotective role of TF and further decreases in hyperactive behavior with TF administration may be due to direct hippocampal protection.

Conclusion

It is concluded that natural terpenoids from *Hygrophila auriculata* show a neuroprotective effect in tGCI induced oxidative stress by protecting brain cells from reactive oxygen species scavenging and increase the endogenous antioxidant capacity of the brain to combat ISH/RF induced oxidative stress. This effect might explain apparent usage of this plant in the folklore claim for stroke.

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Declaration of interest

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