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EFFECT OF FRACTIONS OF *TAXILLUS HEYN EANUS* EXTRACT ON ISCHEMIA-REPERFUSION INDUCED CEREBRAL INJURY IN RATS

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ABSTRACT

The purpose of work is to investigate the effect of ethanol (ETH) and chloroform (CTH) fraction of *Taxillus heyneanus* whole plant extract against cerebral ischemia reperfusion injury in rats**.** For this, animals were pretreated for 30 days with ETH and CTH (200 & 400 mg/kg, p.o) and ischemia was induced by occluding both common carotid arteries for 30 mins followed by 45 mins reperfusion (RF) along with ferric chloride induced thrombosis (FIT). By the end of post treatment for a week, various parameters such as behavioral, neuro-biochemical and histological changes were assessed. The combination of global(BCCAO) and focal (FIT) ischemic model along with reperfusion (RF) has induced prominent ischemic insult indicated by depletion of antioxidant enzymes and elevation of glutamate & lipid peroxidation levels. Pre-treatment with ETH significantly improved the levels of antioxidant enzymes (SOD, GPx, GSH, GST, GR and CAT), total protein levels and decreased the lipid peroxidation, glutamate excitotoxicity and neuronal death. The results established neuro protective role of ETH in cerebrovascular insufficiency states and cerebral ischemic-reperfusion injury which may attribute to presence of polyphenol compound and tannin in it.

Keywords: BCCAO, Reperfusion, *Taxillus heyneanus*, Thrombosis.

INTRODUCTION

Human brain, being command center controls and co-ordinates the body actions. For this, it requires adequate amount of nutrients and oxygen which is being supplied by network of blood vessels [1]. When a cerebral blood vessel that supplies blood, nutrients and oxygen to the brain occluded by thrombosis (clot) results in ischemic stroke. It is the significant source of disability and mortality all over the world. Hemorrhagic stroke (13%) occurs less frequently compared to ischemic stroke. As per 2007 health statistics, it is estimated that stroke occurs every 40 s and results in death every 4 min [2].

Cerebral ischemia confined to a specific region (Focal ischemia) or wide regions (Global ischemia) of the brain occurs due to transient or permanent occlusion of cerebral artery that leads to irreversible damage of cells with in the affected area along with permanent or reversible neurological deficit. Disability following a stroke depends on the part of the brain affected, the extent of damage, the blood vessel occluded and how fast the treatment is given to patients[3].The survivors of stroke require rehabilitation to relearn the skills that are compromised due to ischemic damage. The cost burden for stroke in 2010 was estimated as seventy four billion dollars and may extend to two trillion dollars in 2050 [4].

Ischemic cascade involves various cellular and biochemical changes which are interlinked to each other such as hypoxia induced altered metabolic homeostasis, mitochondrial dysfunction, energy failure, free radical generation, excitotoxicity, elevated cytosolic calcium, activation of various enzymes such as lipases, proteases, kinases, phosphatases, endonucleases which leads to brain edema and apoptosis[5,6]

After understanding the mechanism of ischemic cascade, large number of therapeutic approaches such as NMDA antagonists, antioxidants, thrombolytics and calcium channel blockers has been evaluated in search of ideal drug for stroke. But surprisingly, till now except rtPA within 3 hours of attack, none has shown good improvement in stroke patients and regarded as gold standard [7].This situation necessitate the exhaustive research in the field of drug discovery to develop a promising drug that targets different stages in the ischemic cascade with minimal side effects on long term use [8]. Herbal drugs in ischemic patients exhibited several neuro

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regulative actions such as anti-oxidative, calcium antagonist, anti-inflammatory and anti-apoptotic properties [9].

Taxillus heyneanus Danser from Loranthaceae, a parasitic herb commonly called as badanika was selected for the present study. It grows on shrubs and small trees of dry deciduous forests. Branches are densely covered with white shiny abietiform and stellate hairs. Tawny pubescent branchlets and nodes with prominently ribbed leaf scars are present. Leaves are alternate ovate leaves with attenuate base and crustaceous hairs on both surfaces are seen. Hairy petiole and simple sessile axillary inflorescence. Bracts are obovate to oblanceolate and foliaceous. Petals (5) are united to form a ventricose tube with a median split. Stamens are 5 in number. Oblong anther, turbinate ovary and bifid or depressed stigma are present. Fruits are broadly ellipsoid, crowned by persistent five lobed calyculus. In this study, the effect of ethanolic (ETH) and chloroform (CTH) fraction of *Taxillus heyneanus* extract in cerebral ischemic damage was investigated [10].

MATERIAL AND METHODS

Collection, Identification and Authentication of Plant material

The whole plant of *Taxillus heyneus* was collected in April 2014, from chitturdist. Prof. K. Madhav Shetty, SV University, Tirupathi has identified and authenticated it. A voucher of the material was kept in Anurag Pharmacy College Pharmacognosy Department (ANU/COG/14/01) for reference.

Experimental Animals

Wistar rats (180–200 g) procured from Anurag Pharmacy College, Kodad, India for the experiment. They were kept under standard conditions at 23-25^{0}C and fed with standard diet. Before experiment, the animals were habituated for a week under laboratory conditions. The experiment was conducted in accordance with CPCSEA guidelines and approved by IAEC (Registration no - 1712/PO/a/13/CPCSEA).

Chemicals

All analytical grade chemical agents used in the study were purchased from Himedia (Mumbai) & Sigma Chemicals Co. (USA).

Plant Extraction and Fractionation

Plant material was chopped, washed, shade-dried, pulverized and stored at room temperature (RT) for future use. The dried coarse powder (500g) of plant extract was macerated with absolute ethanol at room temperature for 10 days in a clean and sterilized glass container with frequent agitation and subjected to filtration. The filtrate obtained was concentrated on a water bath at 40°C to dryness and designated as crude extract of TH. The crude extract obtained was subjected to fractionation by suspending in hydroalcoholic (7:3 % v/v) solution and extracted successively with petroleum ether and chloroform using separating funnel. Dried plant extracts of different solvents were weighed and stored for further use. PET ether fraction of the plants formed was very little which is insufficient to carry out analysis. In the present study, except PET ether extracts, the ethanol (ETH) and chloroform fractions (CTH) of crude extract were screened for phytoconstituents and cerebroprotective effect against cerebral ischemia [11]

Phytochemical Screening

ETH and CTH were subjected to phytochemical screening to detect the presence of phyto-constituents using standard methods [12]. Both the fractions showed the presence of reducing sugars, steroids, proteins, tannins, phenols and flavonoids.

Drugs and Extract administration

The ethanolic and chloroform fractions of *T. heyneanus* extract were prepared as a uniform suspension using 1% tween 80 for oral administration in experimental animals.

Acute toxicity studies

ETH & CTH fractions toxicity were tested as per Acute Toxic Class Method described in OECD 423. Prior to the dosing, the animals were fasted overnight. The incidence of mortality was checked for first 24 hours and daily thereafter for 14 days. As the fractions of extract (ETH & CTH) were found safe up to maximum dose 2000 mg/kg with no signs of toxicity or mortality, 1/10 th and 1/5 th of maximum dose i.e.200 mg/kg and 400 mg/kg were chosen for the study[13].

Experiment schedule

Wistar rats of either sex were divided into six treatment groups having eight animals in each group. Treatment Groups received different doses (200 & 400 mg/kg) of ETH and CTH orally once daily for 30 days whereas sham and Ischemic groups received vehicle (1% tween 80). The treatment schedule is mentioned below

Group I (SHAM): Rats were subjected to surgical procedures without occlusion/reperfusion/ferric chloride application

Group II (ISCHEMIC CONTROL): Bilateral common carotid artery occlusion (BCCAO) for 30 mins followed by reperfusion for 45 mins and topical application of ferric chloride to carotid artery.

Group III (ETH1): Rats pretreated with low dose 200 mg/kg of ETH and BCCAO for 30 mins/ reperfusion for 45 /topical application of ferric chloride to carotid artery

Group IV (ETH2): Rats treated with high dose 400mg/kg of ETH and BCCAO for 30 mins/ reperfusion for 45 /topical application of ferric chloride to carotid artery

Group V (CTH1): Rats treated with low dose 200mg/kg of CTH and BCCAO for 30 mins/ reperfusion for 45 /topical application of ferric chloride to carotid artery

Group VI (CTH2): Rats treated with high dose 400mg/kg of CTH and BCCAO for 30 mins/ reperfusion for 45 /topical application of ferric chloride to carotid artery

Induction of stroke

After 30 days of pre-treatment, stroke was induced by the combination of global and focal models i.e occlusion of bilateral common carotid arteries followed by reperfusion and ferric chloride ($Fecl_3$) induced thrombosis. Rats anesthetized with ketamine and xylazine (80 mg/kg i.p. &10 mg/kg i.m.) were placed on the surgical table and ventral midline incision on neck area was made to expose the left and right common carotid arteries. Vagus nerve accompanying carotid artery was carefully separated. Ischemic stroke was induced by occluding both arteries with vascular clips for 30 mins. Then, in the process of reperfusion vascular clamps were clamped and removed for period of 45 mins[14]. Finally, the filter paper (1×1mm) saturated with 25% $Fecl_3$ was applied proximal to the surface of left carotid artery for 15 minutes. During the application, it should not touch other tissue or blood vessels [15]. Temperature conditions were maintained during the surgery. The sutured rats were housed in separate cages for recovery. Sham rats received surgical procedure without ischemia inducing techniques such as occlusion and thrombosis.

Behavioral Parameters

Animals were trained for behavioral studies before surgery and were evaluated at 1 and 3 days after the surgery and given score as per Petullo et al and Garcia et al method [16, 17].

Neuromuscular function test

This involves six sub tests such as torso twisting, circling, inverted angle board, mobility, hind limb placement and forelimb flexion and scores were given as per scoring criteria table for basic behavioural test after stroke by Petullo et al [16]. The sum of scores of all subtests of different groups were calculated and compared. High score indicates high neurological deficit.

Beam balance

Vestibulomotor function of rat is assessed by placing it steadily with all limbs on a 0.75 inch width and 10 inches length narrow beam suspended above a table for 60 secs. Based on how the rat balance, scores were given.

Beam walk

This is complex neuro motor function assessed by placing the rat on a beam with narrow entry darkened goal box on one side and bright light on other side. Noise was generated at the bright end of the beam to motivate the rat to enter the box. The time in seconds taken for all group of rats to enter box were recorded. The scores were given as per the scoring chart of petullo method.

Garcia modified neurological deficit scoring system

This includes various sub tests such as spontaneous activity, movement, proprioception and sensory function. The behaviour score for rats were as per Garcia scoring table [17]. The maximum score is 18 which represents normal activity. Lower the score less is the deficit.

Post treatment

After 3 days of inducing stroke, the treatment schedule was continued for another 7 days with different doses of ETH, CTH and Tween 80. By the end of 7^{th} day, the rat brains were removed by decapitation for neuro-biochemical and histopathology studies.

Preparation of brain homogenate

The separated brains were washed with 0.9% ice cold saline and blotted with filter paper. After that they were weighed and cut into pieces. These pieces were homogenized (10% w/v) with an ice cold 10 mM pH 7.4 tris HCL buffer in a glass homogenizer at a speed of 2500 rpm and centrifuged for 20 mins to get clear supernant. This was separated and maintained at 4^0C in freezer until it is assayed for various biochemical parameters [18].

Super oxide dismutase

SOD activity was determined by Misra and Fridovich method. 0.1ml of 0.4 Mm EDTA, 0.5ml of carbonate buffer of pH 9.7 was mixed with 0.1 ml of the sample aliquot. To this, 1ml of freshly prepared 3mM epinephrine was added to initiate the reaction and absorbance was noted for 3 mins at 30 sec interval at 480 nm [19].

Reduced Glutathione assay (GSH)

Jollow et al method using DTNB as a substrate was used for estimation. One ml of brain homogenate was added to one ml of 4% sulfosalicylic acid and allowed to precipitate at 4ºC for 1 hr. It was centrifuged for 20 minutes. Filtered aliquot (0.1 ml), phosphate buffer (2.7 ml 0.1M, pH 7.4) and (0.2 ml, 100 mM) DTNB forms yellow coloured assay mixture whose absorbance was noted at 412[20].

Glutathione-S-Transferase assay (GST)

2ml of mixture consists of brain homogenate sample (0.3 ml), phosphate buffer (1.475 ml, 0.1 M, pH 6.5), reduced glutathione (0.2 ml, 1 mM) and CDNB (1-Chloro 2, 4-dinitrobenzene, and 0.025 ml of 1 mM). The absorbance of different samples were recorded at 340 nm[21].

Glutathione Reductase assay (GSR)

Carlberg and Mannervik method was used for this. The reaction solution (2ml) composed of homogenate (0.1 ml), phosphate buffer (1.65 ml, 0.1 M pH 7.6), NADPH (0.1 ml 0.1 mM), EDTA (0.1 ml 0.5 mM) and oxidized glutathione (0.05 ml, 1 mM). The activity of enzyme was estimated at 340 nm [22]

Glutathione peroxidase assay (GSH-Px)

It was determined by Mohandas et al method. 2ml mixture consists of homogenate of brain (0.1 ml), phosphate buffer (1.49 ml, 0.1 M, pH 7.4), sodium azide (0.1 ml, 1 mM), glutathione reductase (0.05 ml, 1 IU/ml), (0.05 ml, 1 mM) GSH, EDTA (0.1 ml, 1 mM), NADPH (0.1 ml, 0.2 mM, nicotinamide adenine dinucleotide) and

H_2O_2 (0.01 ml 0.25 mM). Absorbance was recorded at 340 nm [23].

Estimation of Lipid peroxidation

To measure the level of lipid peroxidation MDA was used. In this Slater and Sawyer assay, brain homogenate (0.1 ml) mixed with 10% trichloroacetic acid (1.0 ml) and 0.67% thiobarbituric acid (1.0 ml) and placed on water bath for half an hour and then after that it was kept on crushed ice for 10 mins to develop pink colour. It was centrifuged and absorbance was noted at 532 nm by spectrophotometer [24].

Catalase Activity

Claiborne method was used in which 3 ml of reaction mixture consists of phosphate buffer (1.95 ml of 0.05 M, pH 7.0), hydrogen peroxide (1ml of 0.019 M) and brain homogenate (0.05 ml) and absorbance was read at 240 nm [25].

***Invivo Acetyl* cholinesterase level (AChE) assessment**

The mixture containing brain homogenate (0.4 ml) sample, (100 µl 2.7 mM) DTNB and phosphate buffer (2.6 ml 0.1 M, pH 8) was incubated for 2 mins at 30^0C. ATC (20 µl of 30mM) was added to initiate the reaction. With the change in absorbance the activity of enzyme was determined at 412 nm for a period of 10 mins. Absorbance was noted for every minute [26].

Total protein level

Lowry et al method was used for this determination. Brain homogenate (0.2 ml), acetic acid (1.5 ml of 20 %), sodium dodecyl sulphate (0.2 ml, 4%) and Thiobarbituric acid (1.5 ml of 0.5%) were added and heated for an hour at 95°C in a water bath to give a pink colour which was centrifuged at 3500 rpm for 10 mins to note absorbance at 532 nm [27].

Glutamate Estimation

It was estimated by multiple development paper chromatography as described by Raju et al [28]

Histopathology

The brains of randomly picked animal from each group were fixed with 10% formalin solution and cut into 5 µm thickness sections longitudinally. These sections were stained with haematoxylin and eosin for pathological observation of brain tissues [29].

Statistical analysis

Data of this study expressed as mean ± SEM. Data were considered statistically significant at $P < 0.05$ and highly significant at $P < 0.0001$.Significance of Groups means difference for different parameters was determined by One-way ANOVA followed by Dunnett's multiple comparison test using Graph pad prism 7.03.

RESULTS

Effect of ETH & CTH on SOD, CAT and Protein Levels

Ischemia significantly reduced the total protein and antioxidant enzymes (SOD and CAT levels in control groups compared to sham operated group. Both ethanol and chloroform fraction treated groups significantly ($p\leq 0.0001$) improved the SOD, CAT and total protein which were reduced by ischemic effect [Table 1].

Effect of ETH & CTH on non-enzymatic and enzymatic glutathione Levels

As a result of combined effect of occlusion, reperfusion and thrombosis effect the levels of Glutathione enzymes were significantly decreased in control group. These defensive glutathione enzyme levels were highly ($p\leq 0.0001$) improved by ETH2 treated group which indicate decrease in free radical damage compared to ischemic control group. The ETH1, CTH2 and CTH1 also increased the non-enzymatic and enzymatic glutathione levels. This indicate the antioxidant capacity of fractions [Table 1 and 2].

ETH & CTH effect on LPO and Glutamate level

In ischemic group, glutamate levels and lipid peroxidation ($p<0.0001$) had significantly increased compared to sham operated group which indicate maximum cerebral insult. All the treatment groups significantly reduced LPO ($p<0.001$) compared to ischemic group. ETH1 ($p< 0.001$), ETH2 ($p<0.0001$) and CTH2 ($p <0.001$) had significantly decreased the elevated glutamate compared to ischemic group reducing excitotoxicity whereas CTH1 showed non-significant effect [Table 1 and 3].

Effect of ETH & CTH on Acetyl cholinesterase

Acetyl cholinesterase enzyme level had significantly increased in ischemia induced group than sham group. ETH high dose treated group exhibited significant decrease in AChE levels which indicate improvement of memory functions associated to cholinergic system. ETH1 and CTH2 ($p \leq 0.001$ & $p \leq 0.05$) improved the cholinergic function with decrease in enzyme levels. [Table 3].

Effect of ETH & CTH on Neurologicalfunction deficit

After 24 hrs of surgery all group of animals exhibited similar neurological scores because of post-surgery effect. The differentiated behaviour between animal groups were seen after 3 days of surgery. This behavioral parameters were assessed under three categories of neuromuscular function, vestibulomotor function and complex neuromotor function by David Petullo. Higher the score more is the neurological deficit. Sum of the scores of all neuromuscular subtests of different groups were calculated and compared. The control group ($p\leq 0.0001$) showed significant increase in neurological deficit scores compared to sham. The ETH2 improved all neurological function deficits significantly compared to control group which were indicated by low scores.ETH1 and CTH2 also improved deficits ($P\leq 0.001$& $P< 0.05$) whereas CTH1 showed non-significant improvement in deficits [Fig 1A,1C,1D].

Effect of ETH & CTH on Garcia modified neurological score system

This system consists of various subtests. The sum of all tests maximum score is 18. In this system maximum the score minimum is the deficit. The scores of all the tests as were summed up and compared [Fig 1B]. The control group showed significantly ($p<0.0001$) low scores when compared to sham operated group. The ETH1 and CTH2 showed significant ($p<0.001$) whereas CTH1 exhibited non-significant effect on behavior. The high dose ETH treated groups showed excellent improvement in scores compared to other groups.

Effect of ETH & CTH on Histopathology of brain sections

It was observed that the combination of various techniques such as occlusion, reperfusion and thrombosis produced clear shrinkage and necrosis of brain tissues along vacuolization and inflammatory infiltration seen in fore brains clearly indicate global ischemic effect. Ethanol fraction of extract treated groups had significantly reversed the ischemic damage and decreased the neuronal death which indicates neuroprotective effect of ETH. The chloroform treated groups which showed the congestion of blood vessels, little neuronal death had not repaired ischemic insult comparatively to ETH [Fig 2].

A. Sham rat brain section showing normal architecture B. Control rat brain cortex section showing shrunken, darkly stained small nuclei and increased intracellular spaces. C &D- ETH treated (200 and 400 mg/kg) rat brain sections showing reduced neuronal necrosis indicating less ischemic damage E&F-CTH treated (200 and 400 mg/kg) rat brain sections showing congestion and neuronal necrosis indicating less cerebroprotective effect.

Table 1. Effect of various doses of ETH and CTH on neuro-biochemical parameters in rat

GROUPS	DOSE	SOD (U/mg protein)	CAT (nmol H_2O_2/ min/mg protein)	LPO nmol MDA /mg protein	TOTAL PROTEIN (U/mg protein)
SHAM	% tween 80	8.69±0.20	122±0.88	4.1±0.28	584.53±7.40
CONTROL	% tween 80	4.63±0.27^{a}	75.4±0.47^{a}	9.65±0.74^{a}	100.58±5.68
ETH1	200 mg/kg	7.45±0.21^{b***}	95.9±0.58^{b}	7.17±0.31^{b}	414.92±8.76
ETH2	400 mg/kg	8.11±0.32^{b}	114±2.23^{b}	5.25±0.63^{b}	542.45±6.97
CTH1	200 mg/kg	5.76±0.30^{ns}	78.30±0.95^{ns}	8.8±0.75^{b***}	148.42±2.39
CTH2	400 mg/kg	6.69±0.42^{b**}	90.60±0.69^{b}	7.94±0.80^{b}	205.95±8.67

Values are reported as mean ± SEM (n=8), ^{a} $p<0.0001$ compared to sham, $^{b}p<0.0001$, $^{b***}p<0.001$, $^{b**}p< 0.01$, $^{b*}p<0.05$ and $^{ns}p>0.05$ compared to control

Table 2. Effect of different doses of ETH and CTH on Glutathione related enzyme levels in rat brain

GROUPS	DOSE	GPx (nmol NADPH oxidized/ min/mg protein)	GST (nmol CDNB conjugate/min/mg protein)	GR (nmol NADPH oxidized/ min/mg protein
SHAM	% tween 80	93.90±1.94	80.20±0.51	64.60±0.76
CONTROL	% tween 80	48.20±1.06^{a}	25.10±0.46^{a}	23.00±1.51^{a}
ETH1	200 mg/kg	55.80±0.70^{b***}	35.7±2.63^{b***}	33.70±0.10 ^{b***}
ETH2	400 mg/kg	71.5±0.64^{b}	65.90±0.88^{b}	44.80±2.74^{b}
CTH1	200 mg/kg	50.5±0.59^{ns}	28.10±1.82^{ns}	27.20±0.68^{ns}
CTH2	400 mg/kg	54.20±0.35^{b**}	38.50±0.29^{b}	32.05±1.33 ^{b**}

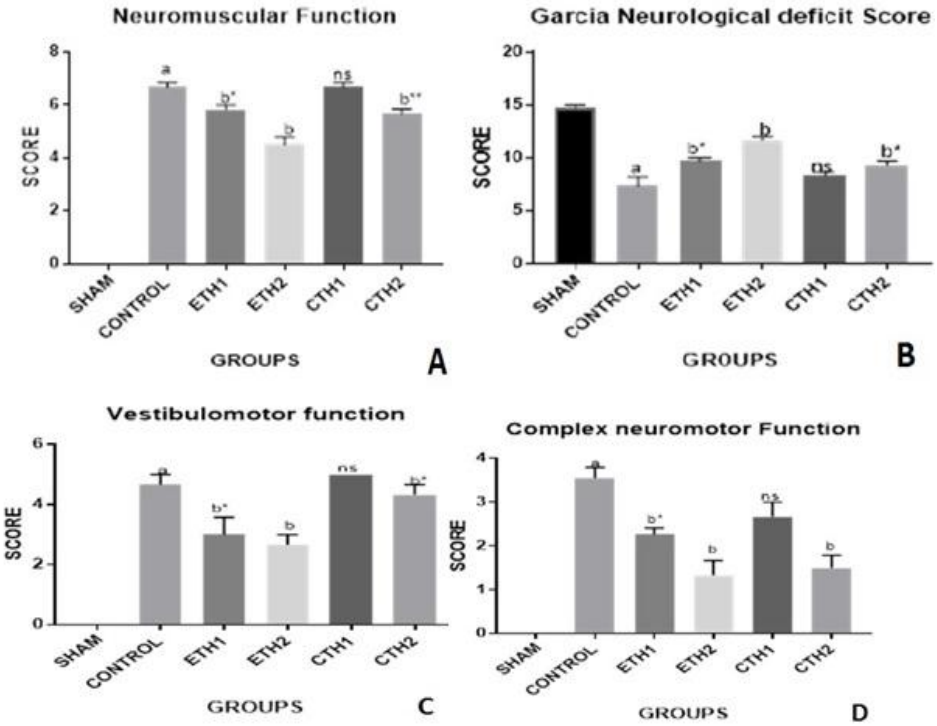
Values are reported as mean ± SEM (n=8), ^{a} $p<0.0001$ compared to sham, $^{b}p<0.0001$, $^{b***}p<0.001$, $^{b**}p< 0.01$, $^{b*}p<0.05$ and $^{ns}p>0.05$ compared to control. Gpx –Glutathione peroxidase, GST-Glutathione S transferase and GR- Glutathione Reductase

Table 3. Effect of ETH and CTH on neuro-biochemical parameters in rat

GROUPS	DOSE	GSH (nmol DTNB oxidized/ min/mg protein)	AChE (µmol/min/mg protein)	Glutamate (µmol /g)
SHAM	% tween 80	1.99±0.05	14.50±0.08	71.15±0.68
CONTROL	% tween 80	0.82±0.02^{a}	20.25±0.23^{a}	82.23±0.63^{a}
ETH1	200 mg/kg	1.15±0.01^{b***}	17.80±0.36^{b}	78.61±0.27^{b***}
ETH2	400 mg/kg	1.48±0.05^{b}	16.59±0.07^{b}	73.96±0.53^{b}
CTH1	200 mg/kg	0.88±0.04^{ns}	19.50.±0.31^{ns}	81.40±0.36^{ns}
CTH2	400 mg/kg	1.13±0.06^{b**}	19.44±0.43^{b*}	79.48±0.23^{b***}

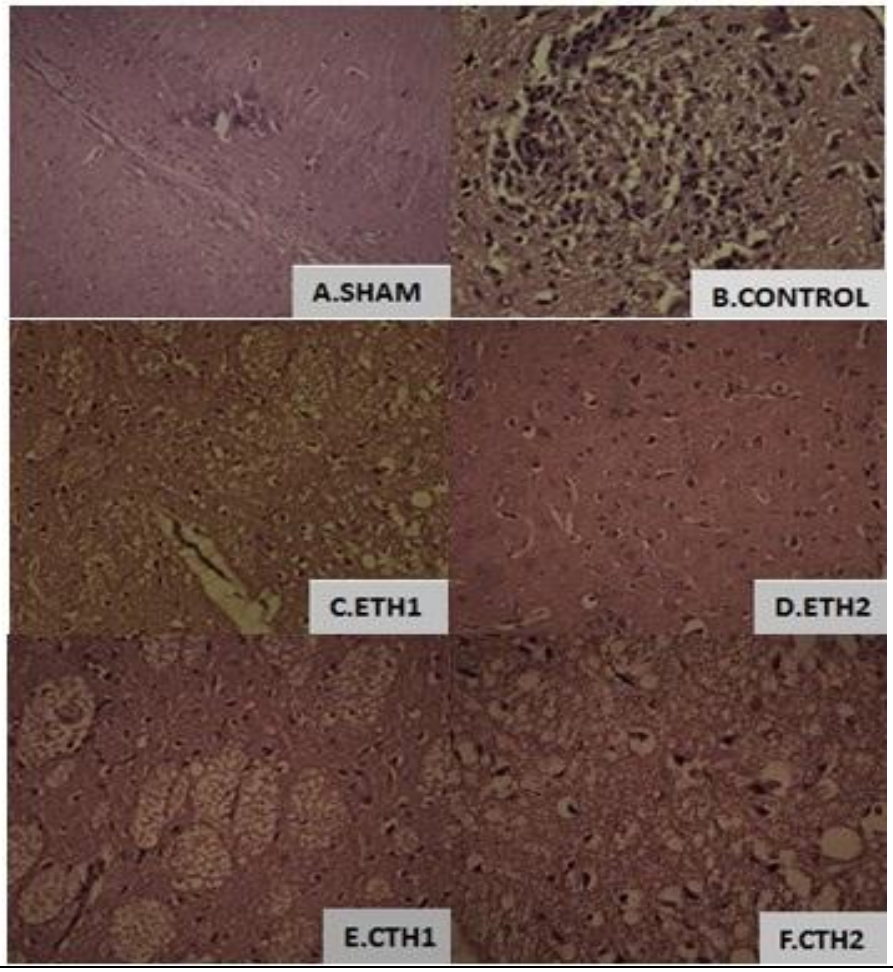
Values are reported as mean ± SEM (n=8), ^{a} $p<0.0001$ compared to sham, $^{b}p<0.0001$, $^{b***}p<0.001$, ^{b**}p, 0.01, $^{b*}p<0.05$ and $^{ns}p>0.05$ compared to control. GSH-Reduced glutathione, AChE-Acetyl cholinesterase

Fig 1 (A-D). Effect of ETH and CTH on various behavioral parameters



Values are reported as mean ± SEM (n=8), [a] $p<0.0001$ compared to sham, [b] $p<0.0001$, [b***] $p<0.001$, [b**] p,0.01, [b*] $p<0.05$ and [ns] $p>0.05$ compared to control

Fig 2. Photographs of Histopathology brain sections of different treatment grouprats



DISCUSSION

Recombinant tissue plasminogen activator (rt-PA) commonly referred as clot buster, is the only thrombolytic drug approved by the FDA to treat ischemic strokes. It must be administered within 3 h of attack for better outcome. Extended use of it results in high risk of intra-cerebral hemorrhage. Therefore, it's been a great challenge for the researchers to develop effective medication that treat stroke induced cerebral damage with minimum side effects [30]. In this study, we examined the potential role of ethanolic and chloroform fractions of *Taxillus heyneanus* in the treatment of ischemic stroke.

For this, we combined Ferric chloride induced arterial thrombosis along with most acceptable model of global ischemia i.e. BCCAO with reperfusion. This caused severe reduction in the blood flow to different regions of brain and act as an adequate preconditioning stimulus to induce early ischemic damage, vascular dementia and neurological deficits [31]. Occlusion along with recirculation leads to production of reactive oxygen species, microvascular dysfunction which impairs endothelial function, plasma protein diapedesis and free radical cellular damage [32]. The topical application of ferric chloride to right carotid artery induced the thrombus formation in exposed common carotid artery causing severe regional injury to vascular wall leaving substantial portion of arterial rim to be structurally impact. This method of ferric chloride application, plays a predominant model in the study of thrombosis pathophysiology, validation of novel antithrombotic drugs and their mechanism of action [33]. Cumulative effect of occlusion, reperfusion and thrombosis produced maximum cerebral insult that mimics many features of stroke in humans and resulted in neurological deficits.

These deficits were assessed by various behavioral tests which is composite of motor, neuromuscular, sensory, complex motor, tactile, proprioceptive, vestibulomotor, vision, reflex and balance tests. The neurological function/deficit was graded by scale mentioned by Petullo et al.and Garcia et al. As per Petullo higher the score high is the deficit where as in the modified Garcia system lower the score higher is the deficit. The ischemic control group animals showed significant neurological deficit compared to sham group animals. These deficits were improved by pre-treatment with ETH and CTH. But among all, high dose of ETH had given prominent results.

Stroke or brain attack is an acute progressive neurodegenerative disorder. The fundamental step in ischemia that leads to cascade of devastating processes is energy failure. This triggers various intricate cellular and molecular mechanisms such as excitotoxicity, oxidative stress, inflammation and apoptosis which are implicated with dysfunction of mitochondria, decreased ATP levels, energy failure, free radical generation and lipid peroxidation [34].

Glutamate is the primary neurotransmitter in the nervous system that is related to excitotoxicity in cerebral ischemia. Imbalanced homeostasis causes increased release of glutamate which binds with NMDA/AMPA/Kainate metabropic receptors and dramatically increases intracellular calcium which triggers many deleterious effect such as activation of proteolytic enzymes. Thus, estimation of glutamate and total protein levels were important to assess the ischemic damage [35]. The ethanol fraction of extract has significantly improved ischemic condition by decreasing elevated glutamate levels and increasing the protein levels.

Oxidative stress is the critical factor of ischemia which occurs when the body's protective antioxidant mechanism is dominated by free radical attack. These reactive oxygen species (ROS) directly damage body's cellular components like lipids, proteins, nucleic acid, and carbohydrates. SOD, CAT and GPx are the first line defense antioxidant enzymes that directly eliminate hydroxyl radicals, superoxide radicals and hydrogen peroxide whereas Glutathione reductase and Glutathione-S-Transferase are secondary enzymes that remove ROS by decreasing peroxide levels or maintain supply of intermediates like glutathione and NADPH essential for main antioxidant enzymes to function. So change in this antioxidants reflects the pathological states which were assessed in our study [36]. The ischemia induced in control group exhibited decline in SOD, GSH, GSR, CAT, GPx and Reduced glutathione which was improved by pre-treatment with different doses of ETH and CTH. This might be attributed to antioxidant phytochemicals in TH.

Lipid peroxidation changes the nature of membrane and membrane bound receptors that change its fluidity and permeability. We assessed the brain tissue lipid peroxidation by measuring malondialdehyde level which is the stable metabolite of lipid peroxidation cascade [37]. Ethanol extract fraction of *T.heyneanus* reversed the increased malondialdehyde levels,

Excessive elevation of intracellular calcium during ischemic cascade seems to increase the activity of Acetyl cholinesterase enzyme (ACE) that leads to hypo function of cholinergic system. This decreased cholinergic activity causes dementia, disorientation in cerebrovascular disease and increase the risk of ischemic stroke [38]. This became evident from elevated ACE levels in control group brain homogenates which was improved in ETH and CTH treated groups.

In our previous studies, the extract fractions of *Taxillus heyneanus* established good antioxidant effect. Therefore, the observed cerebroprotective effect of ETH in ischemic treated groups is associated with the antioxidant potential which may be due to presence of polyphenols like flavonoids and phenolic compounds in it.

CONCLUSION

Both ETH and CTH treated groups had shown dose dependent protective effect by decreasing neurological deficits, lipid peroxidation, AChE and increasing the endogenous antioxidant levels but comparatively ethanolic fraction showed better cerebroprotection than chloroform fraction. The above results suggest that beneficial use of ETH in ischemic stroke treatment and further studies to isolate compounds will be fruitful.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interests regarding the publication of this paper.

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