IJPSR (2020), Volume 11, Issue 10 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 17 October 2019; received in revised form, 06 April 2020; accepted, 27 August 2020; published 01 October 2020

PROTECTIVE EFFECT OF *AMARANTHUS SPINOSUS* FOR ISCHEMIA REPERFUSION INJURY IN RAT BRAIN

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Keywords:

Amaranthus spinosus, Cerebroprotective, Antioxidant, Cerebral ischemia, and neuro deficit score, *etc*.

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ABSTRACT: Cerebral ischemia is a condition in which there is insufficient blood flow to the brain to meet metabolic demands. This leads to poor oxygen supply or cerebral hypoxia and thus leading to the death of brain tissues or cerebral infarction/ischemia stroke. It has been the third most common cause of death in developed countries; stroke can affect walking, talking, speech, vision, spatial awareness, swallowing, bladder control, bowel control, and balance co-ordination. The test drug *Amaranthus spinosus* leaves were defatted with petroleum ether (60-80 °C) and then extracted with hydroalcoholic solvent (Ethanol 95%, v/v: water, 1:1) by soxhlation process. The hydroalcoholic extract of the drug was evaluated for cerebroprotective activity in Wistar albino rats in the doses of 200 and 400 mg/kg by the Induction of cerebral ischemia/reperfusion followed by behavioural tests, infarct size measurement, and antioxidant activity. The test drug ASE produced the cerebroprotective activity in a dose-dependent manner.

INTRODUCTION: Several disorders like advanced age, hypertension, transient ischemic attack, mobile radiation diabetes, high cholesterol, cigarette smoking, and atrial fibrillation leads to oxidative stress [1]. Oxidative stress is one of the primary factors that exacerbate damage by cerebral ischemia [2]. Cerebral ischemia is a condition in which there is insufficient blood flow to the brain to meet metabolic demands. This leads to poor oxygen supply or cerebral hypoxia and thus leading to the death of brain tissues or cerebral infarction/ischemia stroke.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(10).5129-34

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(10).5129-34

It has been the third most common cause of death in developed countries; stroke can affect walking, talking, speech, vision, spatial awareness, swallowing, bladder control, bowel control, and balance co-ordination [1]. The free radical formation has been proved during cerebral ischemia. Several components of reactive oxygen species (ROS) (superoxide, hydroxyl radical, hydrogen peroxide, and peroxynitrite radicals) that are generated after ischemia/reperfusion injury play an important role in neuronal loss after cerebral ischemia. Free radicals have been considered to play an important role in the genesis of tissue injury due to ischemia and reperfusion [3].

There is a lack of scientific data related to cerebroprotective activity for this plant. In this context, the present study was designed to carry out cerebroprotective study of *Amaranthus spinosus* leaves.

MATERIALS AND METHODS:

Preparation of Extract: Plant material of *Amaranthus spinosus* leaves was collected from IFTM University botanical garden. *Amaranthus spinosus* leaves were authenticated by the Botanist, Dr. Beena Kumari, Hindu College, Moradabad, UP, India. A plant specimen (Voucher no. HC.MBD/HAP/BK/2016/01/488) was submitted in the herbarium. The botanical taxonomy of the plants was properly matched with standard floras and also cross-checked with Herbarium files. The drugs were made into coarsely powdered then subjected to the extraction with petroleum ether (60-80^{o}C) followed by with hydroethanolic mixture (Ethanol 95%, v/v: water, 1:1) in a Soxhlet apparatus. The extract was filtered and distilling off the solvent separately and evaporated to dryness using a rotator vacuum evaporator.

Drugs and Chemicals: Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), Glutathione Triphenyltetrazolium chloride (TTC), *etc*. available from sigma Aldrich and other chemicals were of AR grade.

Dose Selection: Two doses of ASE were taken for recent research work after acute toxicity study [4].

Experimental Study: All experimental was in observance with the Animal Ethical Committee, Committee for the Purpose of Control, And Supervision of Experiments on Animals (CPCSEA) and were approved by the University Ethical Committee with an approval number 837/Po/Re/S// 04/CPCSEA. Experiment was carried out on both the sex of Albino Wister rats (80–150g). Sufficient measures were adopted to minimize pain and discomfort with experimental animal procedures.

The bilateral common carotid arteries were subsequently isolated, and 30 min of ischemia was given to the rat by blocking the left internal branch of the common carotid artery was occluded with microvascular clip. After the ischemic period, the neck muscle was stitched, and ointment applied each rat. The test extract was administered to the respective group of rats described below for a period of 7 days. Rats were anesthetized and divided into five groups of six animals each. Sham group (Group I), rats were subjected to surgical procedure, and a thread was passed below both carotid arteries, but the arteries were not occluded. After 30 min, thread was removed, and the animal was sutured back and allowed to recover for 3 h. Control ischemia/ reperfusion (I/R) group (Group II), rats were subjected to 30 min global cerebral ischemia followed by reperfusion for 3 h. Group III and Group IV were treated with ASE (*Amaranthus spinoses* extract) at the doses of 200 mg/kg and 400 mg/kg body weight orally, and rats were subjected to global cerebral ischemia on 7th day. Group V rats were treated with standard drug vitamin E (50 mg/kg, p.o) and rats are subjected to global cerebral ischemia on 7th day employed for the study. Vitamin E suspension was prepared by dissolving it in 4% tween 80 and given orally). Five additional days of post-ischemic survival time were provided. After 5 days, behavioral studies were carried out.

Rats were sacrificed by an overdose of ketamine (75 mg/ml/kg) injected intra-peritoneally as an anesthetic on the 6th day after the completion of behavioral tests. The isolated brains were frozen for biochemical tests.

One animal was taken for infarct size measurement from each group and 5 animals for biochemical estimation and behavioral studies from each group and 2 animals for histopathological studies from each groups [5, 6, 7].

Neurodeficit Score: After ischemia/reperfusion, the animals from one group were taken then neurological condition of the animals was assessed utilizing four categories of neurological findings and noted 0 = no watched neurological deficit; 1 = contralateral forelimb flexion with wrist flexion and shoulder adduction; 2 = reduced resistance to lateral push and 3 = circling movements towards the ipsilateral side [8].

Rota Rod Test: All animals were tried for their capacity to stay on the rotating bar at a speed of 20 rpm on a Rotarod mechanical assembly. Every animal was prepared for at least three trials. Following five post-ischemic days, the animals were tried for motor impairment after the administration of test drugs. Latency to fall off from the rotating rod was noted for every trial with a 5 min maximum to termination of the trials [9].

Beam Walk Test: Beam walk test was employed to evaluate fore and hind limb motor coordination. Each animal was individually placed on a beam

walk apparatus (Inco) made up of a wooden bar 60 cm long and 1.5 cm wide, height 50 cm. The motor performance of rat scored on a scale ranging from 0 to 4. This is a special test for animals subjected to cerebral ischemia and reperfusion. For motor incoordination, a number of foot slip, Number of falls, distance traveled along the beam was studied [9].

Elevated Plus Maze (EPM): The elevated plus maze served as the exteroceptive behavioral model (wherein the stimulus existed outside the body) to evaluate learning and memory in an animal. The apparatus consisted of two open arms (16 cm × 5 cm) and two covered arms (16 cm × 5 cm × 12 cm). The arms extended from a central platform (5 cm × 5 cm), and the maze was elevated to a height 25cm from the floor. Transfer latency (TL) was taken as the time taken by the rats to move into any one of the covered arms with all its four legs. TL was recorded on the 7th day. If the animal did not enter into one of the covered arms within 90 sec., it was gently pushed into one of the two covered arms and the TL was assigned as 90 sec. The rats was allowed to explore the maze for 10 sec and then returned to its home cage. Memory retention was examined 24 h after the 7thday trial on the 8th day [10]. The ‘‘inflexion ratio (IR)’’ using the formula,

$$IR = (L1 – L0) / L0$$

Where, L0 = transfer latency on day-2/day-9 in sec. L1 = initial transfer latency in sec.

Infarct Size Measurement: The brain was removed and frozen at -4 ℃ for 5 min. Coronal slices were made at 1 mm to 2 mm and sections were immersed in 1% of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich Co.) at 37 ℃ for 20 min. Then, the slices were immersed in 4% paraformaldehyde overnight. TTC is converted to red formazone pigment by NAD and dehydrogenase present in living cells. Hence, viable cells were stained deep red. The infracted cells have lost the enzymes and thus remained unstained. After TTC staining slices were photographed with digital camera and were analyzed by image analyzer program (Image analyzer) to determine the extent of the infarct zone. The infarct volume was calculated with the formula;

Infarct volume (%) = (Contralateral hemisphere area- healthy area of ipsilateral hemisphere) × thickness of slice [11]

Biochemical Estimation: After decapitation, ischemia/reperfusion groups, the brain was removed and washed in ice-chilled 0.9% saline, and was kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1 M, pH 7.4) to make a 10% w/v homogenate using a homogenizer. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 ºC, and the supernatant was kept in the deep refrigerator at -80 ℃ for analyzing biochemical estimations (SOD, CAT, MDA) [12].

Histopathological Examination: At the end of experimental procedures, rats were sacrificed, and the brains were dissected out. The brains were transferred to 10% formalin. Sections (<5 µm thick) of the frontal forebrain were prepared and stained by hematoxylin and eosin for microscopical examination.

RESULTS: This test was well-known by short time blocking of the carotid artery for 30 min (ischemia) followed by reperfusion and it can cause sudden loss of vision, memory, coordination, and balance. The cognitive and neurological status of the animals was assessed by neurobehavioral test.

The test drugs improve neurodeficit score, and motor performance in ischemic rats neuro-behavioral tests such as neurodeficit scoring, pole fall beam walk test results showed impairment in neurobehavioral scale in I/R group. The neurobehavioral outcomes [such as neurological deficit score, beam walk test, and TL] were significantly improved by the administration of the test drugs in dose-dependent manner as compared to the I/R group. ASE 400 mg/kg was found to be comparable with the standard vitamin E as the results are seen in **Table 1**.

FIG. 1: BLOCKING THE LEFT INTERNAL BRANCH OF THE COMMON CAROTID ARTERY

TABLE 1: THE EFFECT OF ASE AND VIT. E IN BEHAVIOURAL TEST AFTER I/R

S. no.	Groups	Neurodeficit score	Falling time in sec	Beam walk test	Transverse latency (TL) in sec		Inflexion ratio
					On 7th day	On 8th day	
1	Shame Control	0	3.95±0.74	2.00±0.44	44.50±4.4	40.33±4.93	0.1
2	I/R group	3.66±0.21	1.13±0.24	3.00±0.36	72.75±4.92	68.00±12.57	0.05
3	ASE (200 mg/kg)	2.16±0.30**	3.46±0.39*	2.50±0.42	68.11±2.22	62.45±9.44*	0.09
4	ASE (400 mg/kg)	1.33±2.95***	4.08±0.47**	1.00±0.25**	49.09±6.10	30.03±11.01***	0.34
5	Vit E (50 mg/kg p.o.)	1.66±0.33***	4.02±0.45**	1.00±0.25**	56.21±3.21	41.31±0.92***	0.267

All values are mean ± SEM. Statistical analysis of data was carried out by one-way ANOVA followed by Dunnett's test. ns = not significant, .*P<0.05, **P<0.01 and ***P<0.001 when compared with I/R group.

The test drugs significantly decreased the Infarct volume compared to the I/R control in a dose-dependent manner as the results are shown in **Table 2**.

TABLE 2: THE EFFECT OF ASE AND VIT. E ON INFARCT VOLUME AFTER I/R

S. no.	Groups	Infarct volume (%)
1	Control (vehicle-5ml/kg)	00
2	I/R control (vehicle-5ml/kg)	37
3	I/R + ASE (200mg/kg p.o.)	29
4	I/R + ASE (400 mg/kg p.o.)	24
5	I/R + Vit E (50 mg/kg p.o.)	23

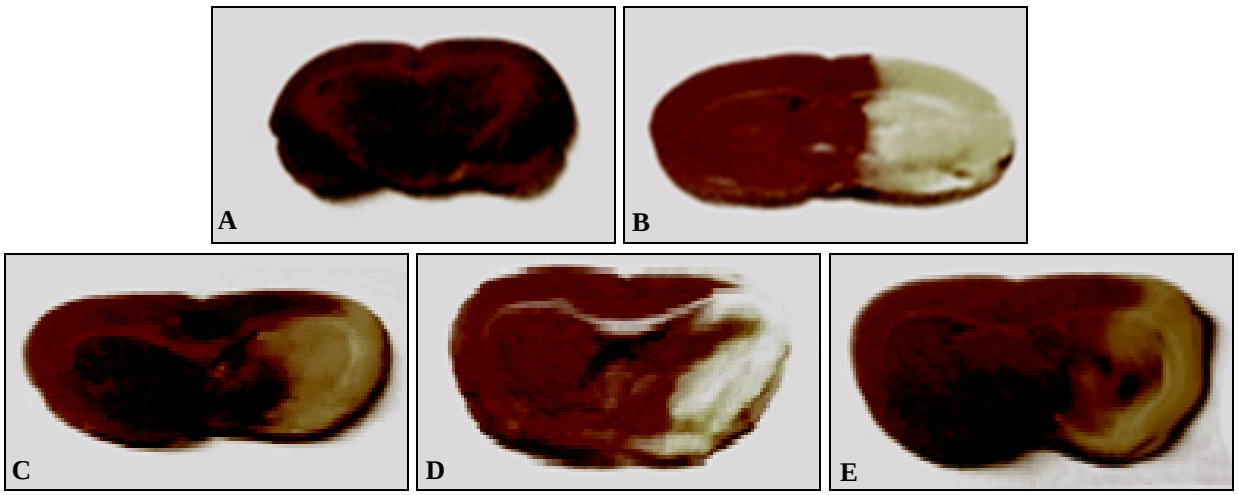

FIG. 2: INFARCT SIZE OF DIFFERENT GROUPS LIKE A = CONTROL GROUP, B = I/R CONTROL GROUP, C = ASE 200, D = ASE 400 AND E= VIT E 50

MDA levels were significantly increased, and SOD, CAT levels were significantly decreased in I/R group of rats as compared to sham control group. The test drugs treated groups, MDA levels were significantly reduced, and SOD and CAT levels were increased significantly as the results are shown in **Table 3**.

TABLE 3: THE EFFECT OF ASE AND VIT. E BY *IN-VIVO* ANTIOXIDANT ACTIVITY

S. no.	Groups	CAT (U/mg of protein)	SOD (U/mg of protein)	MDA (nmole/gm of wet tissue
1	Shame Control	67.40±4.31	34.33±2.65	186.8±4.58
2	I/R group	34.40±2.60	15.50±1.78	366.6±16.85
3	ASE (200 mg/kg)	52±4.33*	29.00±3.86**	272.4±20.85**
4	ASE (400 mg/kg)	61.20±2.51***		176.2±952***
5	Vit E (50 g/kg)	59.40±2.67***		162.0±5.17***

All values are mean ± SEM. Statistical analysis of data was carried out by one-way ANOVA followed by Dunnett's test. ns = not significant, .*P<0.05, **P<0.01 and ***P<0.001 when compared with I/R group.

The histopathology results of I/R study of the brain and extract treated groups are given below, it was observed that section of brain tissue showing swollen neurons, dilated blood vessels with neuronal loss occurred in brain regions of I/R rats induced by BCAO for 30 min followed by 3 h

reperfusion in ischemic control group. While no apparent morphological changes in sham control and brain section showing normal structure. The test drugs treated group of 3 h reperfusion brain section showed significantly prevented the neuron loss by compared with the ischemic control group, as shown in **Fig. 2**.

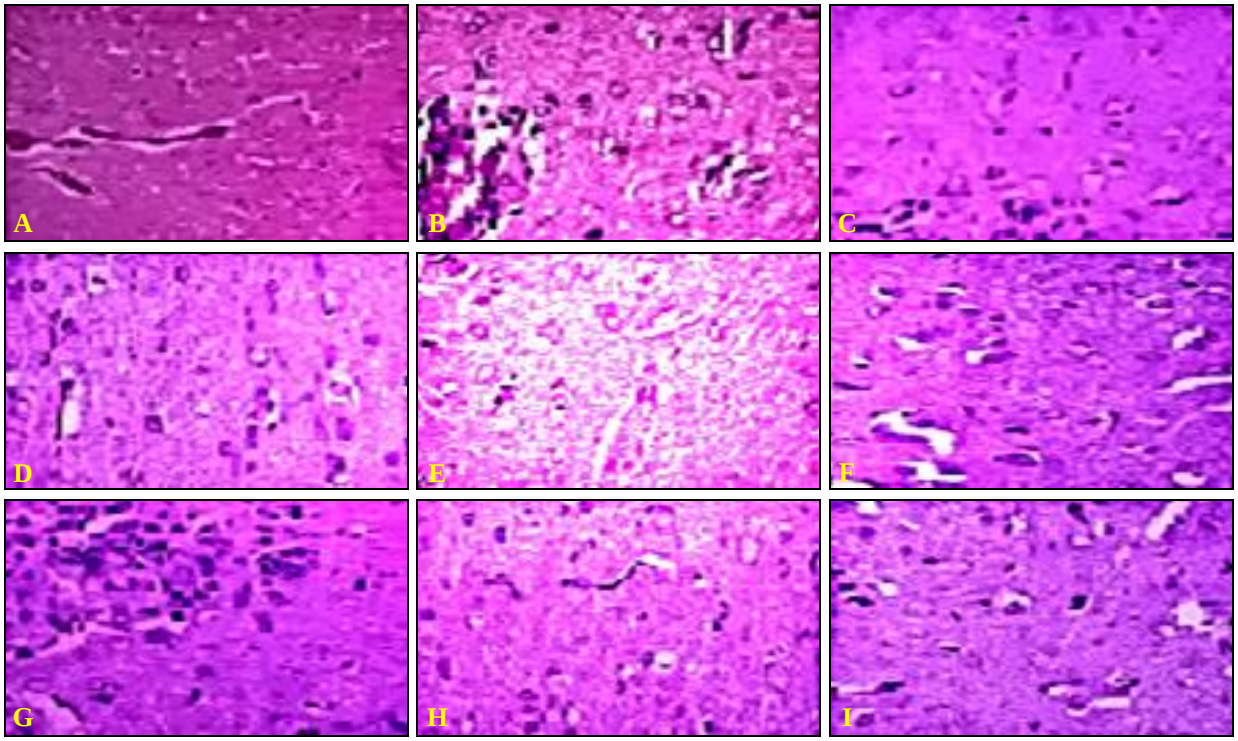

FIG. 3: HISTOPATHOLOGICAL STUDIES. (H&E 20X) OF A = CONTROL GROUP, B = I/R CONTROL GROUP, C = ASE 200, D = ASE 400, E = DDE 200, F = DDE 400, G = C1COMB, F = COMB. C2, AND I = VIT E 50

DISCUSSION: Ischemia is defined as the deficiency of blood in a part of the body, generally due to functional constriction or actual obstruction of the blood vessel and reperfusion injury related to tissue/cell damage caused when blood supply normally returns to the tissue after a period of ischemia. A brain stroke is a sudden loss of brain function usually caused by a blockage or leakage of a blood vessel. It develops from a complex cascade of cellular events that ultimately leads to cerebral infarction[13] and causes a sudden loss of vision, balance, coordination, speech, and memory [14].

The present study was undertaken to evaluate the neuroprotective effect of ASE on ischemia-reperfusion (I/R) brain injury. The neurotoxicity was done by blocking the left internal branch of the common carotid artery, followed by reperfusion. After I/R there is a result of the formation of toxic reactive oxygen species (ROS). Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the capability of antioxidant defenses, such as catalase and SOD enzymes. Severe oxidative stress can cause cell death, and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis. ROS can cause cellular damage by oxidizing membrane lipids, essential cellular proteins [15].

In the absence of substantial results with single phytoconstituent direction, herbal extracts containing several phytoconstituents are being evaluated for neuroprotective and therapeutic effects. The neuroprotective effect of ASE 200 and 400 mg/kg and vitamin E 50 mg/kg as reference was tested in ischemic rats. In this study, it was observed that ASE (200 and 400 mg/kg) significantly improved the ischemia-induced neurological status motor performance and significant changes in memory in neurodeficit score, rotarod test, beam walk test, and elevated plus maze tasks.

This finding is supported by the previous studies where *Bacopa monniera* reversed these effects in ischemia-reperfusion brain injury due to its antioxidant potential [16].

The biochemical investigations of the present study have shown that ASE 200 and 400 mg/kg significantly increased the catalase and superoxide dismutase enzyme activities, which suggests that ASE (200 and 400 mg/kg) may reduce the formation of free radicals. It was also observed that ASE reduced the MDA, which is the marker of lipid peroxidation, suggesting that the antioxidant potential of ASE may have reduced the formation of ROS and subsequently prevented the disastrous chain reaction.

This finding is supported by the previous studies where curcumin, resveratrol, silymarin, green tea extract containing antioxidant potential have counteracted the ROS generation during ischemia-reperfusion induced brain injury [9]. The phytoconstituents present in ASE are flavonoid, phenolic acid, and tannins, *etc*. [4,] which have been already proved to be a potent antioxidant.

Hence, in this study, the neuroprotective effects of ASE 200 and 400 mg/kg were observed. Therefore, we suggest that ASE 200 and 400 mg/kg may be useful in stroke and may prove to be neuroprotective.

CONCLUSION: It was concluded that ASE 200 and 400 mg/kg had attenuated the ischemia-reperfusion induced neurological deficit, decrease in motor performance, beam walk test, and by exerting the antioxidant effects. Further investigations with the isolation of purified active phytoconstituents of ASE (200 and 400 mg/kg) may also validate the neuroprotective and anti-oxidant effects.

ACKNOWLEDGEMENT: The authors are thankful to VC MP. Pandey and Director Dr. Susheel Kumar IFTM University for encouragement, support, and providing the necessary facilities to carry out the research work.

CONFLICTS OF INTEREST: There is no conflict of interest.

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How to cite this article:
Abid M and Khan NA: Protective effect of *Amaranthus spinosus* for ischemia reperfusion injury in rat brain. Int J Pharm Sci & Res 2020; 11(10): 5129-34. doi: 10.13040/IJPSR.0975-8232.11(10).5129-34.