

Effect of *Hemidesmus indicus* on Cerebral Infarct Ischemia-reperfusion Injured Rats by Four Vessel Occlusion Method

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Abstract: Background: To investigate the effect of Methanol root extract of *Hemidesmus indicus* (MEHI) on cerebral infarct by four vessel occlusion method. **Method:** A total of 36 Sprague-Dawley (SD) rats were studied. In the present study the animals were pretreated with MEHI for a period of 1 week (200 and 400 mg kg⁻¹) p.o. Cerebral ischemia was established by permanent occlusion of vertebral artery followed by 15 min occlusion of bilateral carotid arteries. After 24 h of reperfusion the animal behavior were evaluated for neuromuscular function, vestibulomotor function and complex neuromotor function. The treatment was continued for another week after surgery with root extract and the animals were sacrificed and the brain was removed and homogenized. The homogenized content was used for the estimation of anti-oxidant enzymes and various neurotransmitter levels. **Results:** The group treated with 200 and 400 mg kg⁻¹ MEHI showed significant (p<0.01) improvement in neuromuscular, vestibulomotor, complex neuromuscular function when compared with negative control group. Significant increase in brain anti oxidant enzymes was observed in MEHI treated group when compared to negative control. The MEHI treated groups exhibited a significant decrease in the levels of acetyl choline esterase, glutamate and monoamine oxidase-B. Increased levels of dopamine and serotonin were observed in the treated group when compared with ischemic group. **Conclusion:** MEHI can improve the neurological status and may reduce the cerebral infarct in ischemia-reperfusion injured rats.

Key words: Cerebral ischemia, neurotransmitter, *Hemidesmus indicus*, neuromuscular, vestibulomotor, complex neuromuscular, anti oxidant enzymes

INTRODUCTION

WHO (World health organization) states that heart disease and stroke kill some 17 million people a year which is almost one-third of all deaths globally. By 2020, heart disease and stroke will become the leading cause of both death and disability worldwide, with the number of fatalities projected to increase to over 20 million a year and by 2030 to over 24 million a year (Lloyd-Jones *et al.*, 2009). Approximately 80% of strokes or brain attacks, are ischemic. They can develop in major blood vessels on the surface of the brain (called large-vessel infarcts) or in small blood vessels deep in the brain (called small-vessel infarcts) (Brown *et al.*, 2008).

The brain is much complex organ of our body and hence no surprise that only a very few drugs are approved by regulatory authorities for treating multi-factorial ailments in the brain. The agent which cure body and mental diseases, delay old age, increase mental power, generating power, vital energy, eyesight, impart intelligence, memory, aid proper digestion and clear

complexation are Rasayana. In Sanskrit, “medhya” means intellect/cognition and “rasayana” means “rejuvenation”. They nourish the whole body by strengthening the primordial tissue Rasa, the essence of all food we take and which the body can assimilate. If this essence is well distributed in all systems, the body remains healthy. By their physico-chemical action Rasayana purify and promote dhatus (tissue). They augment the body’s disease resistance capacity, as well as the ability for restorative reaction and counteract all the deleterious effects including that of ageing (Puri, 2003).

In India about two thousand medicinal plants are found. *Hemidesmus indicus* (Asclepidaceae) is an important medicinal plant of tropical and subtropical India. Its medicinal usage has been reported in the Indian and British Pharmacopoeias and in traditional systems of medicine such as Ayurveda, Unani and Siddha. The roots are used as antipyretic, anti-diarrheal, astringent, blood purifier, diaphoretic, diuretic, refrigerant and tonic (Anonymous, 1986, 1997). Roots are used in the treatment of biliousness, blood diseases, dysentery, diarrhea,

respiratory disorders, skin diseases, syphilis, fever, leprosy, leucoderma, leucorrhoea, itching, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism (Kirtikar and Basu, 1980). The plant has been indicated in the treatment of brain diseases in Unani system of medicine (Nadkarni, 1989). As *Hemidesmus indicus* has been proved to possess antioxidant, antithrombotic (Mary *et al.*, 2003) and hypothermic effect in experimental animal models and these three properties suggest that *H. indicus* may be useful in the treatment of cerebral ischemia. Hence the present study was designed to study the effect of methanolic root extract of *H. indicus* on cerebral ischemia induced by four vessel occlusion (4VO) in rats.

MATERIALS AND METHODS

Plant material: The roots of *Hemidesmus indicus* were collected from korattur, Chennai. The plant material was identified and authenticated by Dr. Sasikala Ethirajulu, Research Officer (Pharmacognosy) of Central Research for Siddha, Govt of India, Arumbakkam, Chennai, Tamil nadu, India.

Preparation of extract of MEHI: Freshly collected roots of *Hemidesmus indicus* were dried in shade and pulverized to get a coarse powder. A weighed quantity of the powder (960 g) was passed through sieve number 40 and subjected to hot solvent extraction in a soxhlet apparatus using methanol at a temperature range of 60-80°C, respectively. Before and after every extraction the powder bed was completely dried and weighed. The filtrate was evaporated to dryness at 40°C under reduced pressure in a rotary vacuum evaporator. A brownish black waxy residue was obtained. The percentage yield of methanol extract was 16.53% w/w.

Phytochemical screening: Phytochemical screening of the MEHI extract was performed using the reagents and chemicals as follows:

- Alkaloids with Mayer's, Hager's and Dragendorff's reagent (Hultin, 1965)
- Flavonoids with the use of sodium acetate, ferric chloride and amyl alcohol
- Phenolic compounds and tannins with lead acetate and gelatin
- Carbohydrate with Molish's, Fehling's and Benedict's reagent (Harborne, 1973)
- Proteins and amino acids with Millon's, Biuret Xanthoprotein test (Strong and Koch, 1974)
- Saponins test using the hemolysis method
- Sterols with 5% potassium hydroxide
- Steroids with Libermann Burchard's test (Campbell and Farrell, 2005)
- Saponins with foam test
- Terpenes with thionyl chloride
- Glycosides with ferric chloride, acetic acid and concentrated sulphuric acid
- Gum tested using Molish's reagent and Ruthenium red
- Coumarin by 10% sodium hydroxide and Quinones by concentrated sulphuric acid

These were identified by characteristic color changes using standard procedures (Trease and Evans, 1989).

The screening results were as follows: Alkaloids +; Carbohydrates +; Proteins and amino acids +; Steroids -; Sterols +; Phenols +; Flavonoids +; Gums and mucilage +; Glycosides +; Saponins -; Terpenes + and Tannins -ve.

Where + and - indicates the presence and absence of compounds.

Acute toxicity study (Ecobichon, 1997): This was performed for the extracts to ascertain safe dose by the acute oral toxic class method by the Organization of Economic Cooperation and Development (OECD). A single administration of starting dose of 2000 mg kg⁻¹ body weight/po of the MEHI was administered to three female rats and the rats were observed for three days to evaluate considerable changes in body weight and other signs of toxicity (Petullo *et al.*, 1999).

Repeating the experiment with the same dose level of MEHI for more seven days, we observed the body weight change and toxicity sign for totally fourteen days.

Experimental animals: Colony inbred strains of Sprague Dawley rats (male) weighing 250-300 g, obtained from C. L. Baid Metha College of pharmacy was used for the pharmacological studies. The animals were kept under standard conditions maintained at 23-25°C, 12 h light/dark cycle and given standard pellet diet (Hindustan lever, Bangalore) provided *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into six groups of each six animals. Principles of animal handling were strictly adhered to the guidelines and handling of animals was made under the supervision of animal ethics committee of the institute. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

IAEC reference number: IAEC/XIII/04/CLBMCP/2009-2010 dated: 22.04.09

Induction of cerebral ischemia: Experimental protocol for study of cerebral ischemia by Four Vessel Occlusion method (4VO) (Toda *et al.*, 2002).

Day 1: Electrocauterization of Vertebral Artery: Male Sprague dawley rats, weighing 250 to 300 g each were anesthetized intraperitoneally with a combination of Ketamine (40 to 60 mg kg⁻¹) and xylazine (3 to 5 mg kg⁻¹) (Wang-Fischer, 2009). Forebrain ischemia was induced by four-vessel occlusion. A dorsal neck incision is made from the occipital bone to the second cervical vertebra (about 1 cm in length). The paraspinal muscles are separated to expose the second cervical vertebrae. Under the operating lens the visible vertebral arteries at the second vertebra could be easily electrocauterized and completely cut by microscissors to yield complete cessation of circulation of both vertebral arteries.

Day 2: Occlusion of bilateral common carotid arteries: After 24 h, the animals were subjected to 15 min of forebrain ischemia by making a ventral neck incision of 2 cm length and occluding both common carotid arteries (vagus nerve separated) with vascular clips. Rectal temperature was maintained at close to 37°C with a heating pad during and after ischemia.

Experimental procedure: The male SD strain rats were randomized into 6 different groups (n = 6 per group)

Group 1: Animals (Positive control) with sham operation (without occlusion) and treated with saline (p.o)

Group 2: Animals with sham operation (without occlusion) and treated with 200 mg kg⁻¹ of MEHI (p.o)

Group 3: Animals with sham operation (without occlusion) and treated with 400 mg kg⁻¹ of MEHI (p.o)

Group 4: Animals (Negative control) with 4VO and treated with saline (p.o)

Group 5: Animals with 4VO and treated with 200 mg kg⁻¹ of MEHI (p.o)

Group 6: Animals with 4VO and treated with 400 mg kg⁻¹ of MEHI (p.o)

Behavior examination: This was done according to the method of Alexis *et al.* (1995) and Petullo *et al.* (1999).

Neuromuscular function test

The test consisted of 6 subtests: forelimb flexion, twisting, resistance to lateral push, circling, hindlimb placement, inverted angle board gripping. Each subtest was described below (See the scoring chart below).

Forelimb flexion: When held by the tail above a flat surface a normal rat will extend both forelimbs toward the surface, rats with an infarction will consistently flex the paralytic forelimb. Flexion would vary from mild wrist flexing and shoulder abduction to severe flexion encompassing the entire forelimb.

Torso twisting: When held by the tail above a flat surface a normal rat will extend the entire body toward the surface, rats with an infarction show signs of body rotation. This rotation consists of mild twisting of the body to a severe body movement bringing the head and forelimbs into the vicinity of the hindlimbs. Twisting was always toward the paralytic side.

Lateral push: A normal rat will show equal resistance when held behind the shoulders and pushed either to the left (lateral) or right (contralateral) sides. Rats with an infarction show either weaker or no resistance when pushed toward the contralateral side.

Circling: Rats normally do not circle during normal gate. Animals having infarction sometimes circle toward the contralateral side.

Hindlimb placement: A normal rat will immediately replace a hindlimb to the surface top if the leg is removed from the surface, injured rats show either a delay in placement or no replacement at all.

Inverted angle board: Normally a rat can be trained to turn 180 degrees and proceed to the top of an angled board. Injured animals cannot make this turn and proceed up the board. The degree and severity were graded accordingly.

Neuromuscular function criteria	Score	Description
Fore limb Flexion	0.0	No flexion
	0.5	Mild
	1.0	Moderate to severe
Torso twisting	0.0	No signs
	0.5	Mild
	1.0	Moderate to severe
Lateral push	0.0	Equal resistance
	0.5	Weaken resistance
	1.0	No resistance
Hind limb placement	0.0	Immediately replaces
	0.5	Delay in replacing
	1.0	No replacement
Fore limb placement	0.0	Immediately replaces
	0.5	Delay in replacing

Continued:

	1.0	No replacement
Inclined board	0.0	Turns 180° goes to top
	0.5	Able to stay up but not go to top
	1.0	Tries to stay up but eventually slides down
	1.5	Tries to stay up but immediately slides down
	2.0	Not able to hold body up at all, immediately slides to bottom
Mobility	0.0	Normal
	1.0	Spontaneous movement reduced
	1.5	Needs stimulus to move
	2.0	Unable to walk
Maximum score	9.0	

Vestibulomotor function (beam balance): Beam balance is sensitive to motor cortical insults. This task was used to assess gross vestibulomotor function by requiring a rat to balance steadily on a narrow beam. The test involves three 60 sec training trials 24 h before surgery. The apparatus consists of a 3/4-inch-wide beam, 10 inches in length, suspended 1 ft above a table top. The rat was positioned on the beam and must maintain steady posture with all limbs on top of the beam for 60 sec.

Vestibular Function	Score	Description
Balance beam	0.0	Balance 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
	3.0	
	4.0	3 limbs slip off beam
	5.0	
	6.0	Attempts to balance but falls off Animal drapes on beam then falls Falls without attempting to balance
Maximum score	6.0	

Complex neuromotor function (beam walk): This is a test of sensorimotor integration specifically examining hindlimb function. A 1-inch-wide beam, 3 ft in length, was suspended 3 ft above the floor in a dimly light room. At the far end of the beam was a darkened goal box with a narrow entryway. A white noise generator and bright light source at the start of the beam motivate the animal to traverse the beam and enter the goal box. Once inside the goal box, the stimuli were terminated. The rat's latency to reach the goal box (in seconds) and hindlimb performance as it traversed the beam (based on a rating scale) were recorded. Each rat was trained for 3 days before surgery to acquire the task and to achieve normal performance on three consecutive trials (See the scoring chart below).

Complex neuromotor function	Score	Time on beam
Beam walk	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
Maximum score	5.0	

Neurological deficit score-modified garcia scoring system (Garcia et al., 1995): The Garcia scoring system is used to rank the neurological deficit in stroke induced rats. This test involves the following parameters.

Spontaneous activity: The animal was observed for 5 min in its normal environment (cage). The rat's activity was assessed by its ability to approach all four walls of the cage. Scores indicate the following: 3, rat moved around, explored the environment and approached at least three walls of the cage; 2, slightly affected rat moved about in the cage but did not approach all sides and hesitated to move, although it eventually reached at least one upper rim of the cage; 1, severely affected rat did not rise up at all and barely moved in the cage and 0, rat did not move at all.

Symmetry in the movement of four limbs: The rat was held in the air by the tail to observe symmetry in the movement of the four limbs. Scores indicate the following: 3, all four limbs extended symmetrically; 2, limbs on left side extended less or more slowly than those on the right; 1, limbs on left side showed minimal movement and 0, forelimb on left side did not move at all.

Forepaw outstretching: The rat was brought up to the edge of the table and made to walk on forelimbs while being held by the tail. Symmetry in the outstretching of both forelimbs was observed while the rat reached the table and the hind limbs were kept in the air. Scores indicate the following: 3, both forelimbs were outstretched and the rat walked symmetrically on forepaws; 2, left side outstretched less than the right and forepaw walking was impaired; 1, left forelimb moved minimally and 0, left forelimb did not move.

Climbing: The rat was placed on the wall of a wire cage. Normally the rat uses all four limbs to climb up the wall. When the rat was removed from the wire cage by pulling it off by the tail, the strength of attachment was noted. Scores indicate the following: 3, rat climbed easily and gripped tightly to the wire; 2, left side was impaired while climbing or did not grip as hard as the right side and 1, rat failed to climb or tended to circle instead of climbing.

Bodyproprioception: The rat was touched with a blunt stick on each side of the body and the reaction to the stimulus was observed. Scores indicate the following: 3, rat reacted by turning head and was equally startled by the stimulus on both sides; 2, rat reacted slowly to stimulus on left side and 1, rat did not respond to the stimulus placed on the left side.

Response to vibrissae touch: A blunt stick was brushed against the vibrissae on each side; the stick was moved toward the whiskers from the rear of the animal to avoid entering the visual fields. Scores indicate the following: 3, rat reacted by turning head or was equally startled by the stimulus on both sides; 2, rat reacted slowly to stimulus on left side and 1, rat did not respond to stimulus on the left side.

The score given to each rat at the completion of the evaluation is the summation of all six individual test scores. The minimum neurological score is 3 and the maximum is 18.

In vivo pharmacological examination:

- **Motor activity:** This was done according to the method of Kulkarni (2004)
- **Cylinder test (forepaw asymmetry):** This was done according to the method of Schallert *et al.* (1997)
- **Tape removal test:** This was done according to the method of Albertsmeier *et al.* (2007)
- **Water maze navigation:** This was done according to the method of Hartman *et al.* (2005)

Cued water maze task: This task (visible platform) was used to assess sensorimotor (e.g., swimming ability, vision) and/or motivational deficits that could affect performance during the spatial (Place/Learning Set) water maze tasks. The rats were given 20 trials (2 blocks of 10 consecutive trials) during 1 day of testing where each trial involved a different platform location. Rats were allowed to remain on the platform for 30 sec after each trial.

Place water maze-easy protocol: This spatial reference memory-based task involved learning the location of a submerged platform that remained the same across all trials within a given experiment.

This protocol involved 20 trials (2 blocks of 10 consecutive trials) per day for 2 days. Several salient spatial cues were hung on the walls around the room in an effort to facilitate spatial learning. Rats were allowed to remain on the platform for 30 sec after each trial. Retention was evaluated with probe trials which consisted of removing the escape platform and subjecting the rats to a 60 sec “free swim” 2 h after the last place trial on each day. Time spent swimming in the target quadrant and the number of crossings over the former platform location were quantified.

Estimation of neurotransmitters and metabolic enzymes:

- Acetylcholine esterase estimation (Ellman *et al.*, 1961)
- Estimation of glutamate levels by multiple development paper chromatography: This was done according to the method of Raju *et al.* (2004)
- **Preparation of tissue extracts:** This was done according to the method of Schlumpf *et al.* (1974)
- **Estimation of dopamine:** This was done according to the method of Schlumpf *et al.* (1974)
- **Estimation of serotonin:** This was done according to the method of Kepe *et al.* (2006)
- **Estimation of monoamine Oxidase B:** This was done according to the method of Charles and McEwen (1977)

Estimation of anti oxidant enzymes:

- **Assay of Superoxidedismutase (Sod):** This was done according to the method of Marklund and Marklund (1974)
- **Estimation of Catalase (CAT):** This was done according to the method of Sinha (1972)
- **Estimation of Glutathione Peroxidase (Gpx):** This was done according to the method of Rotruck *et al.* (1973)
- **Estimation of Glutathione Reductase (GR):** This was done according to the method of Staal *et al.* (1969).
- **Estimation of lipid peroxidation:** This was done according to the method of Ohkawa *et al.* (1979).
- **Estimation of total proteins:** Total protein was estimated in brain using the method described by Lowry *et al.* (1951).

Statistical analysis: All values are expressed as Mean-SEM. Data were analyzed by non-parametric ANOVA followed by Dunnett’s multiple comparison tests and other data were evaluated using Graph Pad PRISM software. A p-value<0.05 was considered significantly different.

RESULTS

Effect of MEHI on neuromuscular function: Scores of neuromuscular function was found to be significantly (p<0.001) reduced in the negative control group when

compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ showed significant (p<0.01) improvement in Neuromuscular function when compared with negative control group (Table 1).

Effect of MEHI on vestibulomotor function: Scores of vestibulomotor function was found to be significantly (p<0.001) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ showed significant (p<0.01) improvement in vestibulomotor function when compared with negative control group (Table 1).

Effect of MEHI on complex neuromotor function: Scores of complex neuromotor function was found to be significantly (p<0.001) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ showed significant (p<0.01) improvement in complex neuromotor function when compared with negative control group (Table 1).

Effect of MEHI on neurological deficit score: Neurological deficit scores was found to be significantly (p<0.001) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) reduced the neurological deficit scores when compared with negative control group (Table 1).

Effect of MEHI on motor activity: Motor activity was found to be significantly (p<0.01) decreased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and

400 mg kg⁻¹ significantly (p<0.01) increased the neurological deficit scores when compared with negative control group (Table 1).

Effect of MEHI on cylinder test: Scores of cylinder test was found to be significantly (p<0.001) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 mg kg⁻¹ of MEHI did not show significant (ns) improvement when compared with negative control group where as group treated with 400 mg kg⁻¹ of MEHI showed significant (p<0.01) increase in scores of cylinder test when compared with negative control group (Table 1).

Effect of MEHI on tape removal test: Latency to remove the tapes was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) decreased the latency to remove the tapes when compared with negative control group (Table 1).

Effect of MEHI on cued water maze task: Escape latency was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) decreased the latency to find the platform when compared with negative control group (Table 2).

Effect of MEHI on place water maze-easy protocol: Escape latency was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and

Table 1: Effect of MEHI on Neuromuscular function, Vestibulomotor function, Complex Neuromotor function, Neurological Deficit Score, motor activity, cylinder test and tape removal test

In-vivo behavior examination	Group					
	1	2	3	4	5	6
Effect of MEHI on neuromuscular function (Score)	0.0	0.0	0.0	6.40±0.166a**	5.30±0.123b**	4.45±0.112b**
Effect of MEHI on vestibulomotor function (Score)	0.0	0.0	0.0	3.16±0.307	1.66±0.210b**	1.50±0.223b**
Effect of MEHI on complex neuromotor function (Score)	0.0	0.0	0.0	3.50±0.223a*	2.33±0.210b*	1.50±0.223b*
Effect of MEHI on neurological deficit score (Score)	14.8300±0.1667	14.67±0.2108	14.83±0.1667	11.17±0.166	12.50±0.223b*	12.67±0.210b*
Effect of MEHI on motor activity (No. of beam cutoff)	460.0±3.204	462.8±3.381	460.5±3.253	276.5±7.543a**	321.5±3.263b**	342.0±4.933b**
Effect of MEHI on cylinder test (Preference score)	0.50±0.01	0.45±0.022	0.433±0.021	0.16±0.021a***	0.233±0.021b*	0.250±0.022b*
Effect of MEHI on tape removal test (Latency to remove the tape (Sec))	27.50±0.763	28.83±0.477	27.33±0.421	177.2±0.792a**	158.2±1.909b**	153.5±1.258b**

*p<0.05, **p<0.01, ***p<0.001, Values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Group 1 vs. Group 4 and b. Group 4 vs. Group 5 and 6. Symbol represents the statistical significance done by One-way ANOVA, followed by Dunnett's multiple comparison test. Group 1: Sham (saline), Group 2: Sham (200 mg kg⁻¹), Group 3: Sham (400 mg kg⁻¹), Group 4: Ischemia (saline), Group 5: Ischemia+MEHI (200 mg kg⁻¹), Group 6: Ischemia+MEHI (400 mg kg⁻¹)

Table 2: Effect of MEHI by *In-vivo* Behavior Examination

<i>In-vivo</i> behavior examination	Group					
	1	2	3	4	5	6
Effect of MEHI on cued water maze task (Latency sec)	44.93±1.95	43.92±1.15	43.33±0.65	57.01±0.050a**	48.67±0.30b**	48.75±0.69b**
Effect of MEHI on place water maze-easy protocol (Latency sec)	46.53±0.5231	46.83±0.4010	46.50±0.6055	53.92±0.2986a**	48.92±0.3005b**	49.08±0.5388b**

*p<0.05, **p<0.01, ***p<0.001, values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Group 1 vs. Group 4 and b. Group 4 vs. Group 5 and 6. Symbol represents the statistical significance done by One-way ANOVA, followed by Dunnett's multiple comparison test. Group 1: Sham (saline), Group 2: Sham (200 mg kg⁻¹), Group 3: Sham (400 mg kg⁻¹), Group 4: Ischemia (saline), Group 5: Ischemia+MEHI (200 mg kg⁻¹), Group 6: Ischemia+MEHI (400 mg kg⁻¹)

Table 3: Effect of MEHI on acetylcholine esterase level

Micromole/min/mgprotein	Group					
	1	2	3	4	5	6
	17.58±0.058	17.62±0.096	17.58±0.058	21.10±0.187a**	18.66±0.081b**	17.58±0.037b**

*p<0.05, **p<0.01, ***p<0.001, Values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Group 1 vs. Group 4 and b. Group 4 vs. Group 5 and 6. Symbol represents the statistical significance done by One-way ANOVA, followed by Dunnett's multiple comparison test

Table 4: Effect of MEHI on Neuro transmitter level

Neuro transmitter level	Group					
	1	2	3	4	5	6
MEHI on glutamate level (Micromoles/gram)	76.20±0.374	76.10±0.187	76.10±0.291	86.60±0.187a**	81.10±0.367b**	76.10±0.331b**
Effect of MEHI on dopamine level (Picogram/mgtissue)	660.6±0.927	659.6±1.327	658.6±1.327	539.4±1.208a**	599.0±0.836b**	647.4±1.449b**
Effect of MEHI on serotonin level (Picogram/mgtissue)	824.4±2.561	825.2±2.035	825.6±2.293	730.6±2.400a**	787.6±1.600b**	814.8±2.083b**

*p<0.05, **p<0.01, ***p<0.001, Values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Group 1 vs. Group 4 and b. Group 4 vs. Group 5 and 6. Symbol represents the statistical significance done by One-way ANOVA, followed by Dunnett's multiple comparison test

400 mg kg⁻¹ significantly (p<0.01) decreased the latency to find the platform when compared with negative control group (Table 2).

Effect of MEHI on acetylcholine esterase level: Acetylcholine esterase level was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) decreased the level of acetylcholine esterase when compared with negative control group (Table 3).

Effect of MEHI on glutamate level: Glutamate level was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) decreased the level of glutamate when compared with negative control group (Table 4).

Effect of MEHI on dopamine level: Dopamine level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) increased the level of dopamine when compared with the negative control group (Table 4).

Effect of MEHI on serotonin level: Serotonin level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) increased the level of serotonin when compared with the negative control group (Table 4).

Effect of MEHI on MAO-B level: MAO-B level was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) decreased the level of MAO-B when compared with negative control group (Table 5).

Effect of MEHI on Superoxide Dismutase (SOD) level: SOD level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) increased the level of SOD when compared with the negative control group (Table 5).

Effect of MEHI on Catalase (Cat) level: CAT level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) increased the level of CAT when compared with the negative control group (Table 5).

Table 5: Effect of MEHI on anti oxidant enzyme and protein level

Anti oxidant enzyme and protein level	Group					
	1	2	3	4	5	6
Effect of MEHI on MAO-B Level ($\mu\text{mol}/\text{min}/\text{mg}$)	4.187±0.050	4.223±0.033	4.198±0.041	5.437±0.094a***	5.365±0.057b**	5.338±0.049b*
Effect of MEHI on Superoxide Dismutase (SOD) Level (Units/mg protein)	9.10±0.187	9.30±0.300	9.50±0.418	6.80±0.1225a**	8.10±0.187b**	9.10±0.291b**
Effect of MEHI on Catalase (Cat) level ($\mu\text{mol H}_2\text{O}_2/\text{decomposed}/\text{mgprotein}/\text{min}$)	2.52±0.020	2.54±0.050	2.54±0.024	1.73±0.020a**	2.12±0.037b**	2.51±0.024b**
Effect of MEHI on Glutathione Peroxidase (GPx) level (Units/min/mgprotein)	37.20±0.255	37.00±0.224	37.20±0.300	28.20±0.300a**	32.40±0.187b**	37.20±0.300b**
Effect of MEHI on Glutathione Reductase (Gr) level (Nanomoles of NADPH oxidized Units/min/mg protein)	33.50±0.273	33.80±0.300	33.80±0.122	25.60±0.187a**	29.30±0.122b**	33.70±0.122b**
Effect of MEHI on lipid peroxidation level (Nanomoles of TABRS/mgprotein)	2.24±0.067	2.26±0.024	2.28±0.037	4.56±0.0509a**	3.30±0.044b**	2.26±0.0509b**
Effect of MEHI on protein level (Gram dL^{-1})	1.85±0.027	1.89±0.036	1.86±0.018	1.14±0.029a**	1.54±0.018b**	1.80±0.022b**

*p<0.05, **p<0.01, ***p<0.001, Values are expressed as Mean±SEM of 6 animals. Comparisons were made between a. Group 1 vs. Group 4 and b. Group 4 vs. Group 5 and 6. Symbol represents the statistical significance done by One-way ANOVA, followed by Dunnett's multiple comparison test

Effect of MEHI on glutathione peroxidase (GPX) level:

GPx level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg^{-1} significantly (p<0.01) increased the level of GPx when compared with the negative control group (Table 5).

Effect of MEHI on Glutathione Reductase (GR) level:

GR level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg^{-1} significantly (p<0.01) increased the level of GR when compared with the negative control group (Table 5).

Effect of MEHI on lipid peroxidation level:

Lipid peroxide level was found to be significantly (p<0.01) increased in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg^{-1} significantly (p<0.01) reduced the level of lipid peroxides when compared with the negative control group (Table 5).

Effect of MEHI on protein level:

Total protein level was found to be significantly (p<0.01) reduced in the negative control group when compared with the sham operated group. The MEHI treated groups 200 and 400 mg kg^{-1} significantly (p<0.01) increased the level of total protein when compared with the negative control group (Table 5).

DISCUSSION

The present study revealed the neuroprotective effect of the plant *Hemidesmus indicus* in cerebral ischemia induced by four vessel occlusion in rats. Four Vessel Occlusion is an accepted model of global cerebral ischemia in rats. This method is a two stage procedure

which involves the permanent occlusion of the vertebral arteries and following the next day, occlusion of the carotid arteries for 15 min. By combining tasks from many sources we achieved an overall behavioral scheme best suited for our model. For the long term study it was discovered that behavior could be broken down into at least three different levels of function: Neuromuscular function which included leg flexion, twisting, circling, lateral push and inclined angle board; Complex neuromuscular function involves the animal's ability to balance, grip strength and coordinated movements by walking on a balance beam; Vestibulomotor function as tested by the beam-balance. Also the neurological deficit was evaluated using Modified Garcia Scoring system.

There was an increase in Score of Neuromuscular, Vestibulomotor, Complex neuromuscular functions 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} MEHI showed significant (p<0.01) improvement in neuromuscular, vestibulomotor, complex neuromuscular function when compared with negative control group. Further the increase in the neurological deficit score of the treated group when compared with ischemic group showed the protective effect of MEHI in neurological deficit parameters. The forepaw asymmetry was evaluated using the cylinder test which showed a significant reduction of scores in negative control group. The scores were found to be increased in the MEHI treated groups.

The tape removal test is a very sensitive test that quantifies the sensorimotor deficit due to global cerebral ischaemia upto 7 days. Increased latencies in the tape removal test are associated with lesions of the caudal fore limb region of the somatic sensorimotor cortex, the rostral

forelimb region and the anteromedial cortex (Barth *et al.*, 1990). In addition, striatal damage has been identified as a distinct component that is chiefly responsible for the time difference from application of the tape to first contact with the teeth (Grow *et al.*, 2003).

Results of the water maze navigation tests support the earlier reports of 4VO induced deficits spatial learning and memory (Jaspers *et al.*, 1990). Chronic reduction blood flow secondary to 4VO has been reported to cause progressive dysfunction resulting in cognitive deficits (Tsuchiya *et al.*, 1993). Stroke induced animals consistently took longer time to find both the submerged (Place water maze) as well as visible (Cued water maze) platforms. The results obtained shows that MEHI attenuated these alterations significantly suggesting its potential in improving spatial learning and memory in ischemic rats.

It is well known that the cholinergic system in the central nervous system plays an important role in learning and memory function (Sarter and Bruno, 1994) and the cholinergic hypofunction causes dementia with symptoms such as memory loss and disorientation in cerebrovascular disease (Coyle *et al.*, 1983). The AChE activity has been shown to be increased in stroke patients. The calcium influx followed by oxidative stress is involved in the increase in activity of AChE leads to decreasing cell membrane order and ultimately leading to the exposure of more active enzyme (Ban *et al.*, 2006).

It has been reported that hypoxia induces reduction of memory and judgement that is associated with a decrease in acetylcholine synthesis (Gibson and Duffy, 1981). We observed from our *in vivo* experiments like water maze navigation tasks that the ischemic group took more time to detect the goal box and platform suggesting the impairment of memory. Also our biochemical datas show a raise in the level of acetylcholine esterase which is an important enzyme in the metabolism of acetylcholine. These results confirm the relationship of cholinergic system with memory. We also observed reduced levels of AchE in the MEHI treated group which may be correlated with the results of MEHI treated group in Swater maze tasks. Hence the memory enhancing effect of MEHI could be due to the inhibitory action on AchE.

The concentration of both Dopamine (DA) and its metabolite Dihydroxyphenylacetic Acid (DOPAC) were lower in neuro degenerative condition. The MEHI treated groups 200 and 400 mg kg⁻¹ significantly (p<0.01) increased the level of dopamine when compared with the negative control group.

Glutamate is the primary excitatory neurotransmitter in the central nervous system. Under normal conditions, glutamate is released into the synaptic cleft and binds to glutamate receptors resulting in the propagation of an action potential. The modulation of this synaptic activity occurs both by modulation of glutamate receptors and by the removal of glutamate from the synaptic cleft by glutamate transporters. An over activation of glutamate receptors has been particularly emphasized as the key step in the induction of ischemic brain damage (Choi, 1987). Glutamate, released as a consequence of the rapid energy depletion, causes neuronal injury by activating NMDA receptors (Ankarcrona *et al.*, 1995). MEHI treated group showed reduced levels of glutamate when compared with the stroke induced group suggesting that MEHI may have antagonized the release of excess glutamate during ischemic injury.

5-HT is released into the extracellular space during cerebral ischemia (Damsma *et al.*, 1990) and the level of 5-HT extracellular increases with the duration and the severity of the ischemic insult (Richards *et al.*, 1993). It has been reported that 5-HT suppresses the release of glutamate and neurotoxic aminoacid, elicited by membrane depolarization, through the activation of 5-HT1 and 5-HT2 receptors (Maura *et al.*, 1988). We observed a reduction in the level of 5-HT in the stroke induced animals which was reversed in the MEHI treated groups. Hence the increased level of 5-HT could have caused the reduction of glutamate in the extract treated groups.

Monoamine oxidase-B is an important enzyme which catalyses the metabolism of dopamine, noradrenaline and 5-hydroxytryptamine. Increased levels of MAO during dementia have been reported (Zubenko *et al.*, 1987). Increased levels of MAO-B and decreased levels of dopamine and serotonin were observed in the negative control group confirming this process. MEHI treated group exhibited a decrease in the level of MAO-B which may be compared with the increased levels of dopamine and 5-HT in the extract treated groups (Maia *et al.*, 2004). An potent MAO-B inhibitor and other MAO inhibitors (Matsui and Kumagae, 1991) has shown to be protective against cerebral ischemia in rats.

The cell membrane is made up of phospholipids that are composed of polyunsaturated fatty acids, prime targets of ROS. The presence of a double bond adjacent to a methylene group makes the methylene C-H bond of polyunsaturated fatty acid weaker and therefore, the hydrogen becomes more prone to abstraction. Production of free radicals such as hydroxyl radical (^oOH), alkoxy radicals (RO-^o) and peroxy radicals (ROO-^o) results in initiation of the lipid peroxidation sequence (Zhu *et al.*, 2004).

The high metabolic rate of the brain, the low concentration of antioxidant enzymes and the high proportion of polyunsaturated fatty acids, makes the brain a tissue particularly susceptible to oxidative damage. Super Oxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Catalase are three main enzymes involved in cellular protection against damage due to oxygen-derived free radicals. The position that GSH takes part in the redox-sensitive signaling cascade has been receiving growing support. One of the most important GSH-dependent detoxifying processes involved is Glutathione Peroxidase (GPx) which plays a central role in the removal of hydrogen and organic peroxides and leads to the formation of oxidized glutathione (GSSG). GSSG is reduced back to its thiols form (GSH) by the ancillary enzyme glutathione reductase (GRD), leading to the consumption of NADPH which is mainly produced in the pentose phosphate pathway. GSH also takes part in xenobiotic conjugation with the assistance of several glutathione S-transferase isoenzymes. GSH conjugates or GSSG can be eliminated from the cell by the family of ATP dependent transporter pumps (D'Almeida *et al.*, 1996).

The results of biochemical parameters show that 4VO causes ischemia-reperfusion injury. The observed decrease in the lipid peroxide level and proportionate increase in the SOD, CAT, GPx and GR levels in the extract treated groups shows that *H. indicus* 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 *H. indicus* in various conditions of oxidative stress (Ravishankara *et al.*, 2002; Sultana *et al.*, 2003).

CONCLUSION

In present study cerebral ischemia was induced by Four Vessel Occlusion (4VO) method. In this model the major arteries supplying blood to the brain namely vertebral arteries and carotid arteries were occluded to reduce the blood supply to the brain, thereby leading to ischemia.

The results of this study confirmed that MEHI protects rats from ischemia induced brain injury. This protection was evident from, the significant improvement in neuromuscular, vestibulomotor, complex neuromotor and *in-vivo* behavioral tests and decrease in the elevated levels of malondialdehyde (lipid peroxidation) and increased the levels of SOD, CAT GPx, GR and decreased the elevated levels of acetylcholine esterase, glutamate, MAO-B and reversal of decreased dopamine and serotonin levels.

ACKNOWLEDGMENTS

The authors are grateful to Dr. S. Venkataraman (Director of C.L.BAID METHA Foundation for Pharmaceutical Education and Research, Chennai) for his technical and secretarial assistance. The authors have no conflict of interest to report.

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