Annals of Clinical Pharmacology & Toxicology

ISSN: 2642-9233

Research Article

Cerebroprotective Effect of *Talinum Triangulare* in Rats Subjected to Cerebral Ischemia-Reperfusion Injury

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Abstract

Aim: The study was aimed to evaluate the cerebroprotective activity of hydroalcoholic extract of *Talinum triangulare* leaves against cerebral ischemia/reperfusion induced oxidative stress in wistar rats.

Methods: Adult Wistar rats were anesthetized with thiopentone and subjected to global cerebral ischemia by occlusion of unilateral common carotid artery for 30 minutes followed by reperfusion. Neurobehavioral parameters, Infarct size, SOD, MDA, CAT and GSH levels were assessed after 48 hrs of reperfusion.

Results: Treatment with 200 mg/kg and 400 mg/kg doses of *Talinum triangulare* for 15 days prior to the ischemic-reperfusion resulted in significant reduction in neurobehavioral deficit, infarct size, MDA levels and significant increase in SOD, CAT and GSH levels.

Conclusion: TTE extract with 15 days prophylactic treatment prior to ischemia-reperfusion in Wistar rats has shown significant cerebroprotective effect and its effect is attributed partially due to its antioxidant properties.

Keywords: ***Talinum triangulare*; Cerebral ischemia reperfusion; Oxidative stress; Cerebroprotection**

Introduction

Ischemia is the condition which causes acute ischemic stroke, where the cells are deprived of oxygen, glucose and other nutrients which are essential for the survival of the cells. In recent years the incidence of ischemic stroke was increased due to the growth of older global population [1]. Stroke is the serious health condition and it is the third leading cause of deaths in western world. Pathogenically, various heterogenous processes involves stroke. Occlusion of the blood vessels is the most common cause of ischemia in brain, called "cerebral-ischemia" [2]. However studies revealed that the damage occurred in the ischemic episode, progresses even after the revival of the blood flow [3]. The injury formed after revival of the blood flow is called "ischemia reperfusion injury" which can lead to permanent disability in patients [4]. Reperfusion of ischemic tissue produces an influx of inflammatory cells and of oxygen that can cause increases in oxygen-derived free radicals causes oxidative stress in brain. Free radicals are also important in prolonged ischemia [3].

Oxidative stress is an imbalance between the generation and removal of reactive oxygen species [2]. After ischemia- reperfusion injury several Reactive Oxygen Species (ROS) generated play an important role in neural cell damage due to ischemia [1,4]. Enzymes such as Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and catalase, and molecular antioxidants such as reduced glutathione (GSH), ascorbic acid, and α-tocopherol, act as endogenous antioxidant defense of the brain which detoxifies free radicals produced in normal course of metabolism [5]. However this defense fails in pathological situations like ischemia reperfusion due to over production of the ROS than the neutralizing capability of endogenous antioxidants [6]. Transient global ischemia impairs behavioral performance associated with cognitive and motor disorders in rodents [7] and cognition in humans [8].

Therefore strengthening of the brain endogenous antioxidant defense by prophylactic treatment of the natural antioxidant rich diet is one of the approaches to minimize the reperfusion injury. *Talinum triangulare* is commonly known as water leaf, which is a good source of natural antioxidants such as flavonoids and phenolic compounds [9,10]. It is a terrestrial herbaceous plant, cultivated as a medicinal as well as food crop in Sri Lanka, India, South America, other parts of Asia, and Nigeria [11,12]. Its extract was shown to have antioxidant and immunomodulatoy effects by *in vitro* studies. In Taiwan, *T. triangulare* has been used in the treatment and prevention of hepatic ailments and cancer in folk medicine [13]. Experimental as well as epidemiological studies demonstrate that bioflavonoids are neuroprotective in models of cerebral ischemia-reperfusion injury [14,15]. In present study we have attempted to evaluate the cerebroprotective activity of hydroalcoholic extract of *Talinum triangulare* leaves and to establish its antioxidant role in the cerebroprotection in rats.

Materials and Methods

Chemicals and reagents

All the chemicals were purchased from Sigma Chemical, St. Louis, Mo, USA and are of analytical grade. Malondialdehyde (MDA), Superoxide Dismutase (SOD), Catalase (CAT) and reduced glutathione analysis kits were purchased from Allied Scientific Products, Kolkata, India.

Citation: Ghantasala VBR, Nalini M, Raghavullu BV, Annapurna A, Venkateswarlu M. Cerebroprotective Effect of *Talinum Triangulare* in Rats Subjected to Cerebral Ischemia-Reperfusion Injury. Ann Clin Pharmacol Toxicol. 2022;3(1):1023.

Publisher Name: Medtext Publications LLC

Manuscript compiled: Mar 15th, 2022

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Wistar albino rats of either sex (230 g to 300 g) were procured from Raghavendra enterprises, Bangalore. Animals were housed in groups of 6 to 7 in colony cages at an ambient temperature of 25°C ± 2°C and 45% to 55% relative humidity with 12 h light/dark cycle. They had free access to pellet chow (Pranav Agro Limited) and water *ad libitum*. Experimental protocol was approved by the Institutional Animal Ethical Committee of Sri Padmavathi School of pharmacy (Approval No: 1016/a/06 /CPCSEA/04/2012 dt 09-12-2012) under the regulation of Committee for the Purpose of Control and Supervision of Experiments on Animals, (CPCSEA), New Delhi, Government of India. Fresh plant materials were collected, air dried and pulverized leaves were subjected to cold maceration in 100 ml of hydro-alcoholic (30:70; Water: Ethanol) mixture for 72 hours. The filtrate was concentrated in a water bath for solvent elimination; the residue was used for the study.

Dose selection

The doses were selected based on the acute toxicity studies as well as previous experimental studies in animals with *Talinum triangulare*. The acute toxicity studies of the *Talinum triangulare* Leaves Extracts (TTLE) showed no symptoms of toxicity or behavioral changes at the maximum dose (4000 mg/kg). Hence 1/10th of the maximum dose *viz*. 400 mg/kg b.w and sub-maximal dose 200 mg/kg were set as dose for present studies.

Experimental protocol

- All rats are provided normal diet and water ad libitum.
- Group-I (Sham control): 1% Sodium CMC for 15 days
- Group-II (Infarction control (I/R)): 1% Sodium CMC for 15 days
- Group-III (Low dose TTLE): 200 mg/kg TTLE in 1% Sodium CMC for 15days
- Group-IV (High dose TTLE): 400 mg/kg TTLE in 1% Sodium CMC for 15 days.

Induction of cerebral ischemia

Animals were anaesthetized with thiopentone sodium (45 mg/kg), through i.p route. Anesthesia levels are checked intermittently (e.g toe pinch) and adjusted accordingly. The animal is placed by its supine position. The ventral neck region is shaved out and cleansed the area with 70% alcohol. Body temperature was maintained at 37 °C ± 1°C during the surgery by heating lamp. A middle incision was made on the ventral side of the neck to expose the left common carotid artery and carefully it was separated from the other tissues and vagus nerve. Global cerebral ischemia was induced by occluding the left common carotid artery by a knot. After 30 min of global cerebral ischemia, the cotton thread was removed with the help of two knot releasers to allow the reperfusion of blood through carotid arteries. The incision was closed by suture and animals were allowed to regain consciousness. All rats were killed after 48 h of reperfusion and their brains are isolated for the estimation of various parameters.

Determination of infarction size

Determination of infarct size in rats was carried out as described in earlier studies [16]. After 48 h of reperfusion, animals were subjected to cervical dislocation, and immediately brains were removed and frozen for 5 min at -4°C. Thereafter coronal sections were made to have 1 mm to 2 mm sections which were immersed in 1% 2,3,5-Triphenyltetrazoliumchloride (TTC) for 30 min at 37°C. The enzymes, NAD and dehydrogenase present in viable cells, convert TTC into red formazone pigment. Therefore, living cells were stained deep red. The infarcted cells remain un-stained due to exhaustion of these enzymes. The slices of the brain were weighed. The unstained infarcted portions were separated from the slices by dissection and weighed to express it in % total weight of the brain.

Preparation of brain tissue for estimation of biochemical parameters

Since the stained tissue is not suitable for estimating the oxidative stress biomarkers, a separate group of animals were used for estimating these enzymes. After 48 h of reperfusion, the brain of each animal was isolated, washed in cold saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1 M, pH 7.4) using homogenizer. The homogenate was centrifuged at 1000 rpm 4°C for 3 min and the supernatant divided into two portions, one of which was used for measurement of Malondialdehyde (MDA). The remaining supernatant was again centrifuged at12,000 rpm at 4°C for 15 min and used for the measurement of Superoxide Dismutase (SOD), Catalase (CAT) and reduced glutathione.

Estimation of MDA level

Procedure: MDA levels in the brain homogenate were measured by the method developed by Ohkawa et al. [17]. To a sample of 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid solution, and 1.5 mL of 0.8% aqueous solution of TBA were added. The mixture was made up to 5 mL with distilled water and then heated in an oil bath at 95°C for 60 min using a glass ball as a condenser. After cooling with tap water, 5 mL of mixture of n-butanol and pyridine (15:1 v/v) was added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer 85 was taken and its absorbance was measured at 532 nm. The tissue MDA levels were measured from the standard curve and expressed as nmol/g tissue.

Estimation of Superoxide Dismutase (SOD)

Procedure: Superoxide Dismutase (SOD) activity was determined by the method developed by Kakkar et al. [18]. Brain tissue was homogenized with homogenizer in ice cold phosphate buffer (0.1M, pH 7.4) to produce a 10% w/v homogenate. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. Aliquot of 0.1 mL supernatant was added to 1.2 mL of 0.052 M sodium pyrophosphate buffer (pH 8.3) followed by addition of 0.1 mL of 186 µM phenazine methosulphate, 0.3 mL of 300 µM nitroblue tetrazolium, 0.2 mL of 780 µM NADH. Reaction mixture was incubated for 90 sec at 30°C, and the reaction was stopped by the addition of 0.1 mL of glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 mL of n-butanol and centrifuged at 4000 rpm for 10 min. The absorbance of organic layer was measured at 560 nm. A control was prepared using 0.1 mL of distilled water devoid of 0.1 mL of homogenate. One unit of the enzyme activity is defined, as enzyme concentration required inhibiting the absorbance of chromogen production by 50% in control sample under the assay conditions. The SOD level was expressed as Units/ mg protein.

Estimation of catalase

Procedure: Catalase activity was measured by the method of Aebi [19]. The brain tissue was homogenized with a homogenizer in ice cold phosphate buffer (0.1M, pH 7.4) to produce a 10% w/v homogenate. The homogenate was centrifuged at 12,000 rpm at 4°C for 15 min supernatant 0.1 mL was added to cuvette containing 1.9

mL of 50 Mm phosphate buffer. To this mixture, 1.0 mL of freshly prepared 30 mM H2O2 was added and changes in absorbance for 3 min at 240 nm at an interval of 30 sec. A control was prepared using 0.1 mL of distilled water devoid of 0.1 mL of homogenate. Activity of catalase was expressed as µm of H2O2 metabolized/mg protein/min.

Estimation of reduced glutathione (GSH) level

GSH level was estimated by the method described by Ellman et al. [20].

Procedure: The equal quantity of homogenate (w/v) and 10% TCA were mixed and centrifuged to separate the proteins. To 0.01 ml of this supernatant add 2 ml of phosphate buffer (pH 7.4), 0.5 ml 5,5'-Dithiobisnitro Benzoic Acid (DTNB) and 0.4 ml of double distilled water. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. 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}. GSH values were expressed as µ moles of GSH/mg protein [20].

Statistical analysis

The results were expressed as (Mean ± SEM).Differences in infarct size, MDA, SOD, Catalase and Glutathione were determined by factorial one way ANOVA. Individual groups were compared using Tukey's test. Differences with $p<0.05$ were considered statistically significant.

Statistical analysis was performed using Prism software (Version 5.0).

Results

Effect of TT extract on % infarct size after ischemia-reperfusion

Results of percentage of infarct size are presented in Table1 and Figure1. Significant cerebral damage was observed in I/R control group (47.52 ± 2.45) when compared to the sham control (1.57 ± 0.178). The % reduction of infarctions is38.14 ± 2.13 and 30.54 ± 2.57 for the doses 200 mg/kg and 400 mg/kg of TT extract respectively when compared to the I/R control which shows the protective action of the extract. The reduction of infarction is dose dependent as shown in Figure 1 and the TTC stained brain sections were shown in Figure 2.

Effect of TT extract on Superoxide Dismutase (SOD) levels (U/mg Protein)

The results of SOD levels are presented in Table 2 and Figure 3. SOD level was significantly decreased in I/R control rats (0.438 ± 0.157) when compared to sham control rats (2.390 ± 0.46). Significant elevation of SOD levels was observed in animals treated with TT extract and the effect is dose dependent as shown in the Figure 3.

Effect of TT extracts on Catalase (CA) levels (µM H_2O_2 consumed/mg Protein)

The results of CA levels are presented in Table 2 and Figure 4. CA level was significantly decreased in I/R control rats (1.487 ± 0.219) when compared to sham control rats (4.62 ± 0.27). Significant elevation of CA levels was observed in animals treated with TT extract and the effect is dose dependent as shown in the Figure 4.

Effect of TT extracts on Glutathione (GSH) levels (µM GSH consumed /mg Protein)

The results of GSH levels are presented in Table 2 and Figure 5. GSH level was significantly decreased in I/R control rats (0.813 ± 0.14) when compared to sham control rats (3.44 ± 0.45). Significant elevation of GSH levels was observed in animals treated with TT extract and the effect is dose dependent as shown in the Figure 5.

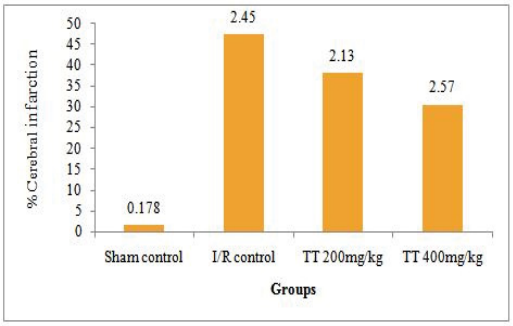

Figure 1: Effect of TTE extract on percentage infraction of rat's brain to follow by reperfusion. The values are expressed as mean ± SEM (n=6) and the results are compare with ischemic com control group.

Table 1: Showing the effect of TTE on cerebral infraction in rats subjected to global ischemia followed by reperfusion in all the four groups.

Group (n=6)	Cerebral infarction	% Reduction of infarction
Sham control	1.57 ± 0.178	-
I/R control	47.52 ± 2.45	-
TT extract (200 mg/kg)	38.14 ± 2.13	19.74
TT extract (400 mg/kg)	30.54 ± 2.57	35.75

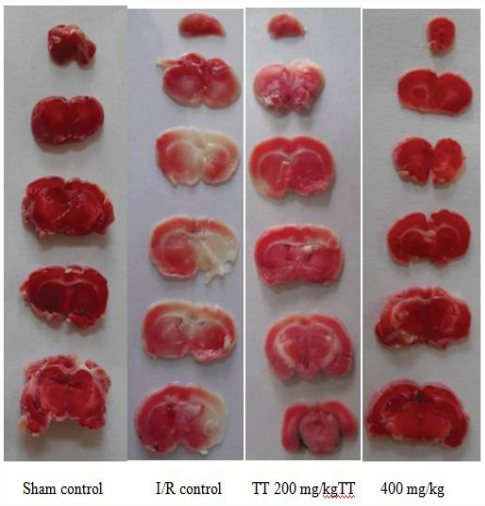

Figure 2: Showing the effect of TTE stained sections of brain tissue in rats subjected to global ischemia followed by reperfusion: I/R group has pale staining with TTC compared to other groups which determines the neural damage.

Effect of TT extract on Malondialdehyde levels (MDA) (nM of MDA/g wet tissue)

The results of MDA levels are presented in Table 2 and Figure 6. MDA is an index of lipid peroxidation. MDA level was significantly increased in I/R control rats (7.417 ± 1.282) when compared to sham control rats (2.533 ± 0.34). Significant reduction of MDA levels was observed in animals treated with TT extract and the effect is dose dependent as shown in the Figure 6.

Discussion

Various pathways are responsible for cause of brain injury in ischemia-reperfusion. Several past studies demonstrate that oxygen

Table 2: Showing the effect of TTE on SOD, Catlase, GSH and MDA levels in rats subjected to global ischemia followed by reperfusion in all the four groups.

S.No	Groups	SOD (U/mg protein)	Catalase (μM H2O2 Consumed/mg Protein)	GSH (μM of GSH/mg protein)	MDA (nM/g wet tissue)
I	Sham control	2.390 ± 0.46	4.620 ± 0.270	3.44 ± 0.450	2.533 ± 0.340
II	I/R control	0.438 ± 0.157	1.487 ± 0.219	0.813 ± 0.140	7.417 ± 1.282
III	TTE200mg/kg	0.970 ± 0.30	2.086 ± 0.86	1.296 ± 0.26	2.032 ± 0.413
IV	TTE400mg/kg	1.540 ± 0.201	3.521 ± 0.13	2.643 ± 0.81	2.130 ± 0.486

P<0.05, all values expressed in mean ± SEM (n=6). I/R indicates ischemia-reperfusion, SOD (Superoxide Dismutase), CAT (Catalase), GSH (Reduced Glutathione), LPO (Lipid Peroxidation), MDA (Malondialdehyde) and TT (Hydro-alcoholic extract of *Talinum triangulare* leaves)

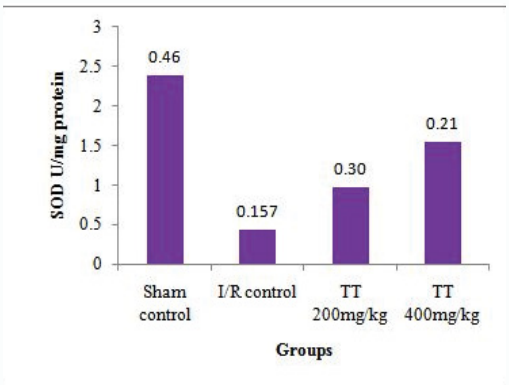

Figure 3: Showing the effect of TTE on SOD levels of rat brain in ischemic reperfusion tissue, where the values are expressed as mean ± SEM (n=6) and the results are compared to ischemic control group.

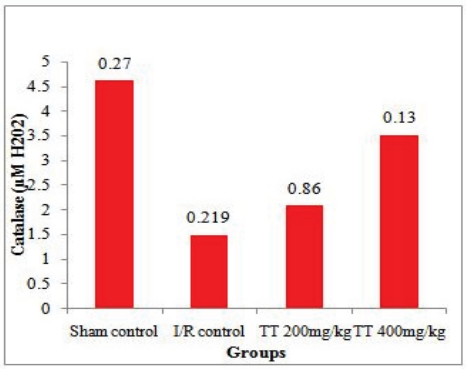

Figure 4: Showing the effect of TTE on Catalase levels of rat brain in ischemic reperfusion tissue, where the values are expressed as mean ± SEM (n=6) and the results are compared to ischemic control group.

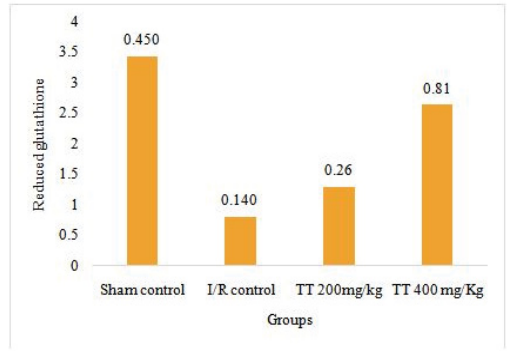

Figure 5: Showing the effect of TTE on reduced glutathione levels of rat brain in ischemic reperfusion tissue, where the values are expressed as mean ± SEM (n=6) and the results are compared to ischemic control group.

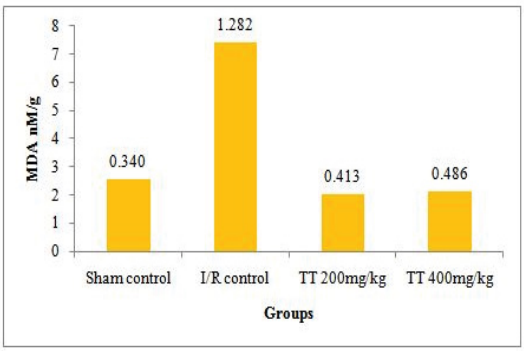

Figure 6: Showing the effect of TTE on MDA levels of rat brain in ischemic reperfusion tissue, where the values are expressed as mean ± SEM (n=6) and the results are compared to ischemic control group.

free radicals play major role in the pathophysiology of ischemia-reperfusion injury and they are elevated in cerebral ischemia-reperfusion [21,22]. In the present study the pre-treatment of hydro-alcoholic extract of *Talinum triangulare* leaves was evaluated after cerebral ischemia and the extract showed a great capability in reducing IR injury. The extent of infarction size is an indication of cerebral damage. Significant cerebral damage was observed in I/R control rats when compared to the sham control. The size of the infarction was significantly reduced in the rats pre-treated with the TTE when compared to the I/R control rats and the protection was dose dependent [23].

A variety of mechanisms such as glutamatergic excitotoxicity, oxidative stress, cytokine effects, and inflammatory injury etc are responsible for damage in reperfusion. Oxidative stress results from failure of endogenous antioxidant defense [24]**.** Since the plant extract had shown antioxidant and lipid peroxidation inhibiting properties in *in vitro* studies, the cerebroprotective effect of the *Talinum triangulare* may be due to its antioxidant role [25,26] .

The cerebro-potective property of the TTE may be attributed to its antioxidant property due to its flavonoid content [27]. To investigate the mechanism of protection of TTE extract against the ischemic reperfusion injury, the parameters such as SOD, CAT and GSH were measured in the brain tissue of rats subjected to ischemia-reperfusion. MDA is a final product of lipid peroxidation of poly unsaturated fatty acids and it is an indication of lipid peroxidation. The results demonstrate an increase in tissue MDA levels in parallel to significant increase in infarct size in I/R control group when compared to sham control group [28]. The MDA levels were significantly reduced in rats pre-treated with TT extract shown in Figure 6 which was also reported by Olarewaju M. Oluba et al. [24] in his study on STZ-induced diabetic rats.

Mammalian cells have antioxidant defense within it to fight against the ROS but their strength is very limited. The enzymes such as SOD dismutase the superoxide into hydrogen peroxide and CAT detoxifies the hydrogen peroxide into water and molecular oxygen [27]. The reduced GSH is an antioxidant or free radical scavenger which is present in all mammalian cells. Since the antioxidant defense is limited in mammalian cell, they exhaust rapidly when there is surge of ROS which occurs in I/R [28-30]. Thus, significant dose dependent alteration in the antioxidant enzyme activities during cerebral ischemia-reperfusion may be responsible for more neuronal death rather than ischemia alone shown in Figures 3-5. The treatment with hydro alcoholic extract of *Talinum triangulare* leaves in our present study resulted in elevation of endogenous antioxidant enzymes SOD and CAT which indicating enhanced biochemical defenses to scavenge the overproduced free radicals which was also observed in several past studies [29].

Conclusion

The current study shows an increase in the activities of SOD, CAT as implicated in I/R brain tissue homogenate of rats treated with TTE which shows that hydro alcoholic extract of *Talinum triangulare* leaves have a potent cerebroprotective effect and the effect may be attributed to its antioxidant activity.

Conflicts of Interest

Authors declare that there exists no conflict of interest.

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