Original Research Article

Evaluation of Stroke Preventive Activity of *Hugonia mystax* Extract in Rats

Abdul Mukit Barbhuiya, S. Sindhura, N. Chenna Keshavulu
Department of Pharmacology, Anurag Pharmacy College, Ananthagiri, Nalgonda, T.S- 508206, India

*Corresponding Author:
Abdul Mukit Barbhuiya
Email: abdulmukit13@gmail.com

Abstract: A stroke is the rapid loss of brain functions due to disturbance in the blood supply to the brain. In the last few years a great deal of research has been carried out to find the effectiveness of herbal drug for the treatment of stroke. Both thrombolytic and neuro-protective properties of herbal drugs may be a novel strategy for effective stroke therapeutics. The animals were divided into five groups of six each. Except the normal and control groups, all other test groups received different doses of aqueous extract of *Hugonia mystax* orally for 45 days as per the treatment schedule.Animals were observed for behavioral parameters after 24 hours of surgery in three different areas, neuromuscular function, vestibule motor function, and complex neuromotor function which consists of various subtests such as forelimb flexion, twisting, resistance to lateral push, circling, hind limb placement, forelimb placement, pinna reflexes and corneal reflexes.The remaining animals in each group were also sacrificed after 72 hours post-surgery by cervical decapitation. The brains were excised and used for the estimation of Lipid peroxidation, Superoxide Dismutase and Catalase.*Hugonia mystax,* a rich source of antioxidant consists of dietary fibers, flavonoids, proteins, vitamins like vitamin B_1 and minerals like iron, copper, magnesium and potassium. It is also an excellent source of molybdenum and folate. The present study results support that *Hugonia mystax* has dose dependent stroke preventive activity in common carotid artery occlusion along with ferric chloride induced thrombosis model.
Keywords: stroke, ischemia, *Hugonia mystax,* brain homogenate, plant extract.

INTRODUCTION

Herbal drugs are defined as a plant or plant part or an extract or mixture of these used in herbal medicine.The pharmacological treatment of disease began long ago with the use of herbs [1]. During recent years herbal medicine has become an increasingly scientifically based system of healing. Due to demands from both the public and medical establishments, studies leading to the scientific explanation of plant therapeutic capabilities are allowing this practice to gain increasing credibility and acceptance within the medical community.

Herbal drugs have been used since ancient times, gained lot of acceptance in the recent years because of their low cost, high therapeutic window, more efficacy and less side effects. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants. Screening active compounds from plants has led to the invention of new medicinal drugs which have efficient protection and treatment roles against various diseases [2].

Rapid changes in lifestyles have occurred with industrialization, urbanization, economic development and market globalization over the past decade have increased the growing epidemic of chronic non communicable diseases including obesity, diabetes mellitus, cardiovascular disease(CVD), hypertension, stroke and some types of cancer which are significant causes of disability and pre mature death in developing and newly developing countries, placing additional burdens on already overtaxed national health budget [3].

Chronic diseases are the leading causes of death worldwide. Disease rates from these conditions are accelerating globally and pervading all socioeconomic classes. Four of the most prominent chronic diseases cardiovascular diseases (CVD), cancer, chronic obstructive pulmonary disease and type 2-diabetes are linked by common and preventable biological risk factors, notably high blood pressure, high blood cholesterol and overweight, and by related major behavioral risk factors: unhealthy diet, physical inactivity and tobacco use. The World Health Report 2002: Reducing risks, promoting healthy life, indicates that the mortality, morbidity and disability attributed to the major chronic diseases currently account for almost 60% of all deaths and 43% of the global burden of disease. By 2020 their contribution is expected to rise to 73% of all deaths and 60% of the global burden of disease. Moreover, 79% of the deaths attributed to these diseases occur in the developing countries. The 1992 international conference on nutrition specifically identified the need to prevent and control the need to

prevent and control the increasing public health problems of chronic diseases by promoting appropriate diet and healthy lifestyles [4].

A stroke (sometimes called cerebrovascular accident), is the rapid loss of brain functions due to disturbance in the blood supply to the brain. There are two kinds of stroke i.e. ischemic and hemorrhagic stroke. Brain damage in stroke results from toxic reaction like free radical generation secondary to the initial death of brain cells. Though medication is available for stroke no single drug treats stroke permanently. Medication combined with alternative and complementary therapies is more beneficial. The most useful alternative therapies include herbal medicine, diet, exercise, massage, hydrotherapy, yoga, homeopathy, music therapy, aroma therapy, remedies, vision light therapy, naturopathic medicine,sound therapy etc. [5].

In the last few years a great deal of research has been carried out to find the effectiveness of herbals in stroke. Both thrombolytic and neuro-protective properties of herbal drugs may be a novel strategy for effective stroke therapeutics. The system of medicine, especially Ayurveda has several medicinal plants with proven beneficial claims towards these pathological conditions [6].

The genus *Hugonia* L., of family Linaceae comprise about 40 species in the world, of which two species namely *Hugonia mystax* L.,(which is known as Kaaki Berra in Telugu) and *H. ferruginea* Wight & Arn., were reported from India. The plant *Hugonia mystax* is a woody evergreen, distributed throughout India in dry topical forest.The plant *Hugonia mystax* aerial parts used as herbal remedies for diabetes androots were used as antihelmentic, astringent and inflammation [7].

Keeping this in view, the present study is aimed to evaluate anti-oxidant and anti-inflammatory activity of *Hugonia mystax* for the potential against cerebral stroke in rats by measuring behavioral parameters.

MATERIALS AND METHODS
Collection of Plant material
The whole plant of *Hugonia mystax* was collected in the month of November- December, 2012 from Chittur Dist. The plant material was authentified by Prof. Madhava shetty, Department of botany, taxonomist, SV University. A voucher specimen was kept in department for reference.

Preparation of extract
The whole plant was dried in shade at room temperature then subjected to size reduction to a fine powder with the help of electric grinder. The grinded plant material was subjected to Soxhlet extraction (45^0-55^0C) employing 95% methanol as solvent. Appearance of colorless solvent in the siphon tube was taken as the termination of extraction. The extract was finally air dried thoroughly to remove all traces of the solvent and the percentage yield was calculated.

Experimental Animals
Albino rats of either sex weighing 150-250 g weight were used in experiment. Animals were obtained from Anurag Pharmacy College, Kodad. Animals were kept under standard conditions at 25 ±20C 12 hour light/dark cycles and given standard pellet diet and water. The animals were accustomed to the laboratory conditions for a week prior to the experimentation. Before using in experiment animals got clearance from IAEC, Anurag Pharmacy College, Kodad (CPCSEA Registration No. 1712/PO/a/13/CPCSEA)

Acute toxicity study
Albino rats (n =6) of either sex selected by random sampling technique were used for the study. The animals were kept fasting for overnight providing only water, after which the plant extract of *Hugonia mystax* were administered orally at the dose level of 2g/kg body weight by oral feeding needle and daily cage side observations included changes in skin, fur, eyes, mucous membrane was observed for 14 days.

Stroke activity
The animals were divided into five groups of six each. Except the normal and control groups, all other test groups received different doses of aqueous extract of *Hugonia mystax* orally for 45 days as per the treatment schedule mentioned in the Table 3.

Induction of stroke
After 45 days of preventive treatment, stroke was induced by the combination of global and focal models i.e. common carotid artery occlusion (CCAO) along with ferric chloride ($Fecl_3$) induced thrombosis. The rats were anesthetized with intraperitoneal injection of ketamine (80-90 mg/kg i.m.) and xylazine (5-10 mg/kg i.m.). Then the skin on the upper central neck area was directly incised in order to expose left common carotid artery which was occluded by ligating with nylon sutures. The filter paper (1×1mm) saturated with 25% $Fecl_3$ was applied proximal to the surface of carotid artery for 15 minutes. Reperfusion was done after 30 min of common carotid artery ligation. The animals were sutured and allowed to recover by housing them in individual cages.

Table 1: Treatment Schedule for Stroke

S.No	Group	Treatment	Purpose
I	Normal	Distilled water	To assess the normal behavioral and antioxidant parameters
II	Control	Distilled water + stroke was induced after 45 days	To asses behavioral and antioxidant parameters in stroke conditions
III	Test I	Aqueous extract of *Hugonia mystax* (250mg/kg p.o)+stroke was induced after 45 days	To evaluate low dose potential of *Hugonia mystax* against stroke.
IV	Test II	Aqueous extract of *Hugonia mystax*(500mg/kg body wt p.o)+ stroke was induced after 45 days	To evaluate moderate dose potential of*Hugonia mystax* against stroke.

48 hours after surgery, neurological functions in all animals were assessed and given scores as per the table 2, 3& 4.

Table 2: Clinical scoring criteria of behavioral parameters

Neuromuscular function Criteria	Score	Description
Forelimb flexion	0.0 0.5 1.0	No flexion Mild Moderate to severe
Torso twisting	0.0 0.5 1.0	No signs Mild Moderate to severe
Lateral push	0.0 0.5 1.0	Equal resistance Weaken resistance No resistance
Hind limb placement	0.0 0.5 1.0	Immediately replaces Delay in replacing No replacement
Forelimb placement	0.0 0.5 1.0	Immediately replaces Delay in replacing No replacement
Mobility	0.0 0.5 1.0 2.0	Normal Spontaneous movement reduced Needs stimulus to move Unable to walk

Maximum score: 9.0

Table 3: Clinical Scoring Criteria

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

Maximum score: 6.0

Table 4:Clinical scoring criteria of behavioral parameters

Complex neuro 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

Animals were observed for behavioral parameters after 24 hours of surgery in three different areas, neuromuscular function, vestibule motor function, and complex neuromotor function which consists of various subtests such as forelimb flexion, twisting, resistance to lateral push, circling, hind limb placement, forelimb placement, pinna reflexes and corneal reflexes. A combined total was taken for each rat with a higher score meaning a higher clinical deficit Procedure for each subtest is described below [8].

Neuromuscular Function includes
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. In our previous work it was found that animals without flexion did not have a detectable lesion histologically, therefore, those without flexion were dropped from the experiment [9].

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 infraction 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 hind limbs. Twisting was always toward the paralytic side [9].

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 contralateralside [9].

Circling
Rats normally do not circle during normal state. Animals having infarction sometimes circle toward the contra lateral side [9, 10].

Hind limb Placement
A normal rat will immediately replace a hind limb 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 [9].

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 [11].

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 [12].

Pinna reflexes and **corneal reflexes**of animal were also observed.

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 in order to identify the area of ischemia 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 [16].

In vivo brain antioxidant and pro oxidant enzymes
The remaining animals in each group were also sacrificed after 72 h post-surgery by cervical decapitation. The brains were excised and used for preparation of brain homogenate as follows

Reagents
1. 0.025 M sucrose solution: 85.87 gm of sucrose was dissolved in 1000ml of distilled water.
2. 10mM buffer solution: 1.2 gm of tris was dissolved in 900 ml of distilled water, pH was adjusted to 7.4 with 1M Hcl and dilute up to 1000ml.

Procedure
Brain was separated and kept in cold condition, were cross chopped with surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted with filter paper. The tissue was minced and homogenized in ice cold 10 mM tris HCL buffer (to pH 7.4) at a concentration of 10% (w/v) with 25 strokes of tight Teflon pestle of glass homogenizer at a speed of 2500 rpm. The prolonged homogenization under hypotonic condition was designed to disrupt as far as possible the ventricular structure of cells so as to release soluble protein and leave only membrane and non-vascular matter in a sedimentable form. It was then centrifuged in cooling centrifuge at 5000 rpm for 20ºC, temperature was maintained at -4 ºC during the centrifugation, and clear supernant was separated and used to estimate SOD, Catalase, and Lipid peroxidation [17].

SUPER OXIDE DISMUTASE (SOD)
SOD was estimated by the method of Misra and Fridovich [13]

Principle
Rate of auto oxidation of epinephrine and the sensitivity of this auto oxidation to inhibition by SOD

were augmented as pH was raised from 7.8 – 10.2, O_2 generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced, the auto oxidation of epinephrine proceeds by at least two distince pathways only one of which is free radical chain reaction involving O_2 and hence inhabitable by SOD.

Reagents
1. Carbonate buffer (0.05 M, pH 10.2): 16.8 gm of sodium bicarbonate and 22 gm of sodium carbonate was dissolved in 500 ml of distilled water and the final volume was made up to with distilled water.
2. Ethylene diaminetetra acetic acid (EDTA) (0.49 M): 1.82 gm of EDTA was dissolved in 1000ml of distilled water.
3. Epinephrine (3 mM): 9.9 mg of epinephrine bitartarate was dissolved in 10 ml of 1M HCL solution.
4. SOD standard: Dissolve 1 mg (1000 units/mg) of SOD from bovine brain in 100 ml of carbonate buffer.

Procedure
0.5 ml of sample was diluted with 0.5 ml of distilled water, to this 0.25 ml ethanol, 0.5 ml of chloroform (all reagents chilled) was added, the mixture was shaken for one minute and centrifuged at 2000 rpm for 20 minute.

The enzymatic activity in supernatant was determined. To 0.05 ml of carbonate buffer (0.05 M, pH 10.2) and 0.5 ml of EDTA (0.49 M) was added. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in optical density/ min was measured at 480 nm. SOD activity was expressed as units / mg protein change in optical density/min. 50% inhibition of epinephrine to adrenochrome transition by

enzyme is taken the enzyme unit. Calibration curve was prepared by using 10 – 125 units of SOD.

Calculation

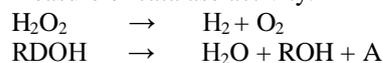$$SOD = \frac{(0.025 - Y)}{Y \times 50} \times 100$$

Where,

Y = Initial reading – Final reading

CATALASE
Catalase was estimated by Hugo E. Aebi method[14].

Principle
In UV range H_2O_2 can be followed directly by the decrease in absorbance (O.D 240) per unit time is a measure of catalase activity.

$H_2O_2 \quad \rightarrow \quad H_2 + O_2$
$RDOH \quad \rightarrow \quad H_2O + ROH + A$

Decomposition of H_2O_2 = Decrease in absorbance at 240 nm.

Reagents
1. Phosphate buffer: (50mM/1; pH 7.0)
   a. Dissolve 6.81 gm KH_2PO_4 in water and make up to 1000 ml.
   b. Dissolve 8.9 gm NaH_2PO_4 $2H_2O$ in water and make up to 1000 ml.
   Mix the solutions (a) and (b) in proportion 1:15 (v/v)
2. Hydrogen peroxide (30 mM/1): Dil 0.34 ml of 30 % hydrogen peroxide with phosphate buffer up to 100ml.

Procedure
Dilute homogenate 20 times with Phosphate buffer pH 7.0

Table 5: catalase procedure

Blank	Test
4 ml of homogenate diluted with 2 ml of phosphate buffer p^H 7, and take absorbance at 254 nm for 3 min. with 30 sec. interval	2 ml of homogenate diluted with 1 ml of H_2O_2 (8.5 micro lit. in 2.5 ml phosphate buffer (50mM/l. pH 7.0) and take the absorbance at 254 nm for 3 min. with 30 sec. interval.

Calculation

$$Catalase = Log\ (A\ /\ B) \times 2297.3$$

Where,
A: Initial absorbance
B: final absorbance (after 30 second)

EVALUATION OF PRO-OXIDANT
Lipid peroxidation (Malondialdehyde formation) was estimated by Slater and Sawyer method [15].

Reagents
Thiobarbituric acid (0.67%) in 1M tris hydrochloride, pH 7, 0.67gm of thiobarbituric acid was dissolved in 100 ml of distilled water.

Trichloroacetic acid (20%): 20 gm of trichloroacetic acid was dissolved in 100ml of distilled water.

Procedure
2 ml of sample was mixed with 2 ml of 20% Trichloroacetic acid and kept in ice for 15 min. The

precipitate was separated by centrifugation and 2 ml of samples of clear supernatant solution were mixed with 2 ml of aqueous 0.67 thiobarbituric acid. This mixture was then heated with on a boiling water bath for 10 minute. It was cooled in ice for 5 minutes and absorbance was read at 535 nm. The values expressed as nm of MDA formed/mg of protein. Values are normalized to protein content of tissue.

Calculation

$$X = \frac{(Y + 0.002)}{0.0026086}$$

Y – Absorbance differences of final (after 3 min) and initial reading of test sample.

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 Dennett's test using computer based fitting program (Graph pad prism.5). Statistical significance was determined accordingly.

RESULTS
Acute toxicity

Acute oral toxicity studies of methanloic extract of *Hugonia mystax* were carried out according to OECD-423 guidelines in wistar female rats and starting at a dose of 2000 mg/kg, p.o. exhibited normal behaviour, without any signs of passivity, stereotypy and vocalization. Their motor activity and secretary signs were also normal and no sign of depression. *Hugonia mystax* even showed no toxicity though repeated. LD 50 cut off value was obtained from the Flow Chart of OECD-423 and dose for Administration were taken as 1/20th and 1/10th of LD$_{50}$ as high and low dose of *Hugonia mystax*.

I*n vivo* antioxidant parameters

Various antioxidant parameters were assessed in the brain at the end of the study.

Effect on Super Oxide Dismutase (SOD)

The first line of defense that the body has against superoxide free radicals is the enzyme known as "Superoxide Dismutase. A significant decrease in the levels of SOD was observed in the control group (G-II), when compared to the normal group (G-I) ($p<0.001$). The group-III receiving low dose (250mg/kg) of *Hugonia mystax* extract exhibited significant increase in the SOD levels, when compared to the control group (G-II) ($p<0.01$).The groups-IV and V treated with different doses (500 mg/kg) of methanolic extract of *Hugonia mystax* also exhibited a significant increase in the SOD levels, when compared to the control group (G-II) ($p<0.001$) (Fig.1).

Effect on Catalase

Control group treated with vehicle alone showed a significant decrease in catalase value when compared to normal group (G-I) upon induction of cerebral stroke ($p<0.05$). The group-III treated with low dose (250 mg/kg) of aqueous extract of *Hugonia mystax extract* had significant increase in the catalase levels, when compared to the control group (G-II) ($p<0.05$).The groups-IV with dose(500mg/kg) of *Hugonia mystax* aqueous extract exhibited a significant increase in the catalase levels, when compared to the control group (G-II) ($p<0.01$).The group V receiving high dose (500mg/kg) of methanolic extract of *Hugonia mystax* showed significant increase in catalase levels, when compared to control group (G-II) ($p<0.001$)(Fig.2).

Acute oral toxicity studies

Table 6: Acute oral toxicity results of *Hugonia mystax* (OECD 423 guideline)

Day	Animals	*Hugonia mystax*	Weight of the body(gm.)		Sign of toxicity
			Before treatment	**After treatment**	
0 to 7th day	1	2g/kg	180g	180g	Nil
	2	2g/kg	150g	150g	Nil
	3	2g/kg	160g	160g	Nil
7th to 14th day	1	2g/kg	160g	160g	Nil
	2	2g/kg	180g	180g	Nil
	3	2g/kg	160g	160g	Nil

Behavioral parameters

The behavioral parameters were assessed after 48 h of inducing stroke. The increase in score indicates increase in neurological deficits (Table 2, 3&4).

Control group treated with vehicle alone showed significant increase in forelimb flexion and torso twisting which indicate neurological deficit upon stroke induction, when compared to normal group (G-I) ($p<0.001$). The group-III receiving low dose (250mg/kg) of methanolic extract of *Hugonia mystax* showed decrease in the score but non-significant when compared to the stroke control group (G-II).

The group-IV treated with dose (500mg/kg) of methanolic extract of *Hugonia mystax* exhibited a

significant decrease in the forelimb flexion and torso twisting when compared to the control group (G-II) (p<0.01 and p<0.001) respectively.

A significant increase in score of lateral push was seen in the stroke control group (G-II) when compared to normal group (G-I) (p<0.01). The group-III receiving low dose (250mg/kg) of methanolic extract of *Hugonia mystax* of had shown decrease in the score but non-significant, when compared to the stroke control group (G-II).Reduction in neurological deficit was observed in group-IV treated with dose (500 mg/kg) of aqueous extract of *Hugonia mystax* which was indicated by significant decrease in the lateral push score, when compared to the control group (G-II) (p<0.05 and p<0.01) respectively.

Score of mobility had been significantly increased in the stroke control group (G-II) when compared to normal group (G-I) (p<0.001). No significant decrease in the score was seen in group-III. The group-IV exhibited a significant decrease in the mobility score, when compared to the control group (G-II) (p<0.05 and p<0.001) respectively.

Control group showed significant increase in scores of beam walk and beam balance when compared to normal group (G-I) (p<0.05 and p<0.001) respectively which indicate deficit in vestibular function caused by cerebral stroke. The group-III receiving low dose (250mg/kg) of methanolic extract of *Hugonia mystax* showed decrease in the score but non-significant. The group-IV treated with dose (500mg/kg) of methanolic extract of *Hugonia mystax* exhibited a significant decrease in the beam walk score and beam balance, when compared to the control group (G-II) (p<0.05 and p<0.001) respectively which indicate improvement in neurological deficits.

A significant increase in scores of hind limb and forelimb placement were seen in the stroke control group (G-II) when compared to normal group (G-I) (p<0.01 and p<0.05) respectively. The group-III receiving low dose (250mg/kg) of methanolic extract of *Hugonia mystax* showed decrease in the score of placements but non-significant which indicate the inefficacy of dose to prevent stroke. The groups-IV and V treated with different doses (500 mg/kg)of methanolic extract of *Hugonia mystax* exhibited a significant decrease in the hindlimb placement score and forelimb placement score, when compared to the control group (G-II) (p<0.01 and p<0.05) respectively.

The pinna pain reflexes and corneal reflexes were observed along with above parameters. There was partial loss of pinna pain reflex in control group when compared to other groups. The corneal reflex was normal in all the groups.

Table 7: Effect of different doses of methanolic extract of *Hugonia mystax* on behavioral parameters in cerebral stroke

S.No	Group	Treatment	Parameter		
			Forelimb Flexion	Torso Twisting	Lateral Push
I.	Normal	Distilled water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
II.	Control	Distill water+stroke was induced after 45 days	$0.91\pm0.08^{\#\#\#}$	$0.91\pm0.08^{\#\#\#}$	$0.875\pm0.125^{\#\#}$
III.	Test 1 (low dose)	Aqueous extract of *Hugonia mystax*(250mg/kg body wt)+stroke was induced after 45 days	$0.58\pm0.08^{*}$	$0.416\pm0.15^{**}$	$0.25\pm0.144^{*}$
IV.	Test 2 (high dose)	Aqueous extract of *Hugonia mystax*(500mg/kg body wt.)+stroke was induced after 45 days	$0.08\pm0.08^{***}$	$0.08\pm0.08^{***}$	$0.25\pm0.25^{*}$

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 controlgroup

Table 8: Effect of different doses of methanolic extract of *Hugonia mystax*on behavioral parameters in cerebral stroke

S.No	Group	Treatment	Parameter		
			Mobility	Forelimb Placement	Hindlimb Placement
I.	Normal	Distilled water	0.0±0.0	0.0±0.0	0.0±0.0
II.	Control	Distill water+stroke was induced after 45 days	$1.08\pm0.20^{###}$	$0.91\pm0.08^{###}$	$0.375\pm0.125^{##}$
III.	Test 1 (low dose)	methanolic extract of *Hugonia mystax* (250mg/kg body wt)+stroke was induced after 45 days	$0.50\pm0.18^{*}$	$0.58\pm0.08^{*}$	0.25±0.144
IV.	Test 2 (high dose)	methanolic extract of *Hugonia mystax* (500 mg/kg body wt)+ stroke was induced after 45 days	$0.25\pm0.17^{**}$	$0.08\pm0.08^{***}$	0.125±0.125*

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 controlgroup

Table 9: Effect of different doses of methanolic extract of *Hugonia mystax*on behavioral parameters in cerebral stroke

S.No	Group	Treatment	Parameter	
			Beam Walk	Beam Balance
I.	Normal	Distilled water	0.0±0.0	0.0±0.0
II.	Control	Distill water+stroke was induced after 45 days	$1.66\pm0.33^{###}$	$1.08\pm0.20^{###}$
III.	Test 1 (low dose)	Methanolic extract of *Hugoniamystax*(250mg/kg body wt)+stroke was induced after 45 days	$0.66\pm0.33^{*}$	0.33±0.21**
IV.	Test 2 (high dose)	Methanolic extract of *Hugonia mystax*(500 mg/kg body wt)+ stroke was induced after 45 days	$0.33\pm0.21^{**}$	0.16±0.16**

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 controlgroup

Table 10:Effect of *Hugonia mystax m*ethanolic extract on oxidative stress parameter

Group	Lipid peroxidation MMDA/gHb	Superoxide Dismutase Units/mg protein	Catalase Units/mg Protein
Normal	2.73±0.371	3.00±0.28	79.0±3.78
Control	$6.63\pm0.318^{###}$	$0.90\pm0.11^{###}$	$45.00\pm1.7^{##}$
Standard	$78.00\pm1.528^{***}$	35.00±0.577	$131.3\pm1.856^{***}$
Low dose	$4.13\pm4.932^{***}$	$2.00\pm0.057^{*}$	$103.00\pm3.51^{***}$
High dose	$1.34\pm2.592^{***}$	$4.630\pm0.296^{***}$	$166.3\pm6.836^{***}$

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 controlgroup

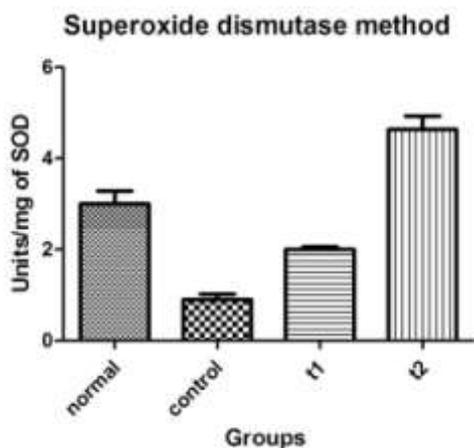**Fig.1: Representing Superoxide dismutase method of *Hugonia mystax***
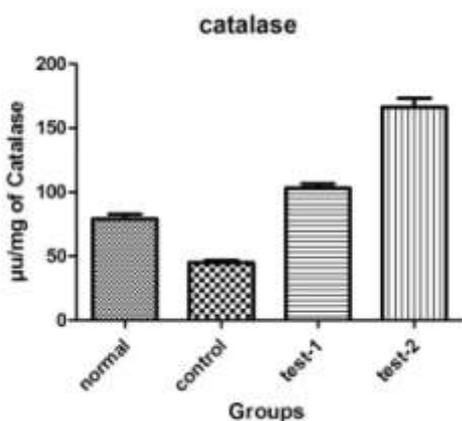

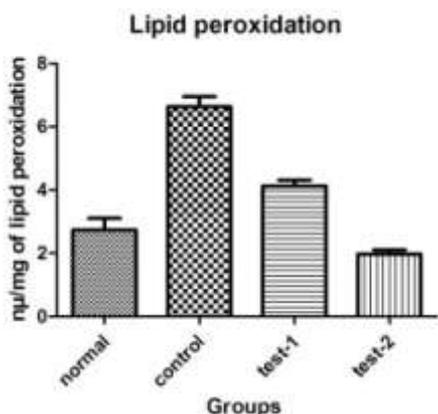**Fig. 2: Representing Catalase method of *Hugonia mystax***

Fig. 3: Representing lipid peroxide method of *Hugonia mystax*

DISCUSSION

Stroke 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. 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 [18].

Therapies to prevent a first or recurrent stroke are based on treating an individual's underlying risk factors for stroke, such as hypertension, atrial fibrillation, and diabetes, or preventing the widespread formation of blood clots that can cause ischemic stroke in everyone, whether or not risk factors are present. Preventive therapy includes diet, changes in life styles, anticoagulation therapy, herbal therapy, yoga, music therapy etc. among which diet is most accepted option, because 5,50,000 strokes, new or recurrent that occur each year are preventable using basic preventive measures which could be easily incorporated into dietary health management with proper stroke prevention education [19].

Hugonia mystax, a rich source of antioxidant consists of dietary fibers, flavonoids, proteins, vitamins like vitamin B_1 and minerals like iron, copper, magnesium and potassium. It is also an excellent source of molybdenum and folate [20].

Rodents are more suitable species for work as they are similar enough anatomically and physiologically to higher species, low cost and high availability. In stroke they are preferred due to their small brain size which allows them for easy fixation process thus making them more acceptable from both ecological and ethical prospective.

In vivo experimental models to induce stroke are either global or focal models, each of them have their own advantages and disadvantages. In order to overcome the disadvantages, in the present study, we had combined both global and focal models i.e. Common carotid artery occlusion along with 25% $Fecl_3$ induced thrombosis [21].

Common carotid artery occlusion (CCAo) is a widely accepted model in gerbils because of simple surgical procedure and absence of significant posterior communicating arteries. Even in rodents it causes delayed white matter lesions and cognitive impairment [22].

Recirculation affects cerebral ischemia and modifies post ischemic events in various ways. Recirculation occurs frequently after spontaneous thrombolysis and break-up of cerebral emboli in a common clinical event. It is difficult to produce reliable recirculation models using the focal models alone because after recirculation cerebral blood flow (CBF) often varies regionally due to spasm or direct

mechanical damage to the occluded vascular wall from clipping or ligation [23].

Topical application of Fecl$_3$ solution is a simple, much used method of inducing localized arterial thrombi formation. Because of its rich platelet content, early studies attributed the exposed subendothelial surfaces and the presence of highly reactive oxygen species account for the thrombus formation. On the side exposed to Fecl$_3$ application a preponderance of electron opaque particles formed in the myocytes, the internal elastic membrane before these diffused particles became concentrated in the vesicle facing the vessel lumen demonstrated that tissue factor elaborated from the arterial wall is a major contributor of the mural thrombus. Tissue factor (TF) primarily expressed by the adventitial cells does not normally enter into the vascular lumen [24].

As shown in our study, topical application caused severe regional injury to the vascular wall including endothelial denudation while leaving a substantial portion of the arterial rim to be structurally intact .During preliminary studies in our lab neither CCAo nor Fecl$_3$ produced cerebral stroke effectively individually. Hence, we attempted to combine both to induce cerebral stroke in experimental animals. Significant cerebral stroke was induced which was supported by staining of brain sections.

Staining of brain sections shown reduced infract size in animals treated with *Hugonia mystax*. Colorless region corresponds to occluded territory. Reperfusion and duration of occlusion influence the severity of ischemia. This decrease in infract area may be due to anti-inflammatory and antithrombogenic properties of flavonoids present in the *Hugonia mystax*. Flavonoids reduce complement activation, thereby decreasing the adhesion of inflammatory cells to the endothelium and in general resulting in a diminished inflammatory response. Another interesting effect of flavonoids on enzyme systems is the inhibition of the metabolism of arachidonic acid [25, 26].

Oxidative stress occurs due to an imbalance between pro-oxidants and antioxidants and consequent excessive production of reactive oxygen species. Reactive oxygen species are biphasic, playing a role in normal physiological processes and are also implicated in a number of disease processes, whereby they mediate damage to cell structures, including lipids, membranes, proteins, and DNA. The cerebral vasculature is a major target of oxidative stress playing a critical role in the pathogenesis of ischaemic brain injury following a cerebrovascular attack. Superoxide, the primary reactive oxygen species, and its derivatives have been shown to cause vasodilatation via the opening of potassium channels and altered vascular reactivity, breakdown of the blood-brain barrier and focal destructive lesions in animal models of ischaemic stroke. However, reactive oxygen species are involved in normal physiological processes including cell signaling, induction of mitogenesis, and immune defense [27, 28].

Antioxidants are substances that protect other chemicals of the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body, hence hindering the process of oxidation. The body has developed several endogenous antioxidant systems to deal with the production of ROS which are divided into enzymatic and non-enzymatic groups. The enzymatic antioxidants include super oxide dismutase, catalase and. The non-enzymatic antioxidants include the lipid-soluble vitamins, vitamin E and vitamin A or provitamin A (beta-carotene), and the water-soluble vitamin C and GSH [29].

The first line of defense that the body has against superoxide free radicals is the enzyme known as "Superoxide Dismutase" or (SOD), which is considered the most effective antioxidant. It eliminates ROS by reducing (adding an electron to) superoxide to form H$_2$O$_2$. It also exhibits its antioxidant activity by reducing O$_2$ that would otherwise lead to the reduction of Fe^{3+} to Fe^{2+} and thereby promote OH$^-$ formation. In brief, SOD keeps oxygen under control. Catalase are responsible for reducing H$_2$O$_2$ to H$_2$O. These enzymes require trace metal cofactors for maximal efficiency i.e. selenium for copper, zinc, or manganese for SOD; and iron for catalase [30].

The respective enzymes that interact with superoxide and H$_2$O$_2$ are tightly regulated through a feedback system which maintains steady low levels of SOD and catalase, as well as superoxide and H$_2$O$_2$ and keeps the entire system in a fully functioning state [13].

In the present study, the stroke control group animals showed decreased amount of defense enzymes and increased amount of lipid peroxidation in brain because of induction of ischemia whereas the group of animals that were treated with aqueous extract of *Hugonia mystax* had shown increased levels of SOD, catalase enzymes and decreased lipid peroxidation. This may be due to the antioxidant principles present in it such as manganese, copper that increases the activity of SOD and iron which increases the catalase activity according to earlier reports. All these enhanced levels of SOD, catalase offered protection and decreased lipid peroxidation in treated groups. Further studies on exact mechanism will be fruitful.

Flavonoids present in *Hugonia mystax* prevent injury caused by free radicals in various ways. One way is the direct scavenging of free radical effects and interaction with enzymes. In direct scavenging, flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with

the reactive compound of the radical because of the high reactivity of the hydroxyl group of flavonoids, radicals are made inactive and can also scavenge the highly reactive oxygen derived radical called peroxynitrite as shown below [31].

Flavonoid (OH) + R• > flavonoid (O•) + RH
Where R• is a free radical and O• is an oxygen free radical.

Potent scavenging of DPPH and reducing power in the present study by *Hugonia mystax* might be due to presence of flavonoids.

Hence, the present study results support that regular consumption of *Hugonia mystax* may not treat cerebral stroke but will definitely decrease the risk of cerebral stroke and prevents morbidity of cerebral stroke which can be attributed to the strong antioxidant activity of *Hugonia mystax*. Further studies to isolate active constituents and establish mechanism of action of *Hugonia mystax*will be fruitful.

CONCLUSION

The present study results support that *Hugonia mystax* has dose dependent anti stroke activity in common carotid artery occlusion along with ferric chloride induced thrombosis model. Further studies have to be carried out to isolate active constituents and establish mechanism of action of *Hugonia mystax*.

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