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Research Article

EVALUATION OF STROKE PREVENTIVE EFFECT OF ETHANOLIC EXTRACT OF *VENTILAGO MADRASASPATANA GAERTN* AGAINST BILATERAL CAROTID ARTERY OCCLUSION (BCAO) INDUCED STROKE IN RATS

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Abstract: The plant *Ventilago madrasapatana Gaertn* commonly known as red creeper belongs to family Rhamnaceae is large wood climber plant distributed in various parts of Southern India. Different parts of this plant are used for treating various conditions such as inflammation, diabetes, jaundice, piles etc. The present study is to evaluate the stroke preventive effect of ethanolic extract of whole plant of *Ventilago madrasapatana Gaertn (EVM)* against the global model of ischemia in rats. In the present study, the animals were pre-treated with different doses (250 mg/kg, 500mg/kg and 750 mg/kg p.o) of EVM for a period of 30 days. After 30 days, stroke was induced by Bilateral Common Carotid Artery Occlusion (BCCAO) for 30 mins followed by 1h and 4h reperfusion. Behavioral, histological and *in vivo* antioxidant parameters were measured. The test groups (500mg/kg and 750 mg/kg p.o.) showed significant recovery in behavioral testing when compared to control group. The levels of antioxidant enzymes in brain such as reduced glutathione, superoxide dismutase and catalase had been increased and the lipid peroxidation had been decreased in the pretreated groups when compared to control. Histological studies performed by TTC staining were used to identify the ischaemic area and intensity of ischaemia. From this, we have observed that the ischaemic area in prophylactically treated groups was reduced. In conclusion, Ethanolic extract of whole plant of *Ventilago madrasapatana Gaertn* produced protective effects in global cerebral ischemia as evident from reduction in behavioral score and neuronal damage.
Key words: Whole plant of of *Ventilago madrasapatana Gaertn*, Bilateral Common Carotid Artery Occlusion, Cerebral ischemia

INTRODUCTION

A stroke (sometimes called an acute cerebrovascular attack) is the rapidly developing loss of brain function(s) due to disturbance in the blood supply to the brain. There are two kinds of stroke i.e. ischaemic and hemorrhagic stroke. Brain damage in stroke results from toxic reaction like free radical generation secondary to the initial death of brain cells [1]. It is the major cause of death and disability globally as it results in damage to many biochemical, molecular and behavioral deficits. The WHO estimates that 5.7 million people die from stroke each year [2]. There are two ways for managing stroke. They are preventive therapy and medication therapy. Treatment of stroke is very expensive and chances of survival and recovery are less. Disadvantages of treatment and the above staggering numbers emphasize the importance of managing preventive aspect of stroke on an emergent basis [3]

Oxidative stress is one of the primary factors that exacerbate damage by cerebral ischemia as the brain is particularly vulnerable to oxidative stress injury because of its high rate of oxidative metabolic activity, intense production of reactive oxygen species metabolites, relatively low antioxidant capacity, low repair mechanism activity, and non–replicating nature of its neuronal cells.[4] .Now a days, great deal of interest has been devoted to neuroprotective therapy, including antioxidant therapy, whose aims are to reduce the vulnerability of brain tissue to ischemia, to extend the therapeutic window for thrombolytic and to increase the efficacy of thrombolytic by reducing the reperfusion injury. The effect of antioxidants is to control damage that is caused by free radicals (also called reactive oxidative species, ROS) such as superoxide anion and hydrogen peroxide.[5] Recently, the antioxidant properties of natural flavonoids and related phenolic compounds extracted from dietary or herb plants have aroused much attention [6]. The plants rich in these compounds are ideal source of natural antioxidants.

The plant *Ventilago madrasapatana Gaertn* of family Rhamnaceae has been reported to possess antioxidant, anti-inflammatory, hepatoprotective and antibacterial activities [7, 8]. Ant inflammatory and Anticancer compounds have been isolated from its parts [9].Keeping this in view the aim of the study is to investigate the stroke preventive effect of ethanolic extract of whole plant of *Ventilago madrasapatana.*

MATERIALS AND METHODS

Collection of plant material and Authentification

The whole plant of *Ventilago madrasapatana gaertn* was collected in the month of November-December, 2012 from chittur dist.The plant material was authentified by Prof. Madhav Shetty, dept of botany, taxonomist, SV University. A voucher was kept in the department for reference.

Preparation of extract

The Plant material obtained were shade dried and pulverized in a mechanical grinder to obtain coarse powder.

The coarse powder was extracted with 95% ethanol in a soxhlet extractor for 18 hours. The solvent was completely removed by distillation and dried in a vacuum desiccator.

Chemicals

All the solvents and chemicals used in the experiment were of analytical grade available. TTC (2, 3, 5 triphenyltetrazolium chloride), 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) ,sodium hydroxide, potassium dihydrogen phosphate, trichloroacetic acid (TCA), Hydrogen peroxide and butylated hydroxyl anisole were procured from Hi-media Pvt Ltd.Other chemicals and diagnostic kits used in this study were procured from Span Diagnostics Ltd., India and Excel diagnostics Ltd., India.

Experimental animals

Rats of either sex weighing about 150-200 g were used in experiment. Animals were obtained from Anurag Pharmacy College, Kodad, kept under standard conditions at 23-25^{0}C 12 hr light/dark cycle and given standard pellet diet and water. Before performing the experiment the ethical clearance was obtained from Institutional Animal Ethics Committee.

Experimental protocol

The Wistar Albino rats of either sex (200-250 gm) were divided into 5 groups of six rats each. Vehicle or drugs were fed once daily for 30 consecutive days prior to the experiment and treated as follows:

Group 1: Normal (n=6). Received vehicle i.e. distilled water.

Group 2: BCCAO control (n=6). Received vehicle i.e. distilled water, BCCAO for 30 min, followed by 1h and 4h reperfusion

Group 3: EVM (250mg/Kg/p.o./day) treated group (n=6), BCCAO for 30 min followed by followed by 1h and 4h reperfusion

Group 4: EVM (500mg/Kg/p.o./day) treated group (n=6), BCCAO for 30 min followed by followed by 1h and 4h reperfusion.

Group 5: EVM (750mg/Kg/p.o./day) treated group (n=6), BCCAO for 30 min followed by followed by 1h and 4h reperfusion

Induction of cerebral ischemia

In the present study, the animals were pre-treated with EVM for a period of 30 days (250,500 & 750 mg/kg) p.o. After 30 days, stroke was induced by occlusion of bilateral Common carotid artery (BCCAO).The rats were anesthetized with intraperitoneal injection of ketamine (80-90 mg/kg i.m.) & xylazine (5-10 mg/kg i.m.) and placed on the back; a midline ventral incision was made in neck. Both carotid arteries were exposed with special attention paid to separating and preserving the vagus nerve fibers. A cotton thread was passed below each carotid artery and a surgical knot was put on both arteries for 30 min to induce ischemia. After 30 min of global cerebral ischemia, the thread was removed to allow the reflow of blood through carotid arteries (reperfusion) for 1 h and 4 h individually. Body temperature of rats was maintained around 37 ± 0.5°C throughout the surgical procedure by heated surgical platform. Normal animals received the same surgical procedures except BCCA were not occluded. After the completion of reperfusion period, the animals were assessed for their behavioral activity and were sacrificed thereafter. The brains were dissected out for determination of biochemical parameter, brain weight, and assessment of cerebral infracts size. [10, 11]

Behavioral assessment:

Animals were observed for behavioral parameters after 72 hrs of surgery in three different areas, neuromuscular function, vestibulomotor function, and complex neuromotor function which consists of various subtests such as Mobility circling, beam walk and beam balance[12,13,14]. The scores were given as per table no 1. A combined total was taken for each rat with a higher score meaning a higher clinical deficit Procedure for each subtest is described below

Circling: Rats normally do not circle during normal state. Animals having infarction sometimes circle toward the contra lateral side.

***Beam balance*:** It measures the animal's vestibulomotor function and examines the rat's ability to balance on a narrow (30 x1.5 cm) beam.

Beam walk: It measures the animal's complex neuromotor function. The animal was timed as it walked a (90 x 4 x 1.5 cm) beam. A box for the animal to feel safe in was placed at one end of the beam. A loud noise was created to stimulate the animal to walk toward and into the box. Scoring was based upon the time it took the rat is go into the box.

Preparation of Brain Homogenate

Following decapitation, the brain was removed and washed in cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized as 10% w/v in cold phosphate buffer (0.05 M, pH 7.4). The homogenate was centrifuged at 10,000 × g for 10 min at 4 ◦C and post-mitochondrial supernant (PMS) was kept on ice until assayed.

Estimation of biochemical parameters

Lipid Peroxidation level

To 0.2 ml of brain homogenate, 0.2 ml of 4% sodium dodecyl sulphate, 1.5 ml of 20 % acetic acid and 1.5 ml of 0.5% Thiobarbituric acid was added. The mixture was heated for 60 min at 95°C in a temperature controlled water bath to give a pink colour. The mixture was centrifuged at 3500 rpm for 10 minute. The absorbance of the supernatant was read spectrophotometrically at 532 nm. [15]

Superoxide Dismutase (SOD) level

Supernatant (0.1ml) of sample was mixed with 0.1 ml EDTA (1x10-4 M), 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of Epinephrine (3x 10-3M). The optical density of formed adrenochrome was read at 480 nm for 3 min at an interval of 30 sec. the enzyme activity has been expressed in terms of U/min/mg protein. One unit of enzyme activity is defined as the concentration required for the inhibition of the chromogen production by 50% in one minute under the

defined assay conditions. The SOD activity (U/mg of protein) was calculated by using the standard plot. [16]

Catalase (Cat) level

A 50µl supernant was added to buffered substrate (50 mM phosphate buffer, pH 7 containing 10 mM H_2O_2) to make total volume 3 ml. The decrease in the absorbance was read at 240 nm for 2.5 min at an interval of 15 sec. The activity was calculated using extinction co-efficient of H_2O_2 0.041/moles/cm^2. Results were expressed as moles of H_2O_2 utilized/min/mg protein. [17, 18]

Glutathion (GSH) level

To 1 ml of sample (brain homogenate), 1 ml of 10%TCA was added. The precipitated fraction was centrifuged and to 0.5 ml supernatant, 2 ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The colour developed was read at 412 nm. Standard curve for GSH was prepared using glutathione. Results were expressed as µmole of GSH/mg tissue. [19]

Staining of brain sections with TTC

Randomly three animals in each group were selected and sacrificed 72 h after surgery by cervical decapitation. The brains were excised and stained with TTC inorder to identify the area of ischaemia and intensity of damage. The brains were kept in freezer at 4˚C until they get hardened to cut into sections which were placed in 1% TTC (2, 3, 5 triphenyltetrazolium chloride) solution for half an hour. Then the sections stained with TTC were scanned for observing induction intensity of stroke. Then the cerebral infarction area was observed and compared between various treatment groups and control group. [20]

Statistical analysis

All the data was expressed as mean ± SEM. Statistical significance between more than two groups was tested using one way ANOVA followed by the Tukey test using computer based fitting program (Graph pad prism.5). Statistical significance was determined accordingly.

RESULTS AND DISCUSSION:

Brain tissue is especially vulnerable to ischemia since it has little respiratory reserve and is completely dependent on aerobic metabolism, unlike most other organs. The processes of ischemic tissue are referred as ischemic cascade. Ischemic brain damage introduced by ischemic stroke or reperfusion, induces production of oxygen free radicals and other reactive oxygen species. Free radicals are liberated from a variety of sources, including inflammatory cells, dysfunctional mitochondria and excitotoxic mechanisms stimulated by increased glutamate and aspartate concentrations. [21, 22]

Table 1: Clinical scoring criteria of behavioral parameters

Behavioral parameter	Score	Description
Neuromuscular Function		
Mobility	0.0	Normal
	0.5	Spontaneous movement reduced
Vestibulomotor Function	1.0	Needs stimulus to move
Balance beam	2.0	Unable to walk
	0.0	Balances with all 4 paws on top of beam.
	1.0	Puts paws on side of beam or wavers
	2.0	1 or 2 limbs slip off beam.
Complex neuro function	3.0	3 limbs slip off beam.
Beam walk	4.0	Attempts to balance but falls off.
	5.0	Animal drapes on beam then falls.
	6.0	Falls without attempting to balance
	0. 0	4 Sec or less
	1.0	5 to 7 sec
	2.0	8 to 10 sec
	3.0	11 to 15 sec
	4.0	Greater than 15 sec
	5.0	Not able to run

In the present study, the stroke control group animals showed decreased amount of defense enzymes (SOD, catalase, GSH) and increased amount of lipid peroxidation in brain because of induction of ischemia where as the group of animals that were treated with different doses of EVM had shown increased levels of SOD, catalase, GSH enzymes and decreased lipid peroxidation (Fig no 1) This may be due to the antioxidant principles such as flavanoids,anthraqinone cpds and phenolic compounds present in it

Since functional recovery of clinical deficits in stroke often correlates with the efficacy of anti-ischemic therapy, we focused on the behavioral recovery. The neurological deficits or changes in behavior were observed in stroke control animals also due to induction of ischemia

in brain regions that are associated with motor and sensory function. [23]

Table 2: Effect of different doses of Ethanolic extract of *V.madrasapatana* on behavioral parameters in cerebral stroke

S.No	GROUP	TREATMENT	PARAMETER		
			MOBILITY	BEAM WALK	BEAM BALANCE
I.	NORMAL	Distilled water	0.0±0.0	0.0±0.0	0.0±0.0
II.	CONTROL	Distill water +stroke was induced after 30 days by BCCAO	1.25±0.25###	1.75±0.25#	4.0±0.0###
III.	TEST 1	EVM (250 mg/kg p.o) + stroke was induced after 30 days by BCCAO	0.75±0.144	3.0±0.577	2.25±0.25*
IV.	TEST 2	EVM (500 mg/kg p.o) + stroke was induced after 30 days by BCCAO	0.125±0.125*	0.625±0.125*	0.25±0.25*
V	TEST 3	EVM (750 mg/kg p.o) + stroke was induced after 30 days by BCCAO	4.25±0.25***	0.0±0.0***	0.0±0.0***

All values are shown as mean ± SEM and n=6.
indicate $p< 0.05$, ## indicate $p<0.01$, ### indicate $p<0.001$ when compared to normal group.
* indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$ when compared to control grouPS

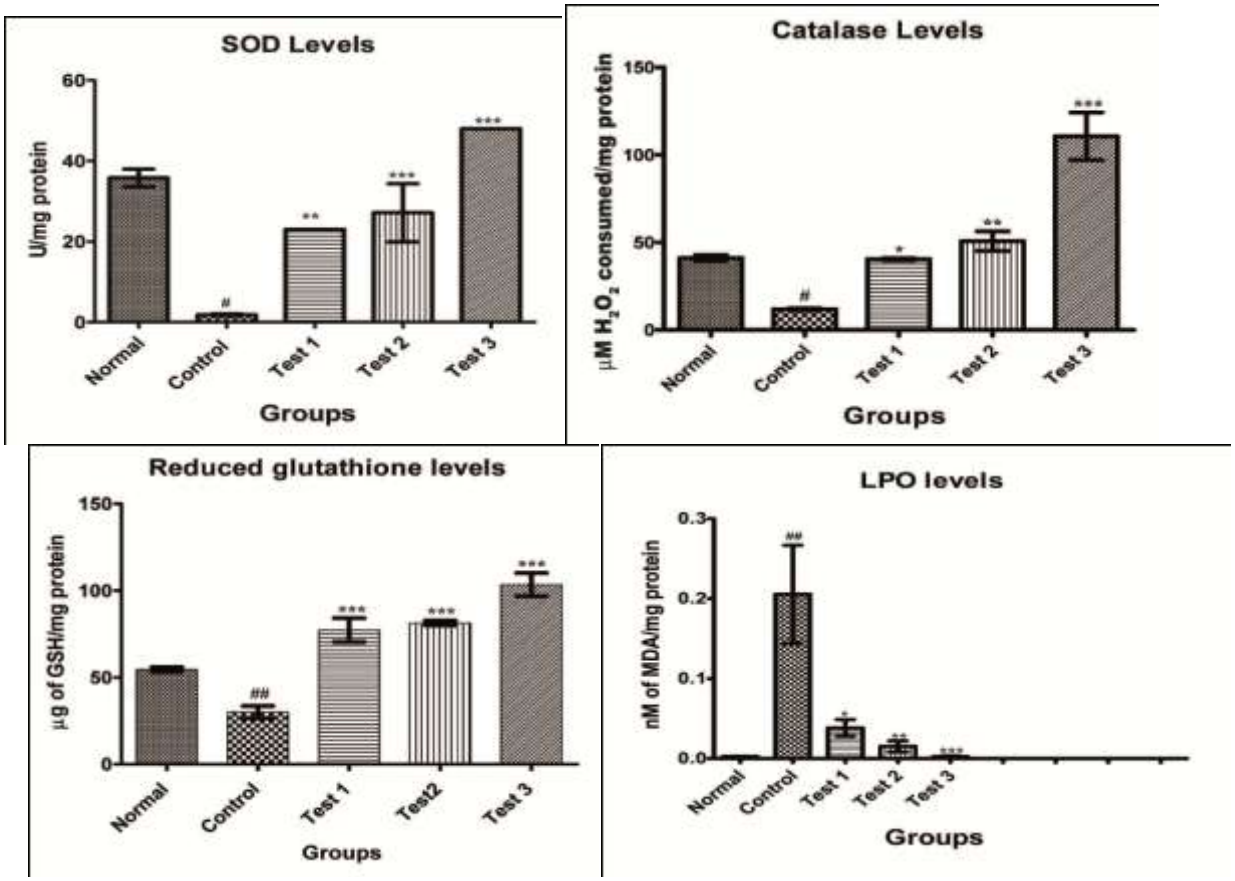

Fig 1: Effect of different doses of Ethanolic extract of *V.madrasapatana* on on SOD, Catalase, Reduced glutathione and Lipid peroxidation levels in brain

All values are shown as Mean ± SEM and n = 6
# indicates p < 0.05, ## indicates p < 0.01, ### indicates p < 0.001 when compared to normal group
***** indicates p < 0.05, ****** indicates p < 0.01, ******* indicates p < 0.001 when compared to control group

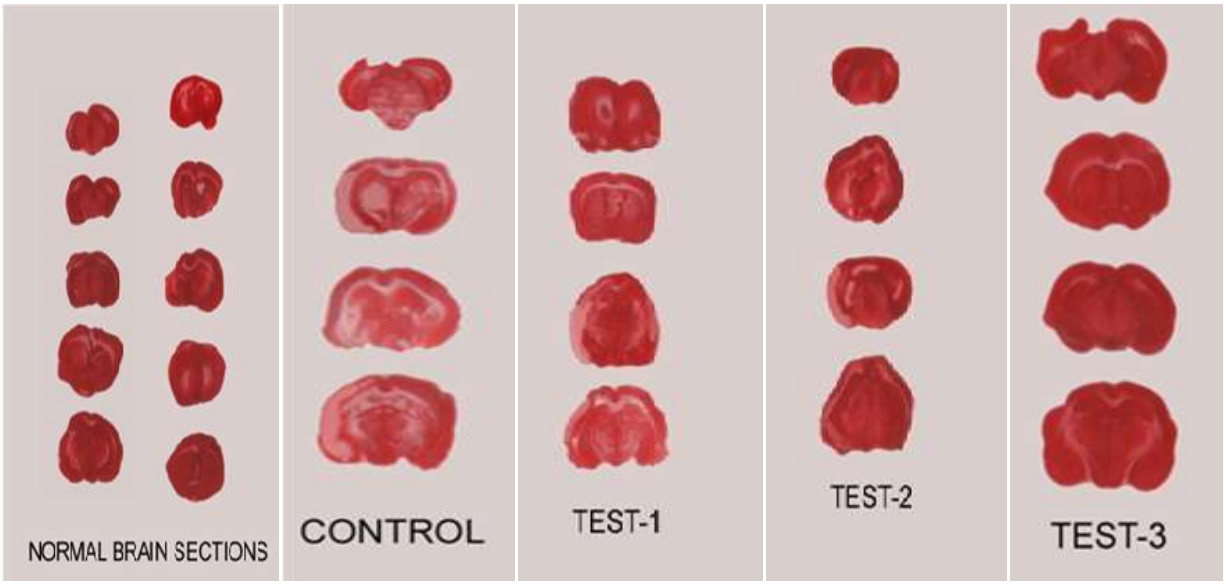

Fig.2: TTC Stained brain section of various treatment groups

Score of mobility, beam walk and beam balance had been significantly increased in the stroke control group (G-II) when compared to Sham group (G-I) ($p<0.001$, $p<0.05$ and $p<0.001$). No significant decrease in the score was seen in group-III. The groups-IV & V exhibited a significant ($p<0.05$, $p<0.05$ and $p<0.001$) decrease in the mobility, beam walk and beam balance score when compared to the control group (G-II) which indicate improvement in neurological deficits. Lack of neurological deficits in test group animals treated with extract may due to presence of flavanoid and phenolic compounds present in it.

Staining of brain sections shown reduced infract size in animals treated with EVM. Colorless region corresponds to occluded territory. Reperfusion and duration of occlusion influence the severity of ischaemia. This decrease in infract area may be due to antioxidant, antiinflammatory and antithrombogenic properties of flavanoids [24, 25] present in the *V.madrasapatana*.

CONCLUSION:

These experimental findings conclude that *V.madraspatana* exhibit neuroprotective effect in cerebral ischemia by potentiating the antioxidant defense system of the brain. Further studies to isolate active constituents and establish mechanism of action will be fruitful.

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