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ASSESSMENT OF NEUROPROTECTIVE ACTIVITY OF ECHINACOSIDE AND ASIATICOSIDE USING THROMBOLYTIC TRANSIENT ISCHEMIC RAT MODEL

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ABSTRACT

Focal cerebral ischemia occurs when a blood clot blocks a cerebral vessel, reducing blood flow to the particular region of the brain and increasing risk of cell death. Echinacoside is a phenylethanoid glycoside which possesses various bioactive properties such as antioxidation, anti-inflammation, anticancer, hepatoprotective, and neuroprotective effects. *Centella asiatica* (L) Urb, commonly known as Indian pennywort, belongs to the family Apiaceae which possesses wound healing, antitumor, memory enhancing, cardioprotective and immunomodulatory properties. The objective of this study is to assess the neuroprotective effect of echinacoside and fractionated asiaticoside enriched extract using a thrombotic ischemic model. The study was conducted using Male Sprague-Dawley rats housed under controlled conditions. After initiating ischemic damage, parameters like body weight, cerebral blood flow, neurological deficit score, grip strength, inflammatory mediators, excitatory and inhibitory amino acid levels were measured to elucidate brain activity. The performance of the echinacoside, asiaticoside and streptokinase treated rats was compared with controls. The results after seven days showed that asiaticoside improved muscle strength (p<0.05). Echinacoside improved skeletal muscle activity (p<0.001) when compared to ischemic rats. Asiaticoside treatment showed a significant increase (p<0.05) in grip strength compared to vehicle treated ischemic rats. Echinacoside showed statistically highly significant (p<0.001) improvement in grip strength compared to vehicle treated ischemic rats. Treatment with echinacoside showed decreased (p<0.001) levels of glutamate and aspartate when compared to vehicle treated ischemic rats. Inhibitory amino acid γ- Aminobutyric acid (GABA) levels increased with

echinacoside, streptokinase (p<0.001) and asiaticoside (p<0.05) treatment indicating neuroprotective activity. It can be concluded that echinacoside and asiaticoside enriched extract exhibitedneuroprotective activity and this effect was attenuated to its cytokinin and excitatory amino acid regulation in the brain after ischemic reperfusion.

Keywords: Echinacoside; Asiaticoside; Cerebral blood flow; Cytokines; Excitatory aminoacids

INTRODUCTION

An ischemic stroke is caused by a thrombotic or embolic event which causes decrease in blood flow to the brain. Both prognosis and outcomes are directly related to the etiology of the stroke making it the cause for 5.5 million deaths and 15 million non-lethal brain injuries worldwide **[1]**.

Echinacoside (ECH) is a phenylethanoid glycoside isolated and purified from the stems of *Cistanche salsa*. It possesses properties that result in antioxidant, anti-inflammatory, anticancer, hepatoprotective and neuroprotective effects. Glutamate excitotoxicity reduction is a potential mechanism involved in neuroprotection while also inhibiting the release of interleukins and caspase-3 activation to inhibit mitochondria-mediated apoptosis **[2, 3]**. It has been found to cross the blood brain barrier and act on the hypothalamus directly inhibiting hypothalamic Androgen Receptor (AR) activity, AR translocation to the nucleus and Hypothalamic-Pituitary-Gonadal (HPG)-axis related gene expression while also protecting against bisphenol A (BPA)-induced reproductive damage **[4]**. ECH protects against high glucose-induced vascular endothelial dysfunction associated with Nrf2/HO-1-dependent antioxidative pathway and regulated by Akt phosphorylation and also plays a role in several other neurological disorders **[5]**.

Centella asiatica, commonly known as Indian pennywort belongs to the family Apiaceae (Umbelliferae). It is chemically composed of asiaticoside, centelloside, madecassoside and Asiatic acid and possesses healing, cytotoxic, antitumor, memory enhancing, cardioprotective and immunomodulatory properties. It was found to inhibit activation of caspase-3 and poly-ADP ribose polymerase (PARP) while increasing expression of Nrf2/HO-1 **[6]**.

The present study aimed to evaluate the neuroprotective activity of Echinacoside and Asiaticoside in the thrombotic rat model of ischemia. Their effect in preventing ischemic damage was determined by measuring cerebral blood flow, inflammatory mediators, and levels of excitatory amino acids (EAA).

MATERIALS AND METHODS

Extraction and Preparation of Asiaticoside

& Echinacoside

Centella asiatica leaves were collected from Coimbatore and authenticated by the Botanical Survey of India in Tamil Nadu Agricultural University (TNAU), Coimbatore (Authentication number: BSI/ SRC/ 5/ 23/ 2019/ Tech-122). Herbs were dried at 35ºC-40ºC and grounded to obtain a coarse powder (<0.7 mm) which was macerated with 95% ethanol at 1:3 w/v ratio. After three days, the supernatant was collected and residue was subjected to the same extraction procedure three more times. The supernatants were pooled and solvent was concentrated under vacuum in a rotary evaporator at 58°C and the final crude extract was obtained. The final crude ethanolic extract of *Centella asiatica* was added in 10 ml n-hexane and kept in a bath sonicator for 10 minutes; the filtered and soluble matter was removed and process was repeated five times. 10 ml ethyl acetate was added to the collected filtrate and kept in a bath sonicator for five minutes. Soluble matter was removed and the filtrate was collected **[7, 8]**. Fractionated ethanolic extract was dissolved in methanol of different concentrations, filtered, and underwent high-performance thin-layer chromatography (HPTLC) analysis with results shown in **Figure 1**. Fractionated methanolic solution of different concentrations of asiaticoside was applied to the layers as 6 mm-wide bands positioned 10 mm from the bottom and 15 mm from the side of the plate, using Camag Linomat V automated thin layer chromatography (TLC) applicator with nitrogen flow providing a delivery speed of 150 nl/s from application syringe as shown in **Figure 1**. These conditions were kept constant throughout the analysis of samples. Following sample application, layers were developed in a Camag twin trough glass chamber which was pre- saturated with mobile phase of Chloroform:Glacial-acetic-acid:Methanol:Water in the ratio 60:32:12:8 for asiaticoside till proper separation of bands up to 8 cm height was obtained. Asiaticoside was scanned at 366 nm using Camag TLC scanner model 3 equipped with Camag Wincats IV software as shown in **Figure 1**. After scanning TLC plates were sprayed with Anisaldehyde sulphuric acid reagent and dried at 100ºC for 10 minutes using a hot air oven, red-violet color was obtained. Echinacoside was a gift sample received from China.

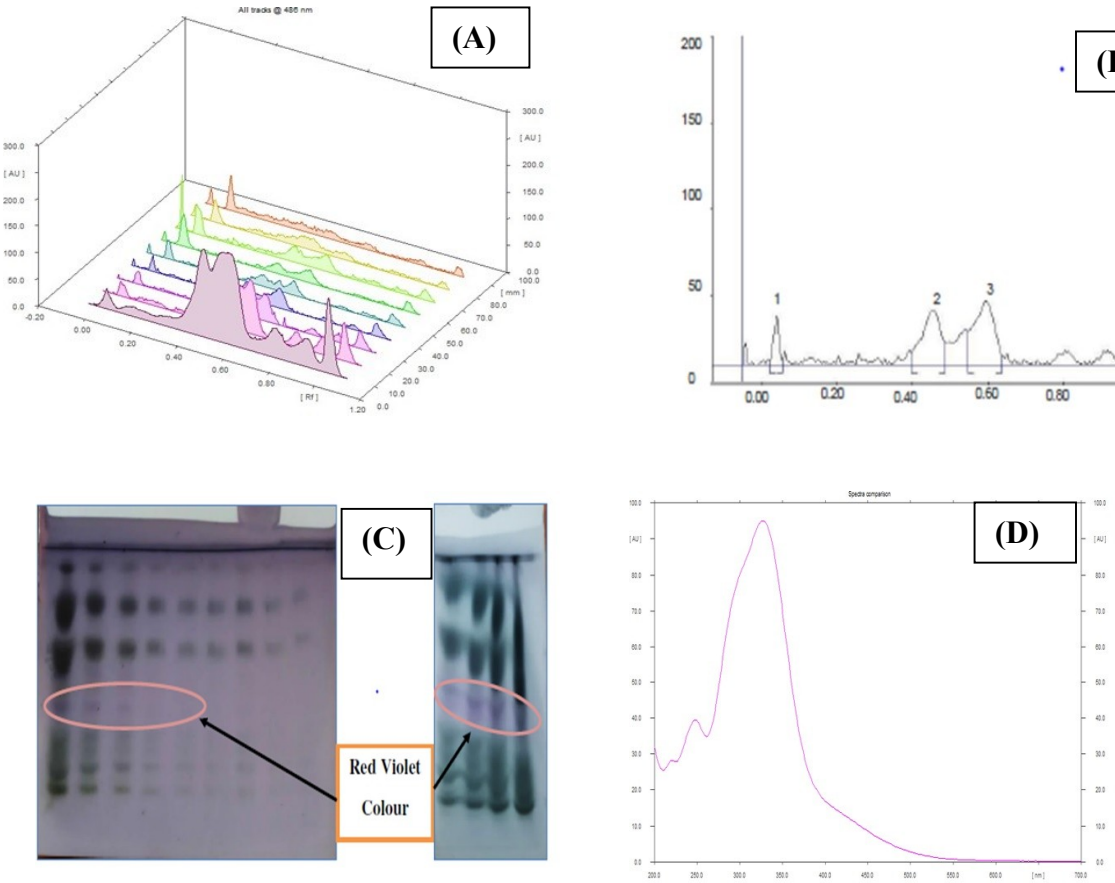


Figure 1: Chromatogram of *C. asiatica* extract (A) and fractionated asiaticoside (B) Development of TLCplate using different concentrations of fractionated asiaticoside and red-violet color was obtained upon spraying anisaldehyde sulphuric acid reagent (C) UV spectra for the determined spot at 326 nm (D)

Surgery

Male Sprague-Dawley rats were used and housed in individual polypropylene cages in a well-ventilated room with an ambient temperature of 25±2 ºC, 50-55% relative humidity and a 12-hour light/dark cycle. They were provided with food and purified water and acclimatized to the laboratory conditions for seven days before experimentation. They were randomly divided into five different groups: Control, Negative control, Streptokinase- 100µl/rat, Echinacoside-5mg/kg and Asiaticoside-100mg/kg. After surgery, post-operative care was maintained. The bodyweight of the rats was monitored before surgery and seven days after treatment. Ethical guidelines as per Indian Council of Medical Research guidelines: "Guide for the care and use of laboratory animals" were followed. The study protocol was approved by the Institutional Animal Ethics Committee with

number 429/IAEC/2019.

Focal thrombolytic cerebral ischemia was induced by injecting thrombin to produce a clot. After anesthetizing with ketamine and xylazine, a midline incision was made to expose the common carotid arteries (CCA) and the external and internal carotid arteries (ECA & ICA) were identified. A 5-0 silk suture was tied loosely at the origin of the ECA and ligated at its distal end. The left CCA and ICA were temporally clamped using a curved microvascular clip. A modified PE-50 catheter (0.3 mm), filled with bovine thrombin (10 NH U/ILI), was attached to a syringe and introduced into the ECA lumen through a small puncture. After ten minutes of withdrawing blood, the right and left CCA were temporarily clipped to reduce cerebral blood flow and the clot in the catheter was injected into the ICA with 30 U thrombin.The clips on the right CCA and left ECA were withdrawn five minutes after injection. The clip on the left CCA was released 15 minutes after injection. After surgery, proper post- operative care was given. The entire procedure was carried out on the operation table and temperature was maintained at 37ºC.

Behavioural Assessment

Neurobehavioral paradigms

Neurological deficit in the ischemic and treatment groups was assessed by the following score pattern at 24 hours and on 7th day after focal thrombolytic cerebral ischemic induction. The scores were assigned from 0-5 based on the observed neurological deficit, 0=No neurological deficit, 1=Failure to extend left forepaw fully, 2=Circling behaviour, 3=Falling to the right, 4=No spontaneous walking with depression, 5=Death.

Grip strength measurement

The motor coordination in the ischemic and drug-treated rats was measured by determining maximal peak force developed at 24 hours and 7th day after surgery. The grip strength test was determined using a Bioseb grip strength meter and expressed in grams **[9]**.

Laser perfusion imaging

After subjecting rats to mild anaesthesia, skin incision was made to expose the skull region to measure the cerebral blood flow using Laser Doppler image analyser (Moor-FLPI, UK). The blood flow intensity was expressed as a flux mean **[10]**.

Preparation of brain samples

The animals were sacrificed 24 hours after the 7th day of thrombolytic focal cerebral ischemicinduction. The brains were removed, immediately frozen, and kept at −70°C. For enzyme- linked immunoassay (ELISA), the

brain tissue (cortex) was homogenized using a homogenizer in 500 µl buffer containing phosphate-buffered saline (PBS) pH 7.2 and centrifuged at 12,000 g for 20 min at 4°C. The supernatant was collected and total protein was determined using Micro BCA Protein Assay Kit. To assess excitatory amino acid, brain samples were homogenized with 0.1 N HCl in 80% ethanol (10 mg tissue/200 µl) and centrifuged at 4500 rpm for 20 min at 25ºC. supernatant was collected to measure excitatory amino acid.

Quantification of inflammatory mediators by ELISA

The levels of cytokines (TNF-α, IL-β, and IL-6) in the ischemic cortex tissue were measured using an ELISA kit (Diaclone, France) specific for rats. The measurement of cytokines was performed as per kit protocol and expressed in pg/ml total protein.

Estimation of excitatory neurotransmitters in rat brain by HPTLC

Excitatory amino acids levels (aspartate and glutamate) in brain samples of ischemic and treatment groups were determined using HPTLC. Standard L-glutamic acid and L-aspartic acid were used as references. Limit of detection (LOD) and limit of quantification (LOQ) wasevaluated.

Histopathology and Immunohistochemistry

Brain samples were sectioned, hydrated with distilled water, and stained with hematoxylin followed by eosin stain and dehydrated. A drop of permount was placed over the tissue in each slide and viewed using a microscope.

Glial fibrillary acidic protein (GFAP)

GFAP expression is increased in severe mechanical or osmotic stress, hypoxia, brain, and spinal cord injury. Brain samples were stained with monoclonal mouse anti-GFAP (Dako, Denmark) (1:300) using immunoperoxidase technique. The paraffin sections of the mouse brain were deparaffinized and hydrated with distilled water. The sections containing antigenic sites were exposed by incubating them in a microwave oven with antigen retrieval solution (trisodium citrate, pH 6.2) for 20 min at 96ºC. Following retrieval, the slides were cooled with distilled water for five minutes. Subsequently, the slides were washed with PBS at each step. The slides were then treated with 3% hydrogen peroxide to decrease the endogenous peroxidise activity and incubated with 1.5% bovine serum albumin (BSA) to block non- specific binding sites. Immune reactivity was detected using an Immunocruz mouse ABC staining kit using biotinylated goat anti-mouse IgG (for GFAP) secondary antibodies at 1:100 dilution and evaluated under a binocular light microscope (40x) **[11]**.

Synaptophysin Immunohistochemistry (IHC)

The integrity of synapses in ischemic brain regions was measured by performing Immunohistochemistry (IHC) staining of synaptophysin. After isolation of the brain, it was fixed with 10% formalin. Paraffin sections of 5 µm thickness were made and IHC was performed using a monoclonal mouse anti-synaptophysin clone SY38 antibody kit (Dako, Denmark). The active synapse was evaluated under a binocular light-microscope (40x) **[12]**.

Statistical Analysis

Data was expressed as mean and standard deviation. The results were analysed using one- way and two-way analysis of variance (ANOVA) followed by Bonferroni post-test to assess the statistical significance of the differences among the study groups using GraphPad Prism 9.0 software (GraphPad Software, Inc. La Jolla, CA, USA). Significance at 95% probability level (p<0.05) was considered as statistical significance.

RESULTS

Effect on Body Weight

The body weight of all rats was measured before and after surgery. After induction of ischemia, the body weight was measured on the 7th day. The vehicle treated ischemic rats showed a significant decrease (p<0.01) in body weight compared to before-surgery group. No significant change was observed for all the treatment groups when compared to control rats **(Figure 2)**.

Effect on Neurological Deficit Score

Inducement of focal thrombolytic ischemia in rats produced significant muscle weakness as observed in the neurological scores after 24 hours and 7 days. After seven days of drug treatment, asiaticoside (100 mg/kg) improved muscle strength (p<0.05) as noticed by decreased neurological score. Similarly, ECH (5 mg/kg) and streptokinase (100 µl) reduced (p<0.001) the neurological score and improved skeletal muscle activity when compared to ischemic rats **(Figure 3)**.

Effect on Grip Strength Measurement

Vehicle treated ischemic rats had a significantly (p<0.001) reduced score indicating lower grip strength activity when compared to control. Seven days of asiaticoside treatment showed a significant increase (p<0.05) in grip strength when compared to vehicle treated ischemic rats. Streptokinase and ECH showed staistically highly significant (p<0.001) improvement in grip strength compared to vehicle treated ischemic rats after seven days of treatment **(Figure 4)**.

Effect on Cerebral Blood Flow

The vehicle treated ischemic rats showed

decreased cerebral blood flow (p<0.001) (more lighter area in the brain tissue) when compared to control rats. After seven days, asiaticoside treated rats showed increased (p<0.05) cerebral blood flow (increased red area) compared to vehicle treated ischemic rats. ECH and streptokinase treated rats exhibited significantly (p<0.001) higher cerebral blood flow when compared to vehicle treated ishemic rats **(Figure 5)**.

Effect on Excitatory and Inhibitory Neurotransmitters

A significant elevation of brain excitatory amino acids, glutamate, and aspartate levels was observed in ischemic rats when compared to the control rats whereas γ-Aminobutyric acid (GABA) level was found to be decreased in comparison to the control group. Treatment with asiaticoside showed no significant change in glutamate level but decreased aspartate level (p<0.05). Treatment with ECH and streptokinase showed decreased (p<0.001) levels of glutamate and aspartate when compared to vehicle treated ischemic rats. Inhibitory amino acid GABA levels increased with ECH, streptokinase (p<0.001) and asiaticoside (p<0.05) treatment indicating neuroprotective activity **(Figure 6)**.

Effect on Inflammatory Mediators by ELISA

Inducement of ischemia significantly increased the TNFα, IL1β, and IL-6 levels in ischemic rat brain tissue compared to control rats. Seven days of asiaticoside treatment did not alter TNFα level but decreased (p<0.05) IL1β and IL-6 levels. Similarly, treatment with ECH and streptokinase reduced (p<0.001) the elevated cytokines level in comparison to vehicle treated ischemic rats **(Figure 7)**.

Effect on Hematoxylin and Eosin staining

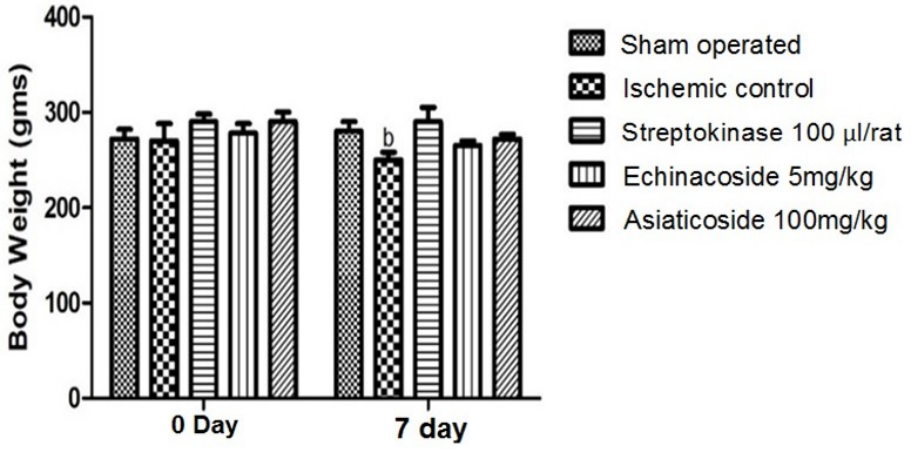
Histopathological changes were not observed in cerebrum, meninges and cerebellum region of tested rats. CA1 region of the hippocampus demonstrated gliosis and focal shrunken eosinophilic neurons in ischemic rat brain. Asiaticoside and ECH treated rats showed normal histology in the CA1 region of the hippocampus **(Figure 8)**.

Effect on GFAP

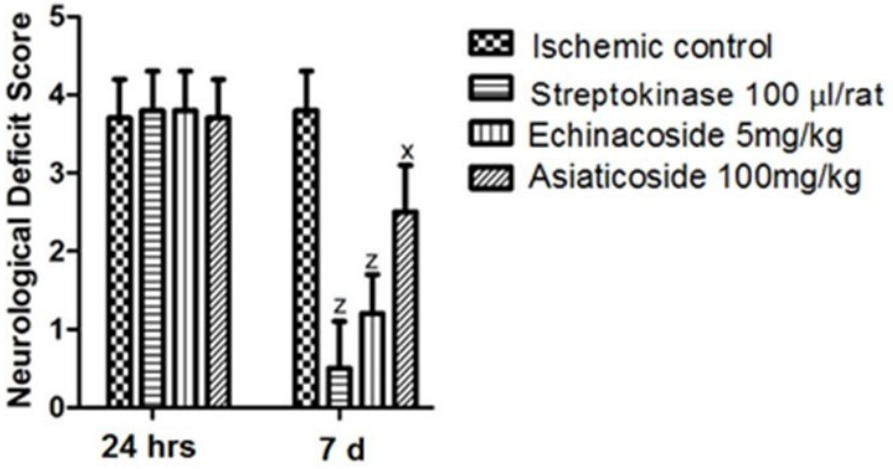
Expression of GFAP in the hippocampal region was found to be significantly increased in ischemic reperfusion rats, indicating reactive changes of glial cells in response to damage. ECH treated rats showed increased expression of GFAP. Asiaticoside treated rats showed decreased expression when compared to ECH **(Figure 9)**.

Effect on Synaptophysin IHC

Onset of ischemia is evidenced by the loss of synaptophysin protein expression in the hippocampal region of the brain observed by the loss of brown color stain. Both asiaticoside and ECH treated ischemic rats showed increased expression of synaptophysin protein in the hippocampus indicating restoration of synapse integrity and neurogenesis **(Figure 10)**.



Figures 2: Effect of Streptokinase 100 µl/rat, Echinacoside 5 mg/kg, Asiaticoside 100 mg/kg on body weight in thrombolytic focal cerebral ischemic rats. Superscript b indicates statistical significance at p<0.01 in comparison to control rats



Figures 3: Effect of Streptokinase 100 µl/rat, Echinacoside 5 mg/kg, Asiaticoside 100 mg/kg on neurological deficit score in thrombolytic focal cerebral ischemic rats. Superscripts x, z indicates statisticalsignificance at p<0.05, p<0.001 in comparison with the vehicle treated ischemic rats

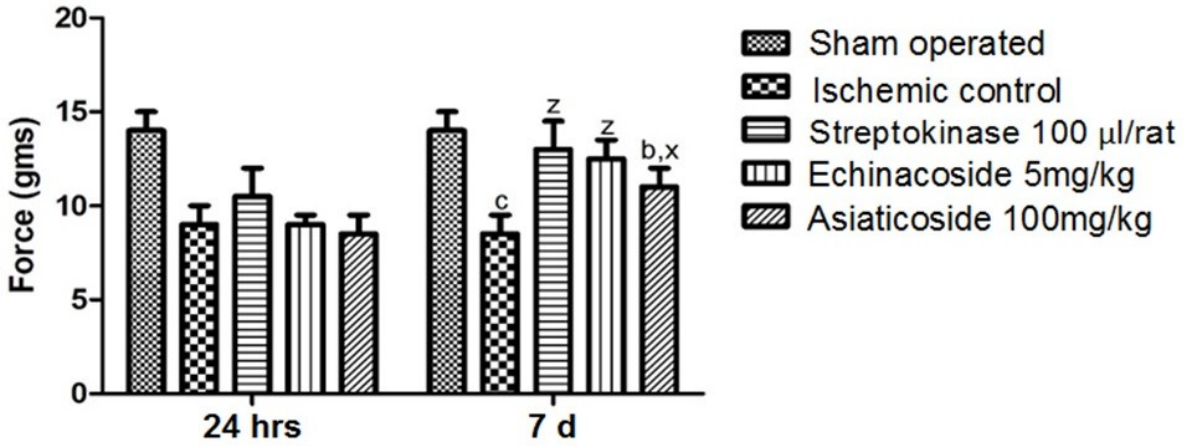
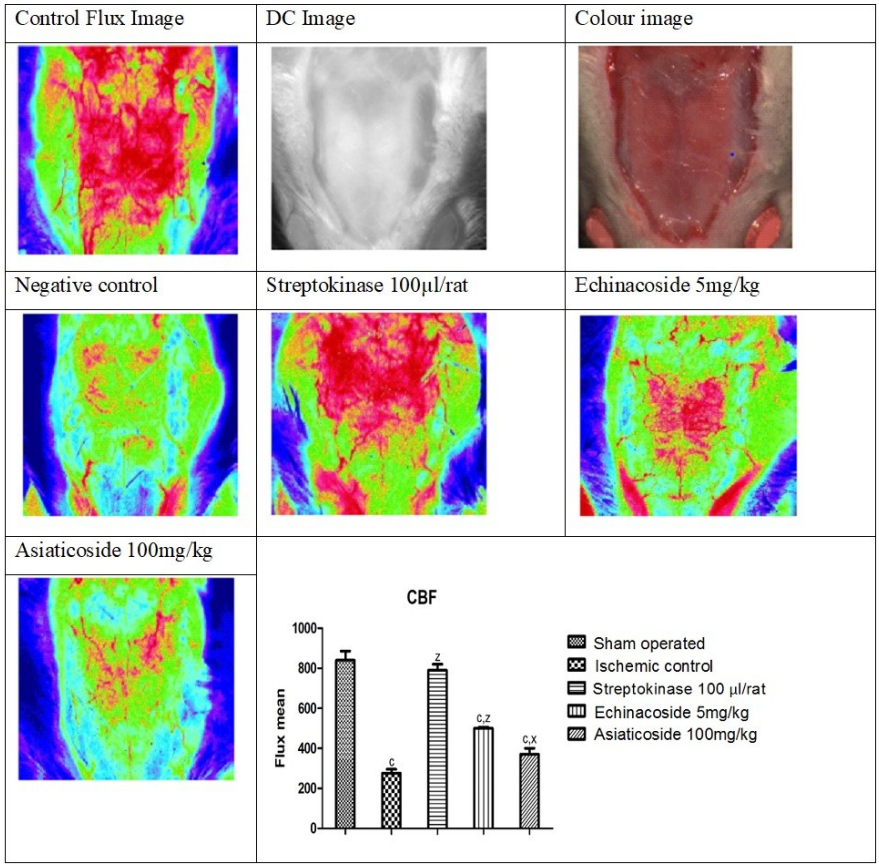
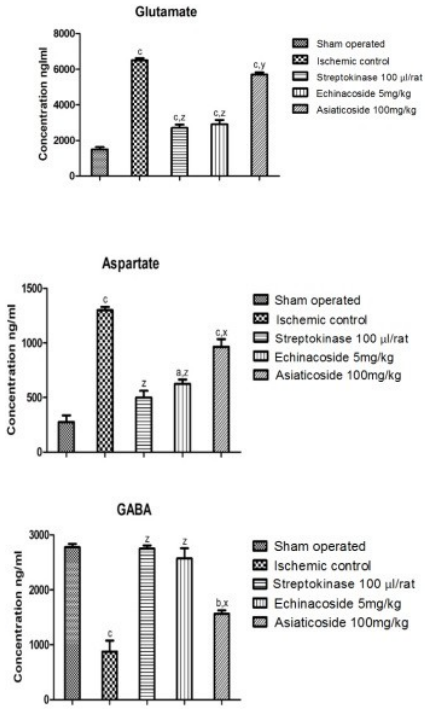


Figure 4: Effect of Streptokinase 100 µl/rat, Echinacoside 5 mg/kg, Asiaticoside 100 mg/kg on assessmentof grip strength in thrombolytic focal cerebral ischemic rats. Superscript b, c indicates statistical significance at p<0.01, p<0.001 in comparison with control rats; x, z indicates statistical significance at p<0.05, p<0.001 in comparison with the vehicle treated ischemic rats



Figures 5: Effect of Streptokinase 100 µl/rat, Echinacoside 5 mg/kg, Asiaticoside 100 mg/kg on cerebralblood flow in thrombolytic focal cerebral ischemic rats and blood flow intensity was expressed as flux mean. Superscript c indicates statistical significance at p<0.001 when compared to control rats; x, z indicates statistical significance at p<0.05, p<0.001 in comparison with the vehicle treated ischemic rats



Figures 6: Effect of Streptokinase 100 µl/rat, Echinacoside 5 mg/kg, Asiaticoside 100 mg/kg on excitatoryand inhibitory amino acids using HPTLC method. Superscripts a, b, c indicates statistical significance at p<0.05, p<0.01, p<0.001 when compared to control rats; x, y, z indicates statistical significance at p<0.05,p<0.01, p<0.001 when compared to vehicle treated ischemic rats

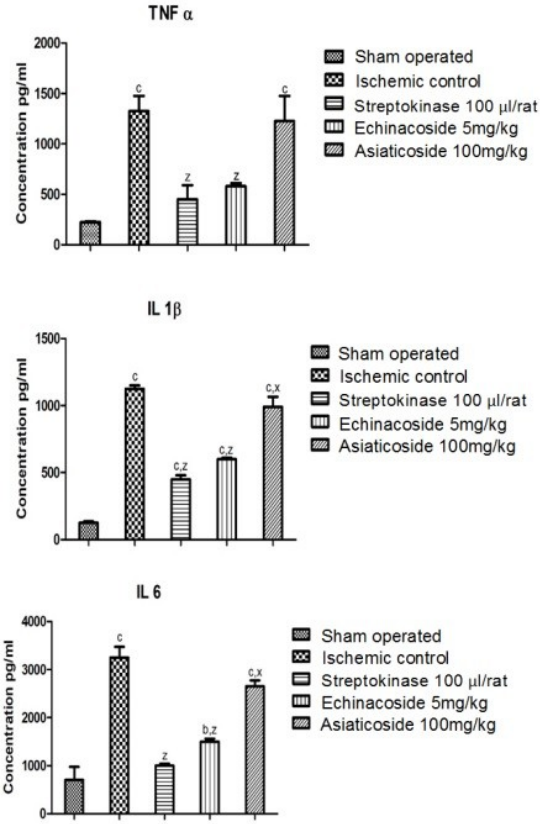


Figure 7: Effect of Streptokinase 100 µl/rat, Echinacoside 5 mg/kg, Asiaticoside 100 mg/kg on cytokine levels by ELISA. Superscripts b, c indicates statistical significance at p<0.01, p<0.001 when compared tocontrol rats; x, z indicates statistical significance at p<0.05, p<0.001 when compared to vehicle treated ischemic rats

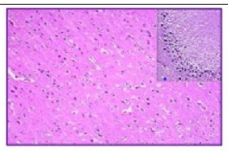
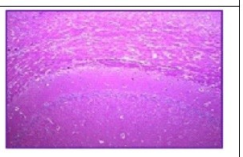
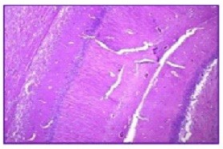
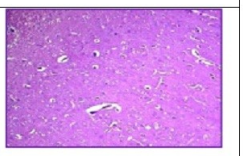
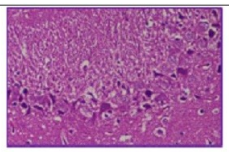
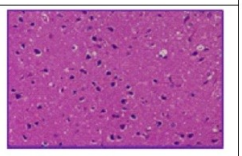
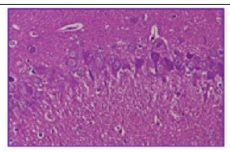
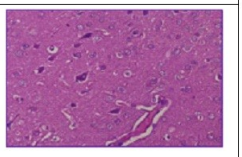
Group	Hippocampus 40x	Cerebrum 40x
Sham operated		
Ischemic control		
Echinacoside 5 mg/kg		
Asiaticoside 100 mg/kg		

Figure 8: Hematoxylin and Eosin staining

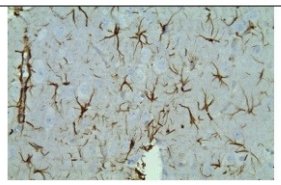
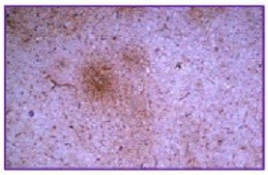
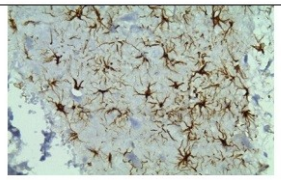
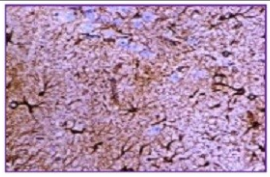
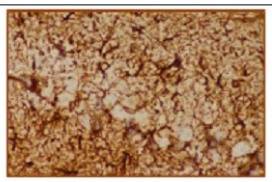
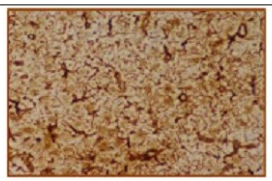
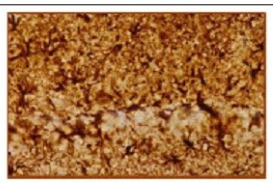
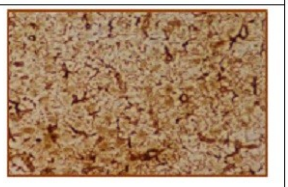
Group	Hippocampus 40x	Cerebrum 40x
Sham operated		
Ischemic control		
Echinacoside 5 mg/kg		
Asiaticoside 100 mg/kg		

Figure 9: Glial fibrillary acidic protein

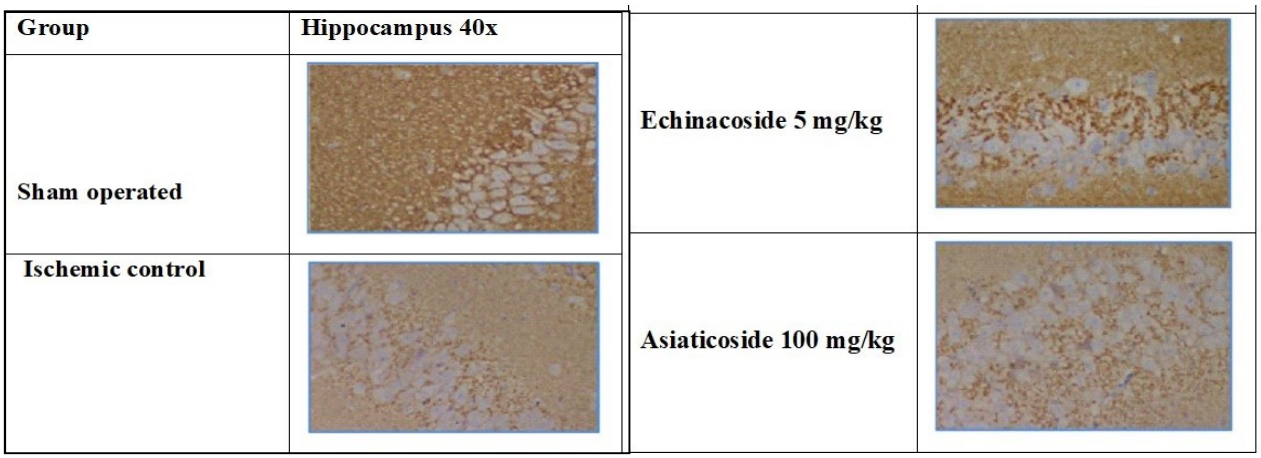


Figure 10: Synaptophysin IHC

DISCUSSION

ECH has been widely studied for its neuroprotective property in Parkinson's and Alzheimer's disease. In the present study, ECH effect was studied using a thrombolytic transient focal ischemic model and resembled a more clinical pathology. ECH intraperitoneal (IP) administration attenuated the ischemic damages and restored cerebral blood flow, excitatory amino acid activities and inflammatory marker levels along with elevated levels of inhibitory neurotransmitter GABA.

Asiaticoside enriched *Centella asiatica* extract was widely studied for its neuroprotective properties. The asiaticoside enriched ethanolic extract exhibited anti-inflammatory activity and controlled excitatory amino acid activity with restoration of cerebral blood flow.

Ischemic damages stimulate the Ca^{2+} mediated glutamate release leading to excitotoxicity followed by gliosis. The hypoxic damage observed during the ischemic reperfusion results in energy imbalance leading to neurodegeneration. The necrotic cells and reactive oxygen species (ROS) stimulate kinase pathways resulting in stimulation of cytokines release through NFκB mechanism **[13]**. In the present study, both ECH and Asiaticoside enriched extract prevented this biochemical release and produced neuroprotective activity. In earlier studies, ECH abolished the glutamate release from rat cerebro-cortical neurons evoked by 4-aminopyridine and this effect was found to be due to voltage dependent Ca^{2+} entry and suppression of protein kinase C activity **[14]**.

ECH upon 5 mg IP administration in normal rats exhibited around 16 mg/ml plasma concentration. On 15 mg oral administration in middle cerebral artery occlusion (MCAo)

rats, it showed 40 ng/30mins. In MCAo model, there will be a blood brain barrier disturbance which might allow the drug to act on brain cells. In experimental model of neurotoxicity, ECH showed decreased 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinson's disease, aspartate, glutamate infarct area, IL6 levels and this effect of ECH was mainly attributed to decreased NFκB activity and p38-MAPK levels **[15]**. Upon administration of Asiaticoside enriched extract, components can cross blood brain barrier and reports also indicated better bioavailability of these components in the brain. In a chemically stress induced rat, Asiaticoside enriched extract lowered the TNFα and elevated Brain-Derived Neurotrophic Factor (BDNF) level in the hippocampus region **[16]**. In the present study, treatment of asiaticoside did not alter TNFα level but decreased IL1β and IL-6 levels after seven days.

The results of the present study are in agreement with a study conducted using an *in vitro* model where pre-treatment of asiaticoside in SHSY5Y cells and murine BV2 microglia cells treated with MPP^+ prevented the neuronal death and this effect was attributed to suppressing the neuroinflammation through the NLRP3 inflammasome activation. This result clearly indicates that one of the triterpenoids glycoside of *Centella asiatica* can prevent neuroinflammation **[17]**. Asiaticoside treatment in the present study showed a decreased expression of GFAP which is associated with glial cell damage. In earlier studies, Asiaticoside enriched extract has exhibited mono-sodium glutamate induced neurotoxicity in rats indicating the ability of extract in the attenuation of glutamate excitotoxicity **[18]**. However in the present study, asiaticoside treatment showed no significant change in glutamate levels but decreased aspartate levels, whereas ECH and streptokinase caused a significant reduction in glutamate and aspartate levels. Most of the Asiaticoside enriched extract neuroprotective effect has been attributed to its regulation on ROS, resulting in mitochondrial protection and activation of inflammatory pathways and this effect of bioenergetic balancing leading to neuronal survival is attributed to NRF2/ ARE and SIRT1/NFκB signalling pathways.**[19]**.

In the present study, the comparison between ECH and Asiaticoside enriched extract has shown that the neuroprotective activity was better with ECH with respect to parameters like aspartate, glutamate, GABA, cytokines levels and behaviour parameters. Asiaticoside enriched extract exhibited

cerebral blood flow restoration along with attenuation of the above parameters in an ischemic model. The superior effect of ECH might be attributed to its direct control over glutamate release and thereby control of inflammatory mechanisms through NFκB and MAPK pathways. It has been observed that Asiaticoside enriched extract mechanism is mediated through TLR4 NFκB, PI3K-AKT/ ERK1/2 signalling pathways. The difference in mechanisms of ECH and Asiaticoside enriched extract might be responsible for differential activity of the two substances **[20, 21]**.

CONCLUSION

Echinacoside and asiaticoside enriched extract exhibited neuroprotective activity and this effect was attenuated to its cytokinin and excitatory amino acid regulation in the brain after ischemic reperfusion. The studied phytomolecules can be further explored with structural modification for better bioavailability to exhibit neuroprotection in thrombolytic transient ischemic model.

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