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Antioxidant activity of Methanolic fruit extract of *Morinda Tinctoria Roxb* in Cerebral Ischemia Induced by Bilateral Common Carotid artery occlusion in rats.

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ABSTRACT

The present study was designed to investigate te antioxidant activity oof methanolic extract of *Morinda Tinctoria Roxb* against (MEMT) fruits in the cerebral ischemia induced animal model system. Global cerebral ischemia was induced by temporary bilateral common carotid artery occlusion for 15 min followed by reperfusion in Sprague Dawley Rats and the animals were pre-treated with MEMT (200 and 400 mg kg-^{1}) for 1 week before induction. After induction of ischemia by BCCAO animals were again treated with MEMT for 1 week and the animals were sacrificed. Homogenized content of brain were estimated in control, sham and treatment groups. The MEMT showed the significance of $p<0.01$ done by ANOVA and Dunetts in the levels of antioxidants such as superoxide dismutase, glutathione peroxidase, glutathione reductase, lipid peroxidation, catalase, protein levels, vitamin C levels. At the 1 week after ischemia a maximum of 85% protection of neurons in CA1 region showed in treatment group at the dose 400 mg kg-^{1}. The results showed neuroprotective nature of MEMT and protection may be due to reduction of oxidative stress which occurs by alteration in levels of antioxidants and MEMT has the potential to use in treatment of global ischemia.

Keywords: Morinda tinctoria, bilateral common carotid artery occlusion, antioxidants, CA1

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INTRODUCTION

From the earlier times, herbs have been prized for their pain-relieving and healing abilities, today we still rely largely on the curative properties of plants. Over the centuries, societies throughout the world have developed their own traditions to make sense of medicinal plants and their uses. India is richly endowed with a wide variety of plants having medicinal value. These plants are widely used by all parts of the society whether directly as folk remedies or indirectly as pharmaceutical preparation of modern medicine[1]. In recent days, focus on plant research has increased all over the world and a large body of evidence has been collected to show immense potential of medicinal plants used in several traditional systems[2].

Stroke is a life-threatening disease characterized by rapidly developing clinical sign of focal or global disturbance of cerebral function due to cerebral ischemia. The incidence of brain infraction, following a reduction of blood flow, is gradually increasing due to relatively recent life style changes, such as consumption of fatty foods, smoking and excess stress. A stroke or brain attack occurs when a clot blocks the blood flow in a vessel or artery or when a blood vessel breaks, interrupting blood flow to an area of the brain. When either of these things are happens, brain cells begin to die[3]. In recent years, several reviews have been published on the effect[4] and potential benefits of traditional Eastern medicine in stroke[5]. It has been suggested that some herbal medicines[3], of their products, may improve microcirculation in the brain and protect against ischemic reperfusion injury possess neuroprotective properties[6]. *Morinda tinctoria* is mainly known for its poly phenol properties. The plant has been shown to assist in the treatment of neurodegenerative disorders and in alcohol abstinence-induced with drawal symptoms. In Ayurvedic, Morinda Tinctoria has been described as a rasayana herb and has been used extensively as an adaptogenic to increase the non-specific resistance of organisms against a variety of stresses.

The whole plant is used in combinations for many herbal remedies. The fruits are used for the treatment of tuberculosis, arthritis, rheumatism, sores, boils, mental depression and muscle aches. The mashed fruit was applied directly to the impaired area including deep cuts, anasarca and broken bones. The shiny green leaves were used as a poultice for wounds, rheumatic joints, fevers, baldness, dysuria (painful urination), dropsy and headaches. The leaves were applied directly to the impaired area to relieve pain. The root is cathartics and astringent fruit and leaves are deobstruent emmenagogue tonic and febrifuge[10]. Since Morinda Tinctoria is said to be a potent antioxidant and anticoagulant as per folklore, based on this claim an attempt has been

made in the present study to evaluate the efficacy of MEMT in the treatment of cerebral ischemia induced by bilateral carotid artery occlusion in rats.

MATERIALS AND METHODS

Animals: The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals). IAEC Reference number IAEC/XXIX/08/CLBMCP/2010 dated on 20/04/2010. Colony inbred strains of male Sprague Dawley Rats of male sex weighing 250-300 g were used for the pharmacological studies. The animals were kept under standard conditions (day/night rhythm) 8.00 am to 8.00 pm., 22±ºC room temperature, in polypropylene cages. The animals were feed on standard pelleted diet and water ad libitum. The animals were housed for one week in polypropylene cages prior to the experiments to acclimatize to laboratory conditions. It was randomly distributed into six different groups with six animals in each under identical conditions throughout the experiments. Food was withdrawn 18 h prior to surgery.

Principles of Laboratory Animals Care and Use (NIH Publication No. 85-23, revised 1985) guidelines were followed throughout the experiment.

Chemical and reagents:

All chemicals and reagents were of the highest analytical grades available locally. Plant material and standardization of extract: The Fruits of Morinda Tinctoria Roxb were collected from Tuticorin, Tamil nadu, India. The plant material was denoted and authenticated by Dr. D. Narasimhan, Associate Professor, Centre for Floristic Research, Department of Botany, Madras Christian College (Autonomous), Tambaram, Chennai-59. Freshly collected fruits of Morinda Tinctoria Roxb were dried in shade and pulverized to get a coarse powder. A weighed quantity of the powder (940gm) was passed through sieve number 40 and subjected to hot solvent extraction in a soxhlet apparatus using methanol at a temperature range of 40-60ºC. Before and after every extraction the powder bed was totally dried and weighed. The filtrate was evaporated to dryness at 40ºC under reduced pressure in a rotary vacuum evaporator. The brownish black waxy residue was obtained. The extract (MEMT) was subjected to preliminary phytochemical screening for the presence or absence of phytoconstituents. These plant extraction and phytochemical screening were carried out in C.L.Baid Metha College of Pharmacy, Chennai, India.

Induction of global cerebral ischemia:

Male Sprague dawley rats, weighing 250 to 300 grams each were anesthetized intraperitoneally

with a combination of Ketamine (40mg/kg) and xylazine (5mg/kg)[8]. Transferred rat to surgery board. Ventral neck area was shaved. The shaved area was washed with 70% ethanol to cleanse, be sure to remove all loose fur and treated with Betadine solution. Temperature measurement was carried out by using thermometer. Started recording temperature and controlled with the help of following method. Body temperature was easily controlled (37ºC) with a heating blanket. An infrared lamp was also effective in maintaining temperature, but it must not be too hot to cause burns. Animal was covered. A small midline skin incision was made in neck (2cm long). The thyroid gland was gently moved with non-traumatic forceps. Both common carotid arteries were isolated. Care was taken to avoid damaging the vagal nerves and isolated with the help of curved forceps. Silk suture was tied under each artery for each access to vessels. A vessel was made free enough to allow easy and rapid placement of clamps. Non-traumatic vessel clamps was applied to each artery for a defined period (10-15 minutes) and after that allowed for reperfusion. Area was infiltrated with the help of warm saline to prevent drying out of tissue during occlusion. After the end of definite period clamps were removed and checked arteries for good reflow. The order of clamp application and removal would be the same and within 10 sec if each other minimize asymmetrical injury. Silk suture was gently removed around each vessel. Incision was infiltrated with a few drops of lignocaine. Incision was sutured and the post surgical care was given to the animal.

Steady state experiment:

For the studies of global ischemia the male Sprague Dawley strain Rats were randomized into 6 different groups (n = 6 per group) Group 1: Animal (POSITIVE CONTROL) with sham operation (without occlusion) and treated with control vehicle (normal saline) only (p.o.). Group 2: Animals with sham operation (without occlusion) and treated with 200 mg $kg^{-1}$of MEMT dissolved in distilled water (p.o.). Group 3: Animals with sham operation (without occlusion) and treated with 400 mg $kg^{-1}$of MEMT dissolved in distilled water (p.o.). Group 4: Animals (NEGATIVE CONTROL) with BCCAO and treated with control vehicle only (p.o.). Group 5: Animals with BCCAO and treated with 200 mg kg^{-1} of MEMT (p.o.). Group 6: Animals with BCCAO and treated with 400 mg kg^{-1} of MEMT (p.o.). The animals were anaesthetized with intraperitoneally with a combination of Ketamine (40mg/kg) and xylazine (5mg/kg) and stroke was induced by occlusion of bilateral common carotid artery (BCCAO) for defined period (10 to 15 min) with aneurism clamps placed on both arteries and later clamps were removed to allow reperfusion and animals were then returned to their cages. The treatment was continued for another week after surgery with fruit extract and the animals were sacrificed with cervical

dislocation and the brain was removed and homogenized. The homogenized content was used for the estimation of anti-oxidant, metabolic enzymes and various neurotransmitter levels. Histopathology of hippocampal CA1 region was carried out. The animals received this extract orally in dosages of 200 and 400 mg kg^{-1} day^{-1} dissolved in distilled water. The particular dose was selected on the basis of our preliminary and acute oral toxicity studies that assessed the ability of MEMT to attenuate the excitotoxicity provoked oxidative damage.

Biochemical analysis:

Assessment of oxidant-antioxidant status of the rat forebrains subjected to global cerebral ischemia was done by measuring the levels of superoxide dismutase (SOD) [17], glutathione peroxidase (GSH-Px)[18], glutathione reductase (GSH-Rd)[19], lipid peroxidation[20], catalase. Estimation of lipid peroxidation was done by measuring the levels of malondialdehyde (MDA), a by-product of lipid peroxidation. 1 week after ischemia, rats from each group were cervical dislocation; the brains were quickly removed and homogenized in ice-cold sodium pyrophosphate buffer (pH 8.3) in a ratio of 50 mg mL^{-1}.

Histopathological examination:

Seven days after ischemia, rats from each group were anaesthetized [21] with sodium Thiopentone (100 mg kg^{-1}). Rats were then transcardially perfused with cold saline followed by 4% formalin in phosphate- buffered saline (0.1 M; pH 7.4). The brains were removed from the skull and fixed in the same fixative for 24 h. Thereafter, the brains were embedded in paraffin and 5 µm thick sections were coronally cut at the level of the dorsal hippocampus by a rotator microtome. The segments of the hippocmpal CA1 region per 1000 µm lengths from bregma -3.3, -3.8 and -4.3 were counted for viable cells.

Tissue sections were stained with hematoxylin and eosin. The hippocampal damage was determined by counting the number of intact neurons in the stratum pyramidal within the CA1 subfield at a higher magnification. Only neurons with normal visible nuclei were counted. The mean number of CA1 neurons per millimetre linear length for both hemispheres in sections of dorsal hippocampus was calculated for each group of animals. An observer who was unaware of the drug treatment for each rat made all the assessments of the histological section 2.10. The slide as photographed under 100 x.

Statistical analysis:

The statistical analysis was carried out using Graph pad prism 4.0 software. All values were expressed as Mean±SEM. Data analysis was done by one-way ANOVA followed by Dunetts Multiple Comparison Tests. Difference level at $p<0.05$ was considered as statistically significant

condition.

RESULTS AND DISCUSSION

Phytochemical Screening:

The percentage yield of methanolic extract was 17.53%w/w. The methanolic fruit extract showed the presence of carbohydrates, alkaloids, proteins, phenols, flavanoids, gums & mucilage, glycosides, terpenes, triterpinoids, sterols and amino acids. Steroids, saponins and tannins were absent.

MEMT treatment blocks global cerebral ischemia induced oxidative stress:

Table 1 shows the effect of MEMT treatment (200 mg and 400 mg kg^{-1} day^{-1} for 14 days) on oxidant-antioxidant status of rat measured after ischemia reperfusion, there was a decrease in the superoxide dismutase (SOD), glutathione per-oxidase (GSH-Px), glutathione reductase (GSH-Rd), catalase and increase in the lipid per-oxidation levels in Stroke induced (negative control) group when compared with the control group and negative control group which showed significance of $p<0.01$ when compared with control group. The group treated with 200 and 400 mg kg^{-1} MEMT showed significant ($p<0.01$) increase in the superoxide dismutase), glutathione per-oxidase (GSH-Px), glutathione reductase (GSH-Rd), catalase and decrease in the lipid per-oxidation level when compared with negative control group. The group treated with 200 and 400 mg kg^{-1} MEMT showed the significance of $p<0.01$ as shown in Table 1 and figure 3 to 7.

Table 1: Effect of MEMT in the levels of antioxidants

Groups	SOD (units/mg protein)	GSH-Px (units/min/ mg protein)	GSH-Rd (units/min/ mg protein)	MDA (nM/mg protein)	Catalase (µmol/mg protein/min)
Sham (saline)	9.00±0.183	37.33±0.168	33.58±0.239	2.267±0.061	2.53±0.021
Sham (200 mg kg^{-1})	9.25±0.250	36.92±0.201	33.75±0.250	2.283±0.016	2.57±0.049
Sham (400 mg kg^{-1})	9.67±0.380	37.33±0.279	33.75±0.112	2.300±0.036	2.53±0.021
Ischemia (saline)	6.75±.0112 a**	28.42±0.201 a**	25.50±0.183 a**	4.550±0.043 a**	1.74±0.020 a**
Ischemia + MEMT (200 mg kg^{-1})	8.00±0.183 b**	32.58±0.154 b**	29.25±0.112 b**	3.300±0.036 b**	2.13±0.033 b**
Ischemia + MEMT (400 mg kg^{-1})	9.08±0.239 b**	37.42±0.201 b**	33.75±0.112 b**	2.250±0.043 b**	2.53±0.025 b**

Values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Sham control Vs ischemia control and b. Ischemia Vs Treatment groups. ** Represents the statistical significance of $p<0.01$ done by ANOVA. Followed Dunetts Multiple Comparison Test. Significant: *P<0.05, **P<0.01, ***P<0.001, ^{ns} Non significant

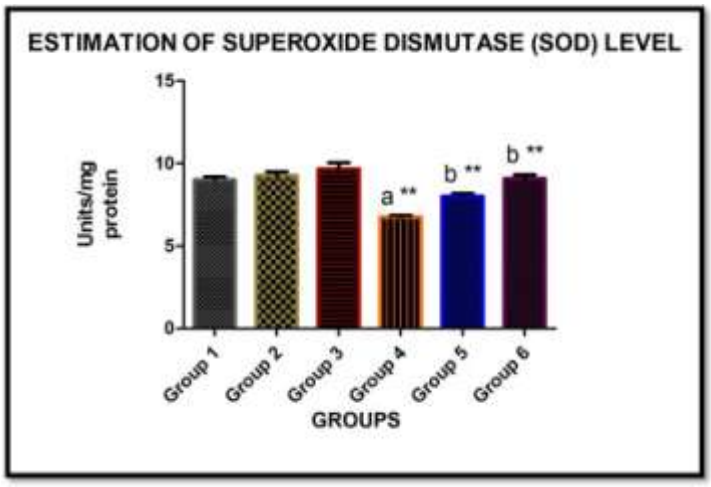

Figure.3 Effect of MEMT on SOD

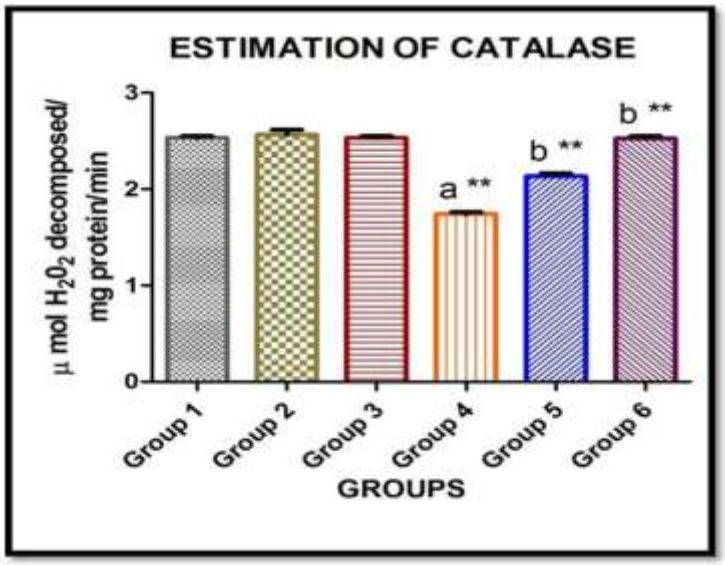

Figure. 4 Effect of MEMT on CAT

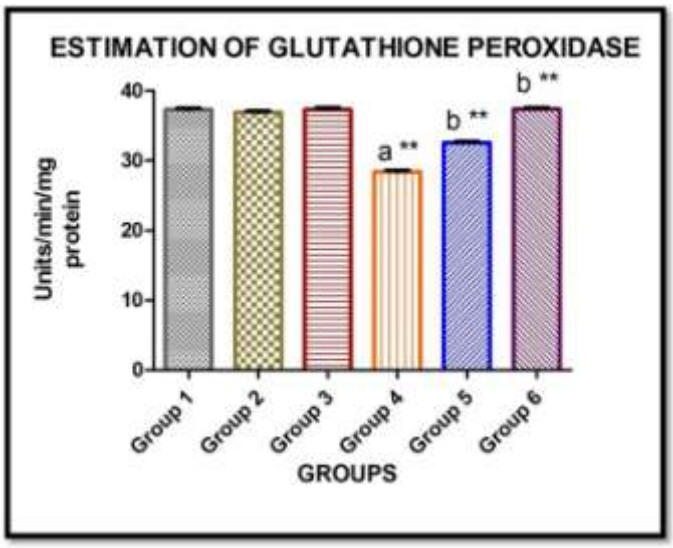

Figure. 5 Effect of MEMT on GPx

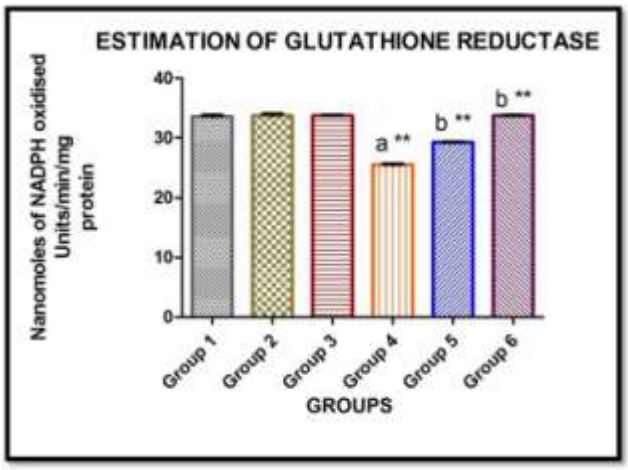

Figure. 6 Effect of MEMT on GRD

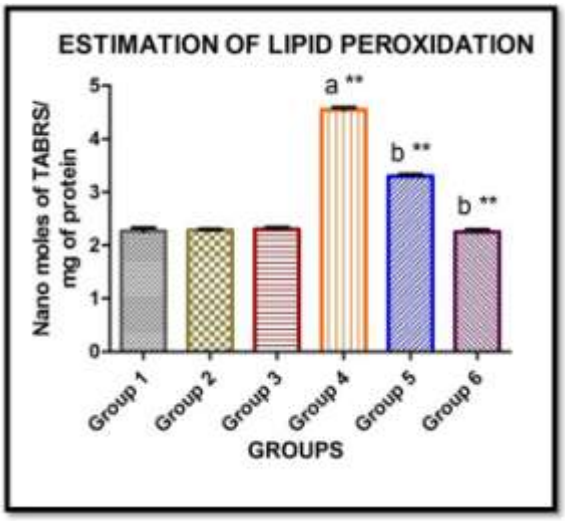

Figure. 7 Effect of MEMT on LPO

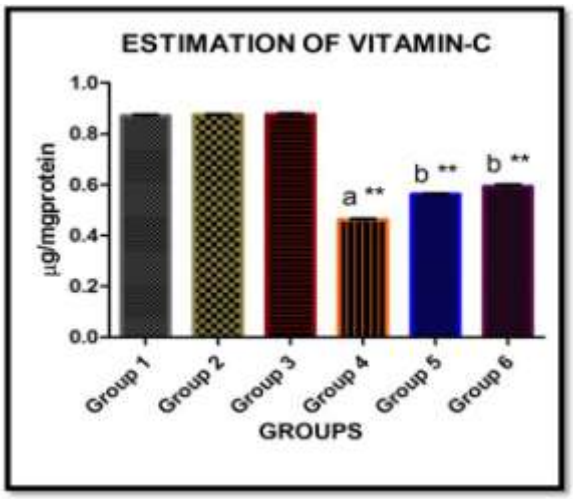

Figure. 8 Effect of MEMT on VIT.C

Figure 3 to 8: MEMT treatment blocks global cerebral ischemia induced oxidative stress and Vitamin C level:

Effect of MEMT on Vitamin C Level:

There was a decrease in the vitamin C level in stroke induced (negative control) group when compared with the control group and negative control group which showed significance of $p<0.01$ when compared with control group. The group treated with 200 and 400 mg kg^{-1} MEMT showed significant $p<0.01$ increase in the vitamin C level when compared with negative control group. The group treated with 200 and 400 mg kg^{-1} MEMT showed the significance of $p<0.01$ as shown Table 2 figure 8.

Table. 2: Effect of MEMT in the levels of Vitamin - C

Groups	1	2	3	4	5	6
µg/mg protein	0.87± 0.0036	0.87± 0.0021	0.88± 0.0021	0.46± 0.0031 a**	0.56± 0.0031 b**	0.59± 0.0061 b**

Values are expressed as mean ±SEM of 6 animals. Values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Sham control Vs ischemia control and b. Ischemia Vs Treatment groups. ** Represents the statistical significance of $p<0.01$ done by ANOVA. Followed Dunetts Multiple Comparison Test. Significant: *P<0.05, **P<0.01, ***P<0.001, ^{ns} Non significant

MEMT treatment shows protection in CA1 region:

There was a decrease in the number of neurons in the hippocampal region in stroke induced (negative control) group when compared with the control group and negative control group which showed significance of $p<0.01$ when compared with control group. The group treated with 200 and 400 mg kg^{-1} MEMT showed significance ($p<0.01$) increase in number of neurons in the hippocampal region when compared with negative control group which was confirmed from photomicrographs of histopathology study. The group treated with 200 and 400 mg kg^{-1} MEMT showed significance ($p<0.01$) as shown in figure. 1

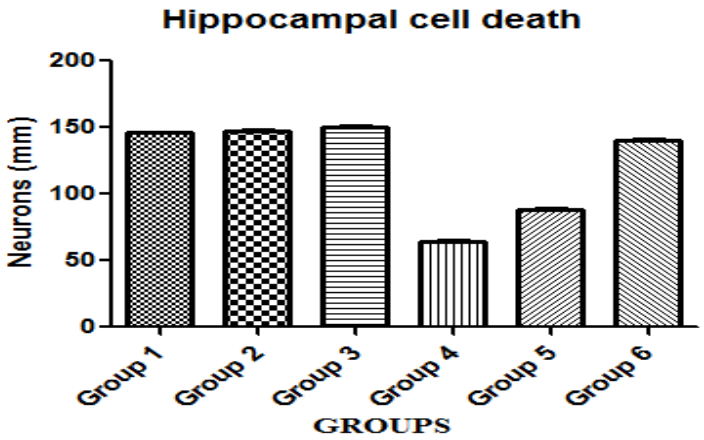

Figure 1: Effect of MEMT on Neuron cell count in CA1 region in rat brain after ischemia.

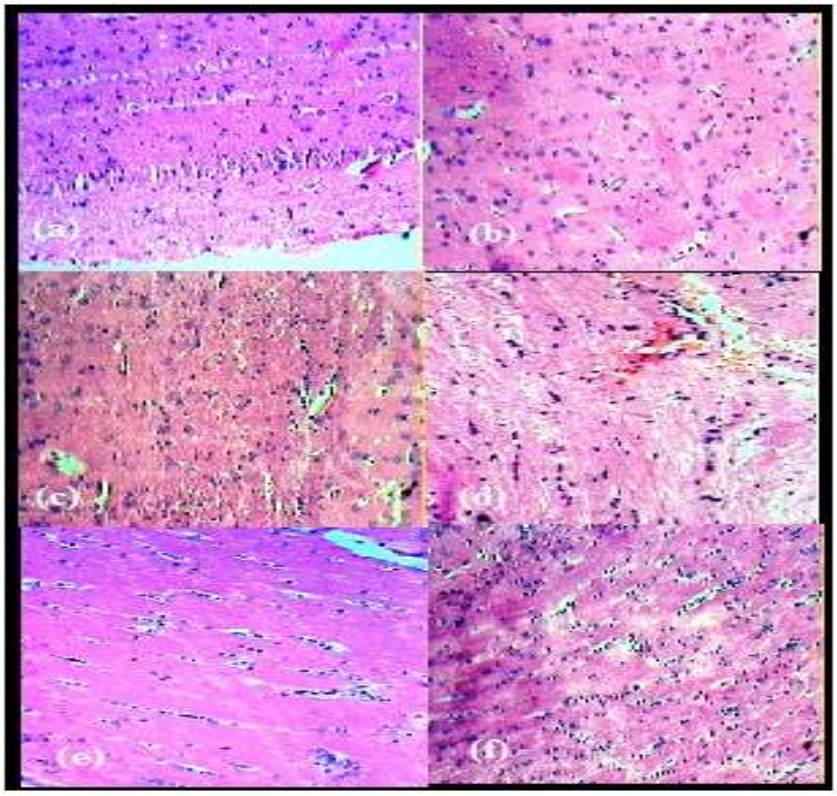

Figure 2: Protective effect of MEMT against ischemia- mediated cell loss in the CA1 hippocampal area 7 days after ischemia in rat. Figure (): illustrates neurons within the CA1 region of the hippocampus stained with haematoxylin and eosin at a magnification of 40x after transient cerebral ischemia. (a) Coronal sections showing intact neurons in the hippocampal CA1 region of the sham control rats; (b) coronal sections showing intact neurons in the hippocampal CA1 region of the sham-treated rats; (c) coronal sections showing intact neurons in the hippocampal CA1 region of the sham-treated rats; administration of MEMT (200 and 400 mg kg^{-1} day^{-1} p.o.) for 14 successive days showed no effect on the number of the intact neurons in the hippocampal CA1 region; (d) most pyramidal cell died in the CA1 subfield 7 days following reperfusion in rats subjected to 10 min ischemia: (e and f) In contrast, administration of MEMT (200 and 400 mg kg^{-1} day^{-1} p.o.) 7 days before ischemia and continued for 7 successive days conferred cerebroprotection by markedly reduced number of damaged pyramidal cells in the CA1 subfield.

Morinda Tinctoria roxb a medicinal plant is well known for its antioxidant, antithrombotic and hypothermic property. Scientific evaluation of these claims using experimental model of Bilateral Common Carotid Artery Occlusion (BCCAO) in rat's induced cerebral ischemia was ascertained in this study. This was supported in our study by several behavioural, biochemical findings and histopathology studies.

The present study revealed the neuroprotective effect of the plant *Morinda Tinctoria Roxb* in cerebral ischemia induced by bilateral common carotid artery occlusion in rats.

Bilateral Common Carotid Artery Occlusion is the basic experimental model for inducing of global cerebral ischemia in animals and common carotid arteries is the main arteries supplying blood to the brain from heart. The occlusion of these arteries for a period of 10 - 15 minutes leads to reduction in blood supply to the brain and the patho physiological events starts and continues followed by reperfusion[7].

The primary elements of the pathophysiologic cascade following concussive brain injury include abrupt neuronal depolarization[22], release of excitatory neurotransmitters, ionic shifts, changes in glucose metabolism, altered cerebral blood flow and impaired axonal function. These alterations can be correlated with periods of post concussion vulnerability and with neurobehavioral abnormalities. While the time course of these changes is well understood in experimental animal models, it is only beginning to be characterized following human concussion.

In the pathophysiology of stroke the neurotoxic pathway [23] which takes place alters the levels of antioxidant enzymes such as superoxide dismutase, malondialdehyde, glutathione peroxidase, glutathione reductase, catalase, vitamin C and neurons levels in animals. Hence estimation of various antioxidant enzyme levels and neurons cell count in hippocampal region was carried out in order to assess these levels in stroke induced animals and treatment groups.

In normal brain tissue, the production of reactive oxygen species (ROS), such as superoxide anion radical, hydrogen peroxide, hydroxyl radical, and peroxinitrite anion, is balanced by endogenous enzymatic (SOD, GSHPx, CAT) and non enzymatic (for example, glutathione, uric acid, vitamins C & E) antioxidative defences[15]. Oxidative damage could arise from an ischemia-induced increase in the synthesis of reactive oxygen species or an impairment of the cellular defences that normally protect against such damage. Evidence has been obtained for a brief burst of production of free radicals and related reactive oxygen species within the first few minutes of recirculation[16]. Previously published studies show that lipid peroxides levels in the hippocampus are elevated 8-24 h after 20min of forebrain ischemia in a rat 2-vessel occlusion model[9].

The results of biochemical parameters show that Bilateral Common Carotid artery occlusion causes ischemia-reperfusion injury. The observed decrease in the lipid peroxide level and proportionate increase in the SOD, CAT, GSHPx and GSH levels in the extract treated groups shows that M.*Tinctoria* roxb acts protective by enhancing the production of antioxidant enzymes and exerting its action as a free radical scavenger. Several studies have reported the antioxidant potential of M.*Tinctoria* roxb in various conditions of oxidative stress.

Ascorbate is the major antioxidant in tbrain and reacts with transition metals and reactive oxygen species. The increased level of vitamin C in MEMT treated group may have exerted significant antioxidant effect thereby reducing free radical generation and preventing neuronal death.

Ascorbate (reduced vitamin C) is an important enzyme cofactor and general reducing agent that is highly concentrated in the central nervous system[24]. The intracellular pool of ascorbate can be released under various conditions including dopaminergic receptor stimulation[25], γ-aminobutyric acid receptor stimulation[26], increased extracellular glutamate[28], and depolarization[29]. The hetero exchange of ascorbate with excitatory amino acids, especially glutamate, may be the most important process underlying the fluctuations in extracellular brain ascorbate in vivo[27&30].

The results of this study confirmed that MEMT protects rats from ischemia induced brain injury. This protection was evident from, the significant decrease in the levels of malondialdehyde (lipid peroxidation), and increased the levels of SOD, CAT GSHPx, GSH and Vitamin C and the significant reduction in neuronal cell death and regeneration of neurons in the hippocampal CA1 region in the M.*tinctoria* Roxb treated groups.

CONCLUSION

The present study exhibited that oral administration of MEMT has protected rats from ischemia-induced brain injury and the protection may be due to the elevated levels of antioxidants enzymes thereby reducing the oxidative stress in the stroke induced animals. These observations suggest that MEMT may be clinically viable and protective against a variety of conditions where cellular damage is a consequence of oxidative stress. In addition, MEMT may have the potential to be used in the prevention of neurodegenerative diseases such as cerebral ischemia. Further studies to find the exact mechanism of MEMT involved in the neuroprotective effect in stroke induced rats is likely to be carried out.

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