Neuroprotective effect of alcoholic extract of *Terminalia arjuna* bark in experimental stroke induced rats

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Abstract: *Terminalia arjuna* Wight & Arn. (Combretaceae) is a tree having an extensive medicinal potential and popular in various Indigenous Systems of Medicine like Ayurveda, Siddha and Unani. Its stem bark possesses glycosides, large quantities of flavonoids, tannins and minerals. Flavonoids are reported to exert antioxidant, anti-inflammatory and lipid lowering effects. Evidence demonstrates that the impaired energy metabolism and the excessive generation of reactive oxygen species (ROS) contribute to the brain injury associated with cerebral ischemia. In the present study, the protective effect of *T. arjuna* bark alcoholic extract (TABE) was investigated in transient middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia–reperfusion injury in rats. Antioxidant activity of TABE was evaluated *in vivo* and *in vitro*. Male albino rats were divided into five groups: Normal, Sham-operated group, Ischemic control group, and TABE-pretreated groups (500 and 1000 mg/kg/p.o.). TABE was administered once a day, for 14 days. The rats were subjected to a 2 h right MCAO via the intraluminal filament technique and 22 h of reperfusion. Pretreatment with TABE significantly reduced the histological changes and neurological deficits. TABE at a dose of 1000 mg/kg significantly reversed the elevated total calcium levels, brain malondialdehyde (MDA) content and restored the decreased activities of Na⁺-K⁺-ATPase, brain superoxide dismutase (SOD), catalase (CAT) and reduced glutathione levels (GSH). TABE when tested against generation of oxygen free radicals *in vitro* at the concentrations (64-1000 µg/ml), counteracted lipid peroxidation and the formation of DPPH, nitric oxide (NO) and superoxide anions (O₂⁻) with IC₅₀ values 646.40 µg/ml, 488.18 µg/ml, 787.29 µg/ml and 1006.81 µg/ml respectively. Results of present study indicated that TABE has a protective potential against cerebral ischemia injury and its protective effects may be due to its antioxidant property.

Key-Words: Stroke, Ischemia, Cerebrovascular diseases, Oxidative stress, *Terminalia arjuna*, Free radicals, Middle cerebral artery occlusion.

1 Introduction
Stroke is an acute and progressive neurodegenerative disorder. It is a medical emergency and can cause permanent neurological damage, complications and death. Stroke is the second most common cause of death worldwide [1] and the leading cause of disease acquired adult disability [2]. In western countries, stroke causes 10-12% of all deaths [3], ranking after heart disease and before cancer [4]. Until now there is no effective neuroprotective therapy for the treatment of stroke. Thus, there is an urgent need to develop therapeutic strategies for the management of stroke.

Ischaemic stroke reduces blood supply, leads to energy shortage-induced
imbalance of ionic gradients across membrane, accumulates calcium, sodium, and reduces pH, which in turn alters membrane transport, mitochondrial function, activates calcium-dependent enzymatic reactions, including DNA-breaking enzymes and generates excessive free radicals and triggers lipid peroxidation and ultimately cell apoptosis. Primary therapeutic strategy in treating cerebral ischemia is to restore blood flow in the shortest time possible in order to preserve neural tissues. Although reperfusion of ischemic brain tissue is critical for restoring normal function, it can paradoxically result in secondary damage, called reperfusion injury. Secondary approach is to ameliorate pathophysiological consequences of stroke caused by free radicals generated during ischemia and reperfusion. Thus, antioxidants or free radical scavenging agents are demonstrated to have protective effect in experimental stroke [5-10]. It is important to note that an anti-ischemic agent aiming at both vascular and parenchymal effects would be more beneficial.

Terminalia arjuna (TA) is widely used by Ayurvedic physicians for its curative properties in organic/functional heart problems including angina, hypertension and deposits in arteries. Its stem bark possesses glycosides, large quantities of flavonoids, tannins and minerals. Flavonoids have antioxidant, anti-inflammatory and lipid lowering effects while glycosides are cardiotonic. Flavonoids and Oligomeric proanthocyanidins provide antioxidant activity and vascular strengthening [11]. It also has mild diuretic, antithrombotic, prostaglandin E2 enhancing and hypolipidaemic activity [12].

2 Materials and Methods

2.1 Plant material
The wet bark of T. arjuna was collected from Tirumala hills during the month of November, 2009 and was shade dried. Stem bark was coarsely powdered and refluxed with 90% v/v methanol for six hours. The extract was filtered and the filtrate was evaporated to dryness by rotary flash evaporator at low temperature under reduced pressure. A dark brownish-red shiny powder-like residue was obtained, which was stored in a desiccator.

2.2 Animals
Male albino rats weighing 200-250 g were chosen to avoid fluctuations due to estrous cycle. The rats were housed in polypropylene cages under 12 hrs light /dark cycle, fed with standard laboratory chow (Hindustan Lever Limited, Mumbai) and water ad libitum. Animals were acclimatized to the laboratory conditions prior to experimentation and all the experiments were carried out according to the study protocol approved by the Institutional Animal Ethical Committee.

2.3 Induction of ischemia
Transient focal cerebral ischemia was produced by intra luminal middle cerebral artery occlusion procedure as reported by Longa et al.,[13] with slight modifications. Briefly the rats were anaesthetized with Thiopentone Sodium (40mg/kg, i.p.). A midline incision was made on ventral side of the neck and the right carotid bifurcation was identified. The external carotid artery was carefully isolated from accompanying nerves and ligated. A small niche was given to the right common carotid artery (CCA) proximal to the carotid bifurcation and a 4-0 monofilament nylon thread (Ethicon, Johnson & Johnson) with its tip rounded by heating quickly by bringing it near a flame was introduced through it into the lumen of internal carotid artery and advanced until a slight resistance was felt which ensured the occlusion of the origin of middle cerebral artery. Two hours after the induction of ischemia, the filament was slowly withdrawn and the animals were returned to their cages for a reperfusion period of 22 hrs, the body temperature was maintained at
37°C with a thermostatically controlled infrared lamp. In sham rats the right CCA was surgically prepared for the insertion of the filament, but the filament was not inserted.

2.4 Study protocol
Rats (200-250g) were randomly divided into five groups of 9 rats in each group, fed with drug or vehicle for 14 days prior to experiment. The first group served as normal and received 1% tween 80 in water orally. The second group served as sham control, received 1% tween 80 in water orally. The third group served as ischemic control, received 1% tween 80 in water orally. The fourth and fifth groups were treated with TABE 500 and 1000 mg/kg/p.o., respectively for 14 days prior to MCAO. After 24 hrs, neurological examinations were performed in all groups. Then six rats in each group were decapitated to obtain brain tissue samples for biochemical analysis and remaining three brains in each group were used for histopathological studies.

2.5 Neurological deficit
Neurological deficit in the vehicle- and drug-pretreated groups were determined after 24 hrs of induction. Neurological findings were scored on a 5-point scale [13] as follows: no neurological deficit = 0, failure to extend left paw fully = 1, circling to left = 2, falling to left = 3, did not walk spontaneously and had decreased levels of consciousness = 4. The above procedures were performed in a blinded manner.

2.6 Biochemical estimations
24 h after induction of ischemia the animals were killed by cervical dislocation and their brains were dissected out quickly. The infarcted area was homogenized in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% w/v homogenate. The homogenate was centrifuged at 10,000 rpm for 10 min at 0°C in cold centrifuge and the resulting supernatant was used for biochemical estimations.

2.6.1 Superoxide dismutase
SOD activity was measured according to the method of Misra and Fridovich [14], at room temperature. 100 µl of supernatant was added to 880 µl of 0.05 M carbonate buffer (pH 10.2, containing 0.1 mM EDTA), and 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture, and the optical density values were measured at 480 nm for 4 min on a Systronics UV–Visible Spectrophotometer; activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50% which is equal to 1 unit.

2.6.2 Catalase
Catalase activity was measured by a slightly modified version of Aebi, [15] at room temperature. 100 µl of supernatant was added to 10 ul of 100% ethanol and placed in ice bath for 30 min. The tubes were brought to room temperature followed by the addition of 10 µl of Triton X-100. In a cuvette containing 200 µl of phosphate buffer and 50µl of above mixture, 250 µl of 0.066M H₂O₂ in phosphate buffer was added. The decrease in optical density was measured at 240 nm for 60 sec in Systronics UV Spectrophotometer. The molar extinction coefficient of 43.6 Mcm⁻¹ was used to determine catalase activity which is equal to the moles of H₂O₂ degraded/mg protein/min.

2.6.3 Reduced Glutathione
Reduced glutathione levels were measured according to the method of Ellman [16] at room temperature. 0.75 ml of supernatant was mixed with 0.75 ml of 4% sulphosalicylic acid and then centrifuged at 1200 rpm for 5 min at 4°C. From this 0.5 ml of supernatant was taken and added to 4.5 ml of 0.01 M DTNB, and absorbance was
measured at 412 nm by using a Systronics UV–Visible Spectrophotometer.

2.6.4 Estimation of lipid peroxidation
MDA levels were measured according to the method of Ohkawa et al., [17] at room temperature. 200 µl of supernatant was added to 50 µl of 8.1% sodium dodecyl sulphate, vortexed, and incubated for 10 min at room temperature. 375 µl of thiobarbituric acid (0.6%) was added and placed in a boiling water bath for 60 min and then the samples were allowed to cool at room temperature. A mixture of 1.25 ml of butanol: pyridine (1.5:1) was added, vortexed, and centrifuged at 1000 rpm for 5 min. The optical density values of colored layer was measured at 532 nm on a Hitachi U-2000 Spectrophotometer against reference blank and the values were expressed in nM of MDA formed for mg protein/min.

2.6.5 Estimation of total Calcium
To 4.5ml of deproteination buffer in a glass centrifuge tube, 0.5ml of the sample was added and was placed in water bath for 3 minutes. Tubes were centrifuged while they were still hot, 0.5ml of supernatant was transferred into clean test tubes, 5ml of working colouring reagent was added to each tube, mixed well and then at 570 nm. Calcium chloride solutions of 2-10 µg calcium/ml were used for the preparation of standard plot [18].

2.6.6 Estimation of Sodium potassium ATPase (Na⁺-K⁺ATPase) activity: 0.25 ml reaction mixture was taken in two sets of test tubes. 0.1 ml of 10 mM Ouabain was added to one set of tubes while to the other, equal amounts of distilled water was added. 50 µl of supernatant was added to both the sets. Total volume of incubation mixture was made up to 0.5 ml with the addition of 50 µl of water to both sets of test tubes (after preincubation period of 5 min), reaction was initiated with the addition of 50 µl of ATP solution. The reaction was terminated after 10 min by adding 0.5 ml of 10% TCA and the tubes were immediately transferred to ice. Enzyme blank was run in the similar way but supernatant was added after the addition of TCA. Incubated in ice for 10 min, all the tubes were centrifuged for 5 min to remove the precipitate. Whole supernatant was taken for phosphate estimation by micromethod. The enzyme activity was expressed as µM of Pi (inorganic phosphate) liberated / hr / mg protein. The difference in the activity in the absence and presence of Ouabain was taken as Na⁺-K⁺ATPase activity [19]. Activity in the presence of Ouabain is Mg²⁺ATPase activity.

2.7 In vitro antioxidant studies

2.7.1 Anti lipidperoxidation assay
The extent of lipid peroxidation in rat brain homogenate was measured in vitro in terms of formation of thiobarbituric acid reactive substances (TBARS). Different concentrations of the TABE (64-1000 µg/ml) in ethanol were individually added to the brain homogenate (0.5 ml). This mixture was incubated with 0.15 M KCl (100 µl). Lipid peroxidation was initiated by adding 100 µl of 15 mM FeSO₄ solution and the reaction mixture was incubated at 37°C for 30 min. An equal volume of TBA (thiobarbituric acid): TCA (trichloro acetic acid), 1:1, 1 ml was added to the above solution followed by the addition of 1 ml butyrated hydroxyl toluene. This final mixture was heated on a water bath for 20 min at 80°C, then cooled and centrifuged. Absorbance of supernatant was read at 532 nm against control blank [20] using a Systronics UV–Visible Spectrophotometer. The percentage inhibition of lipid peroxidation was calculated by using the formula,

\[ \text{Inhibition} \% = \left( \frac{\text{Control} - \text{Test}}{\text{Control}} \right) \times 100 \]
2.7.2 DPPH (1,1- diphenyl, 2-picryl hydrazyl) radical scavenging activity
DPPH scavenging activity was measured by the Spectrophotometric method [21]. To ethanolic solutions of DPPH (200 µM), 0.05 ml of TABE dissolved in ethanol (63-1000 µg/ml) were added. After 20 min the decrease in absorbance of test mixtures (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition was calculated [20].

2.7.3 Scavenging of nitric oxide radical
Nitric oxide (NO) was generated from sodium nitroprusside and measured by Griess’ reaction [22, 23]. Sodium nitroprusside (5 mM) in standard phosphate buffer solution (0.025 M, pH: 7.4) was incubated at 25°C for 5 h with different concentrations (64-1000 µg/ml) of TABE in ethanol. Blank experiments without the test compound but with equivalent amounts of buffer were conducted in an identical manner. After 5 h, 0.5 ml of incubation solution was taken and diluted with 0.5 ml of Griess’ reagent (1% naphthyl ethylene diamine dihydrochloride) and the absorbance of the chromophore formed was read at 546 nm and the percentage inhibition was calculated [21, 24].

2.7.4 Scavenging of superoxide radical
The scavenging activity toward the superoxide radical (O$_2^-$) was measured in terms of inhibition of O$_2^-$ [25] by following alkaline dimethyl sulphoxide (DMSO) method [26]. Potassium superoxide and dry DMSO were allowed to stand in contact for 24 h and the solution was filtered just before use. The filtrate (200 µl) was added to 2.8 ml of an aqueous solution containing nitroblue tetrazolium (56 µl), ethylene diamine tetra acetic acid (10 µl) and potassium phosphate buffer (10 mM) then the test compounds (1ml) at various concentrations (64-1000 µg/ml) of TABE in ethanol were added and absorbance was recorded at 560 nm against a blank to calculate the percentage inhibition.

2.8 Histopathology
Coronal brain sections from normal and experimental groups of focal ischemia were fixed in a mixture of formaldehyde (4%), glacial acetic acid, and methanol (1:1:8, v/v). Brain slices were cut into 4- to 5-mm thickness and embedded in paraffin blocks; brain sections of 4 to 6 µm thickness were stained with hematoxylin and eosin.

2.9 Statistical analysis
All the data was expressed as mean ± S.E.M, statistical significance was determined by one way analysis of variance (ANOVA) followed by Dunnet’s test, using GraphPad prism 5. In all tests, the criteria for statistical significance was P<0.05.

3 Results

3.1 Effect of TABE on neurological deficit
The neurological score after 22 hrs of reperfusion was given in Table 1. Pretreatment with TABE (500 and 1000 mg/kg/p.o.) significantly reduced the neurological deficit (P<0.05 and P<0.001 respectively) [Fig. 1].

<table>
<thead>
<tr>
<th>Groups</th>
<th>Neurological score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Sham control</td>
<td>0</td>
</tr>
<tr>
<td>Ischemic control</td>
<td>2.786 ± 0.2857</td>
</tr>
<tr>
<td>TABE (500 mg/kg)</td>
<td>2.071 ± 0.1304*</td>
</tr>
<tr>
<td>TABE (1000 mg/kg)</td>
<td>1.571 ± 0.1304***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=9); *(p<0.05), ***(P<0.001) Vs Ischemic control group.
3.2 Effect of TABE on brain MDA, SOD, CAT and GSH levels

The effect of TABE on brain MDA, SOD, CAT, and GSH levels in MCAO-induced ischemia-reperfusion rats is shown in Table 2. The content of brain MDA in the ischemic hemisphere was significantly increased (P<0.001) in Ischemic control group as compared to the Normal group. Pretreatment with TABE (500 and 1000 mg/kg/p.o.) significantly reversed the elevated MDA levels (P<0.001) as compared to the Ischemic control group [Fig. 2].

The catalase level in the ischemic hemisphere of Ischemic control group was significantly decreased (P<0.001) as compared to the Normal group. Pretreatment with TABE (500 and 1000 mg/kg/p.o.) significantly increased the decreased catalase levels (P<0.001) when compared with Ischemic control group [Fig. 4].

The level of reduced glutathione in the ischemic hemisphere was significantly decreased (P<0.001) in Ischemic control group as compared to the Normal group. Pretreatment with TABE (500 and 1000 mg/kg/p.o.) significantly increased the reduced glutathione levels (P<0.001) when compared with that of the Ischemic control group [Fig. 5].

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Superoxide dismutase (Units/mg protein)</th>
<th>Catalase (µM/min/mg protein)</th>
<th>Reduced glutathione (µM/mg protein)</th>
<th>MDA (nM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.596±0.05</td>
<td>51.32±0.96</td>
<td>0.097±0.01</td>
<td>0.261±0.02</td>
</tr>
<tr>
<td>Sham control</td>
<td>1.519±0.08</td>
<td>50.47±0.06</td>
<td>0.088±0.01</td>
<td>0.228±0.01943</td>
</tr>
<tr>
<td>Ischemic control</td>
<td>0.291±0.01***</td>
<td>15.43±1.87***</td>
<td>0.029±0.001***</td>
<td>0.674±0.01***</td>
</tr>
<tr>
<td>TABE (500 mg/kg)</td>
<td>0.905±0.03***+++</td>
<td>45.66±0.99***+++</td>
<td>0.067±0.001***+++</td>
<td>0.463±0.01***+++</td>
</tr>
<tr>
<td>TABE (1000 mg/kg)</td>
<td>1.333±0.02***+++</td>
<td>47.68±0.71+++</td>
<td>0.078±0.01***+++</td>
<td>0.306±0.01+++</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6);
* (P<0.05), ** (P<0.01), *** (P<0.001) Vs Normal group;
+++ (P<0.001) Vs Ischemic control group.
3.3 Effect of TABE on Calcium levels
The effect of TABE on brain calcium levels in different groups was given in Table 3. The content of brain total calcium in the ischemic hemisphere was significantly increased (P<0.001) in Ischemic control group as compared to the Normal group. Pretreatment with TABE (500 and 1000 mg/kg/p.o.) significantly reversed the elevated calcium levels (P<0.001) as compared to the Ischemic control group [Fig. 6].

3.4 Effect of TABE on Na⁺-K⁺ATPase activity
Na⁺-K⁺ATPase activity in the brain tissues of Ischemic control group rats was significantly decreased (P<0.001) as compared to that of the Normal group (Table 3). Pretreatment with T. arjuna bark extract (500 and 1000 mg/kg/p.o.) significantly reversed the decrease in Na⁺-K⁺ATPase activity (P<0.05 and P<0.001 respectively) as compared to the Ischemic control group [Fig. 7].

Table 3. Effect of TABE on Calcium levels and Na⁺-K⁺ATPase activity

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Calcium (µg/mg protein)</th>
<th>Na⁺-K⁺ATPase (µM of Pi/h/mg protein)</th>
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3.5 In vitro antioxidant activity
Several concentrations, ranging from 64-1000 µg/ml of TABE in ethanol were found to have a dose dependent free radical scavenging activity in different in vitro models like anti lipidperoxidation assay (Fig. 8), DPPH scavenging activity (Fig. 9), NO scavenging activity (Fig. 10) and superoxide radical scavenging activity (Fig. 11). The maximum percentage inhibition at 1000 µg/ml concentration and IC₅₀ values are as given in Table. 4. On comparative basis TABE showed excellent activity against lipid peroxidation and in quenching DPPH and nitric oxide radicals, but showed moderate activity in quenching super oxide radicals as given in Table 4.
Table 4. In vitro antioxidant studies of TABE

<table>
<thead>
<tr>
<th>In vitro model</th>
<th>% Inhibition (at 1000 µg/ml)</th>
<th>IC$_{50}$ (µg/ml)</th>
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<tbody>
<tr>
<td>Antilipid peroxidation assay</td>
<td>75.38</td>
<td>646.40</td>
</tr>
<tr>
<td>DPPH scavenging activity</td>
<td>98.19</td>
<td>488.18</td>
</tr>
<tr>
<td>NO scavenging activity</td>
<td>62.83</td>
<td>787.29</td>
</tr>
<tr>
<td>Superoxide scavenging activity</td>
<td>50.30</td>
<td>1006.81</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6);
* (P<0.05), ** (P<0.01), *** (P<0.001) Vs Normal group;
+ (P<0.05), ++ (P<0.01), +++ (P<0.001) Vs Ischemic control group.

3.6 Effect of TABE on histopathological studies
Figures A and B show brain sections of Normal and Sham control animals and depicts normal cytoarchitecture of brain tissue and neuronal cells (N) with normal nucleus. Figure C show MCAO, hypoperfusion-induced marked congestion of blood vessels (HCBV), excessive degeneration of neuronal cells (DN), excessive vaculation (EV) and necrosis of neural cells. Pretreatment with TABE 500 mg/kg showed mild congestion (MC), mild degeneration (MD) of neuronal cells, and mild vaculations (MV) in Fig D, where as pretreatment with TABE 1000 mg/kg showed neuronal cells with normal nucleus (Fig E). Figures D and E showed that TABE pretreatment dose dependently reversed the congestion of blood vessels, congestion and necrotic degeneration of neuronal cells compared with Ischemic control group.
4 Discussion

In the present study, pretreatment with TABE significantly reversed the MCAO-induced focal cerebral ischemia in rats. Neurological deficit symptoms and histopathological changes confirm the evidence of the brain damage. Focal cerebral ischemia causes cerebral cell death, resulting in neuronal deficit symptoms and cerebral infarction [27]. Numerous reports indicate the involvement of oxidative stress in focal cerebral ischemia [7, 8, 28]. In the present study GSH, CAT, and SOD levels were decreased and MDA level was increased, indicating the involvement of oxidative stress in MCAO induced reperfusion injury.

SOD dismutates superoxide radicals to form hydrogen peroxide, which in turn is decomposed to water and oxygen by glutathione peroxidase and catalase, thereby preventing the formation of hydroxyl radicals [29]. Therefore, these enzymes act co-operatively at different sites in the metabolic pathway of free radicals. Failure of this antioxidant defense leads to oxidative damage and initiation of lipid peroxidation [30]. Cerebral ischemia up regulates inducible nitric oxide synthase (iNOS), producing excessive nitric oxide and it reacts with superoxide to form peroxynitrite, which directly hydroxylate and nitrate the aromatic residues of amino acids and nucleotides in cytosol and nucleus [31, 32], resulting in cellular machinery dysfunction and neuronal loss after. Focal cerebral ischemia increases MDA and decreases GSH, CAT and SOD levels [7], [8]. In the present study depletion of GSH levels was observed in ischemic rats. Reduced glutathione (GSH) is one of the primary endogenous antioxidant defense systems in the brain, which scavenges hydrogen peroxide and lipid peroxides [33]. Depletion of GSH in ischemia reperfusion injury is attributed to several factors such as cleavage of GSH to cysteine, decrease in the synthesis of GSH and the formation of mixed disulfides, causing their cellular stores to be depleted [34].

Excessive generation of ROS results in the lipid peroxidation of the cell membrane and subsequent damage is reflected by accumulation of MDA, a byproduct of lipid peroxidation [35]. Lipid peroxidation is one of the major consequences of free radical mediated injury to the brain. The peroxidation of the membrane phospholipids (PUFA) reaction continues either until the exhaustion of the substrate or termination of chain propagation by antioxidants. Lipid peroxidation products are widely accepted group of oxidative stress markers, especially MDA levels detected as a stable derivative chromophore of Thiobarbituric acid adducts, is used as a potentially reliable and sensitive marker of reperfusion injury [36, 37]. Several studies demonstrated higher MDA levels in stroke patients [38-42] and infarct size, clinical severity and patient’s outcome are well correlated with MDA levels [41], [42]. Online with previous studies in the present study, ischemic rats showed significant increase in MDA levels indicating reperfusion injury by ROS. Our findings, viz. significantly elevated LPO and depleted protective antioxidant enzymes (GSH, CAT, and SOD) in ischemia/reperfusion groups, are thus in agreement with other reports on the role of oxidative stress in cell injury in general and cerebrovascular disease in particular [7, 43-46]. In the present study, TABE exhibited marked protection against MCAO induced ischemia/reperfusion as evidenced by significant reversal of enzymatic alterations produced by such insult.

Ischemic cascade is initiated by energy failure which quickly leads to dysfunction of energy-dependent ion transport pumps (like Na⁺-K⁺ATPase) and depolarization of neurons and glia [47, 48]. Na⁺-K⁺ATPase, which is responsible for the maintenance of neuronal excitability and the control of cellular volume in the central nervous system [49], is an important parameter to investigate stroke-induced brain damage. Inhibition of the activity of
this crucial enzyme is associated with various neuropathological conditions, including cerebral ischemia, neurodegenerative disorders and spinal cord edema, which also provide evidence for the vulnerability of Na⁺-K⁺ATPase to free radical attacks [50-52]. In the present study, decreased Na⁺-K⁺ATPase activity due to cerebral ischemia, indicates membrane damage and deterioration of membrane fluidity, is in accordance with increased levels of calcium and lipid peroxidation. TABE pretreatment (500 and 1000 mg/kg) increased Na⁺-K⁺ATPase activity when compared to Ischemic control group (P<0.5 and P<0.001). This effect of TABE may be due to its powerful antioxidant properties thereby protecting membrane Na⁺-K⁺ATPase from being attacked by the free radicals produced during ischemia.

In the very early phase of ischemia, membrane potential is lost due to energy depletion consequently presynaptic neurons and glia depolarize [53] to release excitotoxic amino acids (especially glutamate) into extracellular compartment in large amounts. The activation of glutamate receptors leads to a further increase of intracellular Ca²⁺, Na⁺ and Cl⁻ levels. Calcium enters into damaged neurons through voltage-gated calcium channels, via N-methyl-D-aspartate (NMDA) receptor operated channels, and release from intracellular stores. Cytoplasmic calcium activates enzymes and second messenger cascades that contribute to cell death. Activated proteolytic enzymes break down elements of the cytoskeleton, leading to protein aggregation. Calcium-mediated lipolysis damages membranes and along with nitric oxide synthase activation provides nitric oxide and fatty acid substrates for free radical production. Glutamate release is stimulated by calcium-dependent exocytosis and the released glutamate in turn causes Ca²⁺ channels to open, leading to further Ca²⁺ overload. In the present study results demonstrated the elevation of calcium levels in Ischemic control group compared to Normal group rats which is in consonance with the earlier reports [54]. A significant decrease in total calcium level observed in TABE pretreatment groups may be due to either calcium channel blocking or calcium chelation or via antiglutamnergic activity of some phytochemical constituent in the extract. Free radicals are well documented to increase calcium levels [55], [56], [57]. Therefore, the observed decrease in calcium levels with TABE may also be attributed to its anti-oxidant effects.

Search for agents providing protection against lipid peroxidation and enhancing anti-oxidant enzyme defense system is a rational approach for therapy of cerebrovascular ailments. Natural products (i.e. medicinal plants) with intrinsic antioxidant property constitute an ideal choice for maximum therapeutic effects with minimal risk of iatrogenic adverse effects. Recently, several natural products like rutin, sodium selenite, curcumin, isoliquiritigenin (flavanoid), Spirulina, Acorus calamus rhizomes extract, Thymoquinone, Nigella sativa seed oil, and Korean ginseng tea showed their protective effect on MCAO-induced reperfusion injury due to their antioxidant property [7, 8, 10,28, 58-60].

TA bark has active components such as Arjunolic acid, tomentosic acid, β-sitosterol, ellagic acid, (+)-leucodelphinidin [64], arjunic acid [62]; arjunetin [63], arjunenin, arjunglucoside I and II [65], tannins containing catechin, gallo catechin, epicatechin, epigallocatechin [66], arjunolone, baicalein [67, 68], arjunglucoside III [66], terminic acid[69], arjunolitin[70], arjunglucoside IV, V [71], arjunasides A-E [72], 2α, 3β-dihydroxy urs-12, 18 dien-28-oic acid 28-O-β-D-glucopyranosyl ester [73], casuarinian [74], arjunophanoloside [76], terminoside A [74], arjunin [77], terminarjunoside I, II [78].

The phytochemical screening of TABE showed the presence of triterpenoids, tannins and flavonoids which are arjunolic acid, arjunic acid, arjunenin, arjuni tin, arjunglucoside I, arjunglucoside III, arjunoside I, arjunolitin, terminoside A, arjunetoside, lupeol, arjunglucoside II
(triterpenoids), casuarinin, ellagic acid and gallic acid (tannins), leucocyanidin and luteolin (flavonoids). Tannins and flavonoids are well known to have antioxidant properties, anti-inflammatory activity [79], antiproliferative as well as anti-platelet effects [80, 81] and may be responsible for significant rise in the endogenous antioxidants (SOD, GSH and CAT) of treatment groups in the present study. Arjunolic acid (triterpene) a potent principle isolated from TA has been shown to prevent the decrease in levels of SOD, CAT, GSH and ceruloplasmin ascorbic acid and provided significant protection against lipid peroxidation in Isoproterenol induced myocardial necrosis in wistar rats [82]. Arjunic acid was significantly active against the human oral (KB), ovarian (PA1) and liver (HepG-2 and WRL-68) cancer cell lines [83]. Luteolin, a flavone isolated from the butanol fraction of TA was found to be effective in inhibiting a series of solid tumors (Renal A-549, ovary SK-OV-3, Brain SF-295, Leukemia P 388, SKMEL-2, XF-498, HCT 15, Gastric HGC-27). Casuarinin, a hydrolysable tannin isolated from the bark of TA protects cultured Madin Darby canine kidney (MDCK) cells against H2O2 mediated oxidative stress when compared with Trolox (hydro soluble Vitamin E analog) by decreasing DNA oxidative damage and preventing the depletion of intracellular Glutathione (GSH) in MDCK cells [84]. Terminoside A isolated from the acetone fraction of the ethanolic extract of stem bark of TA potently inhibited Nitric Oxide (NO) production and decreased inducible Nitric Oxide Synthase (iNOS) levels in lipopolysaccharide-stimulated macrophages [75]. Arjunaphthanoloside isolated from the stem bark of TA showed potent antioxidant activity and inhibited Nitric Oxide (NO) production in lipopolysaccharide (LPS)-stimulated rat peritoneal macrophages [76]. Arjungenin and its glucoside isolated from the stem bark of TA exhibited moderate free radical scavenging activity in vitro and was found to have moderate activity with an IC50 value of 290.6 lg/ml as compared to standard vitamin C [85]. These might contribute to neuroprotection against cerebral iskemia too.

The results of present study suggests that the TABE pretreatment dose dependently protected the integrity of brain tissue against MCAO-induced ischemia-reperfusion injury as evidenced by histopathological studies, neurological deficit symptoms, brain SOD, CAT, GSH levels, MDA content, total calcium levels and Na+-K+ATPase activity. Pretreatment with TABE 500, 1000 mg/kg/p.o significantly reversed and restored the levels of SOD, GSH, CAT, Na+-K+ATPase activity and reduced LPO and calcium levels in MCAO rats and there by prevented the stroke induced changes in the brain to near normal in the groups pre-treated with TABE. The neuroprotective effect of TA may be due to presence of flavonoids and tannins.

In conclusion, these findings suggest a potential role of Terminalia arjuna in stroke and gain importance in view of the fact that stroke is at present the third leading cause of death worldwide, and cerebravascular disease constitute second most frequent cause of projected deaths. TABE contains many active constituents with potent antioxidant effects. TABE offered significant neuroprotection in MCAO-induced focal cerebral ischemia, and this may be attributed to inhibition of lipid peroxidation and increase in endogenous antioxidant defense enzymes. However, further research is required for clinical use of TA against stroke.

5 References


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Histopathology

**Fig A.** Normal (magnification 40X)

**Fig B.** Sham control (Magnification 40X)

**Fig C.** Ischemic control (magnification 40X)

**Fig D.** TABE 500mg/kg pretreatment in focal ischemia (magnification 40X)
**Fig E.** TABE 1000 mg/kg pretreatment in focal ischemia (magnification 40X)

**Fig. 1.** Effect of TABE on Neurological deficit in experimental stroke induced rats.

Values are expressed as Mean ± SEM (n=9); * (p<0.05), *** (P<0.001) Vs Ischemic control group.

**Fig. 2.** Effect of TABE on MDA levels in experimental stroke induced rats.

Values are expressed as Mean ± SEM (n=6); ***(P<0.001) Vs Normal group; +++ (P<0.001) Vs Ischemic control group.

**Fig. 3.** Effect of TABE on SOD levels in experimental stroke induced rats.

Values are expressed as Mean ± SEM (n=6); ***(P<0.001) Vs Normal group; +++ (P<0.001) Vs Ischemic control group.

**Fig. 4.** Effect of TABE extract on Catalase levels in experimental stroke induced rats.
Values are expressed as Mean ± SEM (n=6); ** (p<0.01), *** (P<0.001) Vs Normal; +++ (P<0.001) Vs Ischemic control group.

**Fig. 5.** Effect of TABE on Glutathione levels in experimental stroke induced rats.

Values are expressed as Mean ± SEM (n=6); * (p<0.05), ** (P<0.01), *** (P<0.001) Vs Normal group; +++ (P<0.001) Vs Ischemic control group.

**Fig. 6.** Effect of TABE on Calcium levels in experimental stroke induced rats.

Values are expressed as Mean ± SEM (n=6); *** (P<0.001) Vs Normal group; +++ (P<0.001) Vs Ischemic control group.

**Fig. 7.** Effect of TABE on Na+K+ATPase activity in experimental stroke induced rats.
In vitro studies

Fig. 8. Effect of TABE on Lipid peroxidation.

Fig. 9. DPPH radical scavenging activity of TABE.

Fig. 10. Nitric oxide scavenging activity of TABE.
Fig. 11. Superoxide radical scavenging activity of TABE.