

Modulation of Oxidative Stress Induced Cerebral Ischemia in Wistar Rats by Hydroalcoholic Extract of *Talinum triangulare*

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ABSTRACT

Background: Neuroprotection is one of considerable interest in search of novel therapies to improve ischemic brain damage in the present scenario. The objective of the present investigation is to study the actions of *Talinum triangulare* in cerebral ischemia/reperfusion induced oxidative stress in Wistar rats and *in-vitro* antioxidant actions. **Methods:** Four different groups of adult Wistar rats are selected for study where group III and group IV are treated for 15 days with TTE (200mg/kg, 400mg/kg) earlier to ischemia, on 16th day rats are dissected through global cerebral ischemia by occlusion of unilateral common carotid artery, prior to the dissection animals are sedated with thiopentone and after ischemia induction that 30 min reperfusion was followed. Following the ischemic injury various parameters such as infarct size, SOD, MDA, CAT and GSH levels were assessed after 48hr of reperfusion. **Results:** Phytochemical screening of TTE indicated the presence of alkaloids, flavonoids, glycosides, carbohydrates, saponins, tannins and resins. TTE also showed a significant *in-vitro* antioxidant activity. Pre-treatment of TTE for 15 days prior to the ischemic-reperfusion, the results reported a huge reduction in reduction in infarct size, MDA levels and significant increase in SOD, CAT and GSH levels compared to ischemic control rats which was statistically significant. **Conclusion:** The present study showed that hydroalcoholic extract of TTE leaves showed a significant *in-vitro* and *in-vivo* antioxidant activity. The present investigation reported that *Talinum triangulare* leaves extract showed dose dependant cerebroprotective effect with the selected dose of TTE 200 mg/kg and 400 mg/kg.

Key words: *Talinum triangulare*, Cerebral ischemia reperfusion, Oxidative stress, Cerebroprotection, Antioxidant activity.

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INTRODUCTION

Ischemia is the condition that 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 the older global population.¹ Stroke is a serious health condition and it is the third driving cause of death in western countries. Pathogenically, various heterogeneous processes involve stroke. Occlusion of the blood vessels is the most common cause of ischemia in the 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.² The injury formed after the revival of the blood flow is called “ischemia-reperfusion injury” which can lead to permanent disability in patients.² Reperfusion of ischemic tissue causes oxidative stress by an increase in oxygen derived free radicals and inflammatory cells influx in the brain. Free radicals are moreover vital in drawn-out ischemia.³



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Oxidative stress leads to an imbalance between the excess generation of free radicals and impaired action of the body's antioxidant system.² After ischemia- reperfusion injury several free radicals generated such as ROS play an important role in neural cell damage due to ischemia.^{1,4} Several endogenous antioxidant defence systems of the brain detoxifies the free radicals produced in the normal course of metabolism protects the brain from oxidative stress. This defence system includes molecular proteins such as Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase. Other antioxidants enzymes include reduced glutathione (GSH), α -tocopherol including ascorbic acid detoxifying free radicals produced in normal course of metabolism.⁵ However this defence fails in pathological situations like ischemia reperfusion due to overproduction 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 and cognition in humans.^{7,8}

Therefore strengthening of the brain's endogenous antioxidant defence by prophylactic treatment of the natural antioxidant-rich diet is one of the approaches to minimize reperfusion injury. *Talinum triangulare* is commonly known as waterleaf, the beneficial constituents are natural antioxidants such as flavonoids as well as phenolic compounds.^{9,10} It is a earthbound herbaceous plant, cultivated as a medicinal as well as food crop in most parts of Asia including Sri Lanka, India as well as in South America and Nigeria.^{11,12} Its extract was shown to have antioxidant and immunomodulatory effects by *in vitro* studies. In Taiwan, *T. triangulare* has been utilized within the treatment and avoidance of hepatic sickness and cancer in people.¹³ Experimental, as well as epidemiological studies demonstrate that plant flavonoids and phenolic compounds reported neuroprotective actions cerebral ischemia-reperfusion injury.^{14,15} In the present study we have attempted to assess the cerebroprotective activity of *Talinum triangulare* leaf extract and to establish its antioxidant role in the cerebroprotection in the rodent model.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals were obtained from Sigma Chemical, St.Louis, Mo, USA, and are of analytical grade. All the bioanalytical kits were procured from Allied Scientific Products, Kolkata, India, such as malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione analysis kits.

Collection, processing and extraction of TTE leaves

S.V.Govt Polytechnic College, Tirupathi Chittoor (dist). A.P., India was the site of plant material collection and plant material was authenticated by Dr. B. Sitaram, Professor/ Senior Consultant, Department of Dravyaguna, S.V. Ayurvedic Medical College, Tirupati Figure 1.

The collected plant material was shade dried and pulverised to powdered form. 500g of pulverised leaves were subjected to cold maceration in 100ml of hydro-alcoholic (30:70; Water: Ethanol) mixture for 72.

The solvent elimination was carried by using concentrated filtrate in a water bath; the leftover residue was stored and used when required.

Antioxidant activity: *In-vitro* method

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

DPPH activity of TTE experiment was carried out by standard procedure described by Braca *et al.*, 2003 with ascorbic acid set as standard and comparing the TTE activity with that of standard, DPPH activity of TTE was assessed after preparing different concentration of the test sample by dissolving the plant extract in dimethyl sulphoxide (DMSO) at different concentrations (15.6 to 1000 μ g/ml). Absorbance was recorded at 517nm.¹⁶

Superoxide radical scavenging

The SOD activity of TTE is estimated by the standard reported method developed by McCord and Fridovich 1969.^{17,18} Using UV absorption light at 560nm, induction of superoxide free radical generation by riboflavin and subsequent reduction by nitroblue tetrazolium (NBT) was calculated.

Hydroxy free radical scavenging

Hydroxyl radical scavenging activity was estimated by the method developed by Kunchandy and Rao *et al.*, the



Figure 1: *Talinum triangulare*.

$\text{Fe}^{+2} \text{H}_2\text{O}_2$ system generates the hydroxyl radicals which are calculated by using UV absorption at 532nm.¹⁹

Selection of animals and toxicology studies

The animals used in the study were procured from Raghavendra enterprises, Bangalore. Wistar rats of both sex's weight range 230-300g were divided into groups of 6-7 and housed in colony cages at suitable conditions such as an ambient temperature of $25 \pm 2^\circ\text{C}$ and 45-55% relative humidity with 12 hr light/dark cycle. The rats are also supplied with standard feed pellet chow (Pranav Agro Limited) and water *ad libitum*. The present experimental study protocol was approved by the ethical committee Institutional Animal Ethical Committee of Sri Padmavathi School of pharmacy (Approval No: 1016/a/06 /CPCSEA/04/2012 dt 09-12-2012) CPCSEA New Delhi, Government of India, under the oversight of the Committee for the Purpose of Control and Supervision of Experiments on Animals. Fresh plant materials were collected, air dried and pulverized leaves were subjected to cold maceration in 100ml of hydro-alcoholic (30:70; Water: Ethanol) mixture for 72 hr. The filtrate was processed for solvent extraction in a water bath; the leftover residue was used for experimental study.

Acute toxicity studies

OECD-423 (Organisation for Economic Co-operation and development, 2001) guidelines were followed to perform acute toxicity studies of TTE. The animals were fasted overnight supplied only water, after which the extract was administered orally at the dose level of 2000mg/kg weight by gastric intubations and animals are kept under observation for 24hr for any change in behavioural profile, neurological profile and autonomic profile. After dosing, any change in the animals was noted initially at least once during the first 30 min and during the first 24 hr for any chances of toxic symptoms or mortality. The acute toxicity studies of the selected plant extracts showed no symptoms of toxicity or behavioural changes at the maximum dose (4000 mg/kg). Hence 1/10th of the maximum dose viz. 400 mg/kg b.wt and submaximal dose 200mg/kg were set as high dose for further studies.

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 (ITLE) showed no symptoms of toxicity or behavioural changes at the maximum dose (4000 mg/kg). Hence 1/10th of the

maximum dose viz. 400 mg/kg b.wt and sub-maximal dose 200mg/kg were set as dose for present studies.

EXPERIMENTAL PROTOCOL

Determination of Infarction Size

Determination of infarct size in rats was carried out as described in earlier studies.¹⁶ Following 48hrs of reperfusion brains of rats were isolated by cervical dislocation and frozen for 5 min at -4°C . Thereafter, coronal sections were made to have sections of 1-2 mm which were drenched in 1% 2, 3, 5-triphenyltetrazolium chloride solution (TTC) at a temperature of 37°C for 30min. TTC was converted into red formazone pigment by the enzymes present in viable cells i.e., NAD and dehydrogenase producing living cells deep red colour and un-stained for infarcted cells due to exhaustion of these enzymes. The slices of the brain were weighed. The unstained infarcted portions were separated from the slices by examination and expressed as percentage of total weight of the brain.

Preparation of Brain Tissue for Evaluating Various Biochemical Parameters

After 48 hr reperfusion, the brain of each animal was isolated and washed with cold saline, after drying the tissue on filter paper, it was homogenized with buffer solution using phosphate buffer 0.1M prepared at pH 7.4 using homogenizer and centrifuged at a speed of 1000 rpm using cold centrifuge at a temperature of 4°C for 3 min. The supernatant portion was divided into two parts; one part was utilized for estimation of MDA. The left over supernatant was again centrifuged at 12,000 rpm, maintaining the above conditions for 15 min which was utilized for the estimation of superoxide dismutase, catalase and reduced glutathione.

Estimation of MDA Level

Procedure

Malondialdehyde levels in brain tissue were estimated by the Ohkawa *et al.*,¹⁷ method. To the brain tissue homogenate TBA solution, water and a mixture of butanol and pyridine ((15:1 v/v)) was added and absorbance was measured at 532 nm. Values of MDA are represented as nmol/g tissue with reference of the standard curve.

Estimation of Superoxide Dismutase (SOD)

Procedure

Superoxide dismutase (SOD) assay was estimated by Kakkar *et al.*, method.¹⁸ Using 0.1M ice cold phosphate buffer brain tissue was homogenized to produce 10%w/v homogenate which was centrifuged at 12,000 rpm

for 15min for 4°C. To 0.1 ml of supernatant sodium pyrophosphate buffer (pH 8.3), phenazine, methosulphate, nitroblue tetrazolium and NADH was added and incubated at 30°C, to this reaction mixture glacial acetic acid was added to stop the reaction to which glacial acetic acid was added and centrifuged, organic layer was separated and absorbance was measured at 560 nm. The SOD level was represented as Units/ mg protein.

Estimation of Catalase

Procedure

Catalase assay was performed according to the method developed by Aebi.¹⁹ Ice cold phosphate buffer (0.1M, pH 7.4) was used to homogenize the brain tissue using homogenizer to produce a 10% w/v homogenate. The obtained homogenate was separated by centrifugation at 12,000 rpm at 4°C for 15 min. 0.1 ml of was added to the cuvette containing 1.9 mL of 50 Mm phosphate buffer. To this solution freshly prepared 30mM H₂O₂ of 1.0mL was added and change in absorbance was observed at an interval of 30sec at 240nm for 3min. A control was prepared using 0.1 mL of distilled water without homogenate solution. Activity of catalase was expressed as μ moles of H₂O₂ metabolized/mg protein/min.

Determination of Glutathione

Glutathione (GSH) levels were assayed according to the method described by Ellman *et al.*, 1959.²⁰ Mixed solutions of homogenate (w/v) and 10% TCA of equal measures were centrifuged to separate the proteins. 0.01ml of this supernatant, 2ml of phosphate buffer (pH 7.4), 0.5 ml of DTNB and 0.4 ml of double distilled water were mixed and absorbance was read at 412nm within 15min. The enzyme level was assayed as nM glutathione oxidized/min/mg protein. Reduced glutathione levels were expressed as μ moles of GSH/ mg protein.²⁰

Statistical Analysis

All the results were expressed as (Mean \pm SEM). Statistical differences between groups were determined by one way ANOVA. Differences between groups were compared using Tukey's test where $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using Prism software (Version 5.0).

RESULTS

The hydro alcoholic extract of TTE was evaluated for its *in-vitro* antioxidant activity compared with the standard ascorbic acid. The *in-vitro* antioxidant effect

was represented in the Figure 2 the graph plotted between log concentration and %inhibition of respective free radicals. The present study leaf extract of TTE showed a significant effect in inhibiting DPPH compared with the standard ascorbic acid which was shown in Figure 2. The hydro alcoholic extract of TTE showed a significant effect on inhibiting lipid peroxidation effect which was also shown in Figure 2. Similarly the TTE extract also showed a significant effect on inhibiting nitric oxide radical which was also shown in Figure 2.

Potency of TTE extract was evaluated with pre-treatment of extract and evaluated after cerebral ischemia. The *in-vitro* results found that TTE significantly improved the inhibition of free radicals which was also confirmed by *in-vivo* methods.

Effect of TT extract on % Infarct Size after Ischemia-Reperfusion

Significant cerebral damage was observed in the I/R control group (47.52 \pm 2.45) when compared to the sham control (1.57 \pm 0.178) which was represented as percent infarct size after reperfusion shown in Figure 3. The % reduction of infarctions is 38.14 \pm 2.13 and 30.54 \pm 2.57 for the doses 200 mg/kg and 400 mg/kg of TTE extract, when compared to the I/R control group TTE showed cerebro-protective action. The reduction of infarction is dose dependent as shown in Figure 3 and the TTC stained brain sections were represented in Figure 4.

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

There was a significant decline in superoxide dismutase levels in I/R control rats (0.438 \pm 0.157) when compared to sham control rats (2.390 \pm 0.46) which was represented

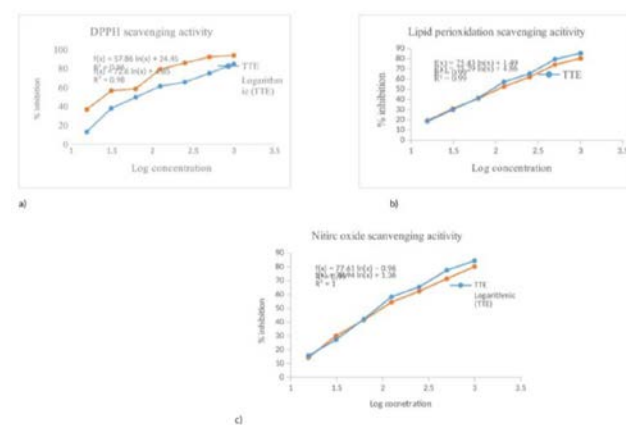


Figure 2: Antioxidant activity of TTE at different doses and ascorbic acid as standard by *in-vitro* method.

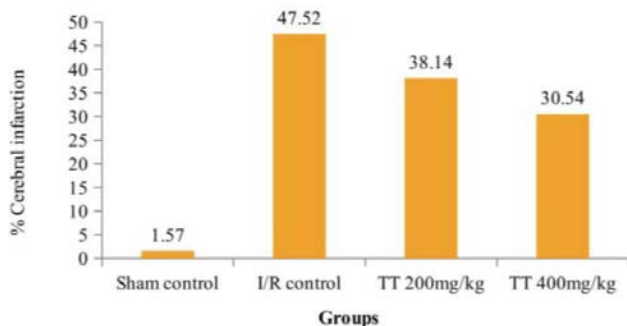


Figure 3: Effect of TTE on infarct size after ischemia reperfusion in wistar rats of all the four groups. The results are expressed as mean±SEM (n=6) and the results are compared with an ischemic control group.

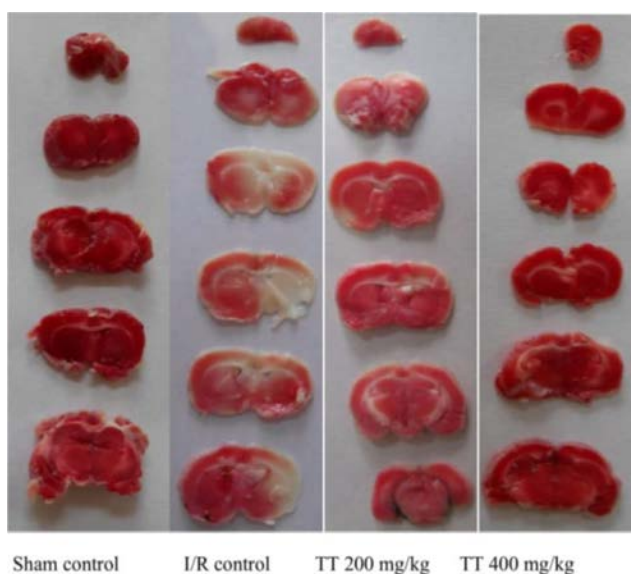


Figure 4: Showing the effect of TTE on stained brain tissue sections after ischemia reperfusion in wistar rats of all the four groups: I/R group has pale staining with TTC compared to other groups which determines the neural damage.

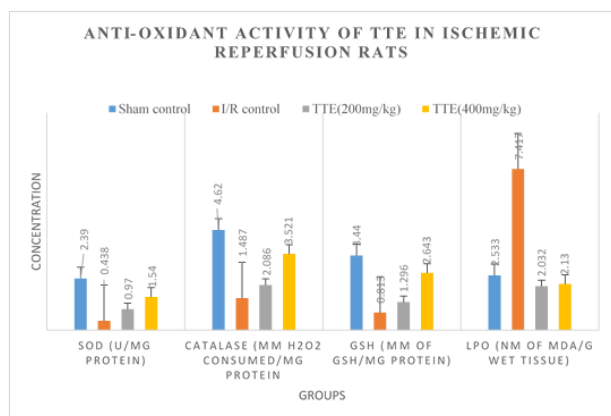


Figure 5: Graph showing cerebral biochemical parameters changes in different groups

in Figure 5. Significant elevation of SOD levels was detected in animals treated with TT extract and the effect is dose dependent as shown in the Figure 5.

Effect of TTE extracts on Catalase (CA) levels (µM H₂O₂ consumed/mg Protein)

The results of CA levels are presented in Figure 5. Catalase levels were significantly decreased in I/R control rats (1.487±0.219) when compared to sham control rats (4.62±0.27). In animals treated with TTE extract showed a significant elevation of catalase levels in a dose dependent manner as shown in Figure 5.

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

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). Figure 5 show the results of MDA in different groups. Significant reduction of MDA levels was detected in animals treated with TTE extract in a dose dependent manner as shown in the Figure 5.

Effect of TTE extracts on Glutathione (GSH) levels (µM GSH consumed /mg Protien)

The levels of Glutathione were 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 reported in groups treated with TTE extract 200mg/kg, 400mg/kg as shown in the Figure 5.

DISCUSSION

New therapeutic agents are developed from compounds extracted and isolated from plants. In this investigation, hydro alcoholic extract of TTE leaves exhibited free radical (DPPH, nitric oxide, lipid peroxide radicals) scavenging activity which may be related to phenolic compounds present in the leaves. As reactive oxygen species are important contributors to several disorders in the body, the hydro alcoholic extract of TTE might be useful for the development of newer and potent natural antioxidants that are useful in preventing oxidative stress related degenerative diseases. In 2015, Dung Y, et al., also reported that stem extracts of TTE showed potent antioxidant activities due to its flavonoid and phenolic compounds.²¹ Based on these results, further studies were carried out on this plant for evaluation of *in vivo* cerebroprotective potential.

The main cause for as many as 100 human diseases including cancer worldwide is oxidative stress. Various pathways are responsible for the cause of brain injury in ischemia-reperfusion. Several past studies demonstrate

that ROS (oxygen free radicals) play the major character in the pathophysiology of ischemia-reperfusion injury and that they are elevated in cerebral ischemia-reperfusion.^{21,22} In this research, the pre-treatment with *Talinum triangulare* leaf extract was evaluated after cerebral ischemia and the extract showed a great capability in reducing I/R injury where *in-vitro* studies showed that TTE has better capability in reducing the expression of free radicals, hence *Talinum triangulare* leaves were tested against cerebral ischemia which causes brain damage due to oxidative stress. The extent of infarction size is an indication of cerebral damage. In contrast, group 1 has significant cerebral damage was observed in I/R control rats, which was observed in Figure 4. The percentage of infarction was significantly diminished in groups treated with the TTE in when correlated 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 lipidperoxidation inhibiting properties in *in vitro* studies, the cerebroprotective effect of the *Talinum triangulare* may be due to its antioxidant role.^{25,26} The cerebroprotective property of the TTE may be attributed to its antioxidant property due to its flavonoid content.^{27,28} 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.^{29,30} MDA is a final product of lipidperoxidation of poly unsaturated fatty acids and it is an indication of lipid peroxidation.

The findings indicate an elevation in tissue MDA levels is in coordinated to increase in infarction size in I/R group relative to the sham control.³¹⁻³³ There was a significant reduction in the lipid peroxidation index in TTE treated groups as shown in Figure 5 which was also reported by Olarewaju M. Oluba *et al.*, 2019 in his study on STZ-induced diabetic rats.²⁴

Mammalian cells have antioxidant defense within it to fight against the ROS but their strength is very limited. Enzymes like SOD inactivate superoxide into hydrogen peroxide, and catalase convert hydrogen peroxide into water and molecular oxygen.²⁷⁻³¹ The reduced GSH is an antioxidant or free radical scavenger which is present in all mammalian cells. Since the antioxidant defence is limited in mammalian cell, they exhaust rapidly when there is surge of ROS which occurs in I/R.^{33,34} Thus, during cerebral ischemia-reperfusion, dose dependent alteration in antioxidant enzyme activity might be

responsible for neural death other than ischemia alone as shown in Figure 4, 5. The treatment with hydro alcoholic extract of *Talinum triangulare* leaves in our present study resulted in elevation of endogenous antioxidant enzymes such as superoxide dismutase and Catalase which suggest increased biochemical protection to scavenge the overproduced reactive oxygen species which was also reported in several past studies.

CONCLUSION

The current study shows an increase in the elevated levels of SOD, CAT as implied from I/R brain homogenate of rats treated with TTE which shows that hydro alcoholic extract of *Talinum triangulare* leaves have a potent cerebroprotective effect which might be due to TTE antioxidant activity.

Compliance with Ethical Standards

The present study has followed all the international, national or institutional guidelines for animal care and use of animals.

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Compliance with Ethical Standards

National, international and/or institutional guidelines for the care and use of animals was followed for the present research. Ethical standards of the institution or practice was followed for the experimental procedure in studies involving animals in the present research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

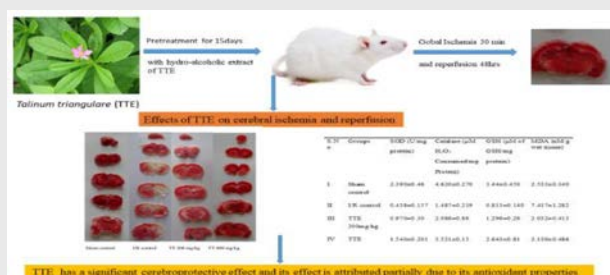
TTE: *Talinum triangulare* extract; **I/R:** Ischemic reperfusion; **GSH:** Glutathione; **ROS:** Reactive oxygen species; **TTC:** Tetrazolium chloride; **DPPH:** 2,2-Diphenyl-1-picrylhydrazylradical; **DMSO:** dimethyl sulphoxides; **SOD:** Superoxide Dismutase; **NBT:** nitroblue tetrazolium; **CAT:** Catalase; **GPx:** glutathione peroxidase; **MDA:** malondialdehyde; **DTNB:** 5,

5'dithiobisnitro benzoic acid; **ANOVA**: Analysis of variance; **SEM**: Standart error of mean.

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PICTORIAL ABSTRACT



About Authors



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SUMMARY

- The Hydroalcoholic extract of TTE reported cerebroprotective effect in albino rats against oxidative stress induced due to ischemic reperfusion in rats
- The release of free radicals caused brain injury was significantly attenuated by treatment with hydroalcoholic extract of *Talinum triangulare* 200mg/kg and 400mg/kg b.wt in rats.
- The *in vitro* antioxidant properties on TTE was proved by DPPH scavenging activity, hydroxyl radical activity, nitric oxide radical activity.
- Benefits of TTE on oxidative stress induced brain damage was proved by decreased lipid peroxidation and increased SOD, catalase and glutathione in rat brain tissues.
- Hydro alcoholic extract of *Talinum triangulare* leaves in our present study resulted in elevation of SOD and CAT which are endogenous antioxidant enzymes indicates the beneficial effects of TTE in ischemic brain damage.

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