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EVALUATE THE NEUROPROTECTIVE EFFECTS OF *WALSURA PISCIDIA* LEAVES AGAINST TRANSIENT GLOBAL CEREBRAL ISCHEMIA IN RATS

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ABSTRACT

Stroke is defined as an acute neurologic dysfunction of vascular origin with sudden (within seconds) or at least rapid (within hours) occurrence of symptoms and signs corresponding to the involvement of focal areas in the brain.Herbal medicine may be beneficial for the treatment and prevention of stroke.The aim of present study is on neuroprotective effects of ethanolic extract of *Walsura piscidia leaves* [EEWP] against transient global cerebral ischemia. *Walsura piscidia* is a traditionally used plant for various ailments. It has many pharmacological actions like bark-stimulant, expectorant, emmenagogue, emetic. Global cerebral ischemia was induced by temporary bilateral carotid artery occlusion followed by reperfusion. The present study indicates that the ethanolic extract of *Walsura pisidia leaves* may be considered for neuro protection. However prophylactic treatment with EEWP could mitigate memory impairment following ischemic stroke. Both low and high doses show dose dependent effects.

INTRODUCTION

Stroke is defined as an acute neurologic dysfunction of vascular origin with sudden (within seconds) or at least rapid (within hours) occurrence of symptoms and signs corresponding to the involvement of focal areas in the brain. Stroke related brain injury is a major neurological disability worldwide, the second most common cause of dementia and the third leading cause of death. It has enormous clinical, social, and economic implications. In practice, stroke refers to a range of conditions that are caused by occlusion or hemorrhage of one of the main arteries supplying blood to cerebral tissues. Ischemia is simply defined as the diminution of cerebral blood flow (CBF) to a critical threshold that propagates brain damage. Global cerebral ischemia entails reduction in CBF over the entire brain, encountered clinically as result during extracorporeal circulation following cardiac arrest from ventricular fibrillation that lasts 5 to 10 minutes. The biochemical cascades during and after brain ischemia are very complex[1,5]. During acute ischemia, energy stores in the brain become entirely depleted within five minutes. This causes membrane pump failure which is permissive to membrane depolarization with a subsequent rise in cytoplasmic calcium ion. The continuation of anaerobic glycolytic metabolism leads to lactate formation and cellular acidification. The acidification activates acid-sensing ion channels which results in

a further influx of calcium[2,6]. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. Several reviews have been published on the effect and potential benefits of traditional Eastern medicine in stroke. It has been suggested that some herbal medicines, or their products, may improve microcirculation in the brain, protect against ischemic reperfusion injury, possess neuroprotective properties and inhibit apoptosis, thus justifying their use in ischemic stroke patients[4,7]. *Walsura piscidia* is a traditionally used plant for various ailments. It has many pharmacological actions like bark-stimulant, expectorant, emmenagogue, emetic. It is also used to kill vermin in the hair. Bark is use in the treatment of skin diseases, expectorant and stimulant. Traditionally the plant is reported for its Anti-microbial agent[9,10]. According to the literature tribal people uses this plant to treat various diseases like skin allergies, astringent and diarrhea. The plant bark is used as astringent to treat diarrhea and other diseases. Plant has been shown antifeedant activity against insect and pests.

Fig 1:*Walsura piscidiaRoxb*

Literature revealed that *Walsura piscidia* has not been reported against transient global ischemia or stroke related disorders earlier. Hence the present study is on neuroprotective effects of ethanolic extract of *Walsura piscidia leaves* [EEWP] against transient global cerebral ischemia.

Materials and Methods

Collection of plant and authentication: The leaves of *Walsura Psidia Roxb* were collected from Seshachalam forest, Tirumala, Chitoor Dt, Andhra Pradesh, India. The plant was authentified by Dr. K. Madhavachetty, Associate Professor, Department of Botany, Sri Venkateswara .University, Tirupati, A.P, India.

Drugs and chemicals: Vitamin C was obtained from Eurokem laboratories Pvt limited Thiruporor. Trichloro acetic acid, 2-thiobarbituric acid, and Triphenyltetrazoliumchloride were obtained from Himedia laboratories, Mumbai. Malonaldehyde (1, 1, 3, 3-tetraethoxy propane)were obtained from Sigma Aldrich, Bangalore. Lignocaine gel and Ketamine i.p were obtained from Neon laboratories Pvt Ltd, Mumbai. NADPH and Glutathione reductase and Glutathione were obtained from Sisco Research Laboratories, Mumbai. Xylazine was obtained from Indian Immunologicals Hyderabad. Adrenaline tartrate was obtained from Hindustn Pharmaceuticals, Barauni. Sodium dihydrogen phosphate, potassium dihydrogen phosphate, Tris buffer, carbonate buffer and all other reagents used were of analytical grade.

Equipments: Electronic balance (Shimadzu, Model no: DS-825J), Remi centrifuge (Remi, Model no: KKLO-9013), Tissue homogenizer (Ever shine, Model no: 607), UV-Visible spectrophotometer, Elevated plus maze, Open field test, Rota- rod, Y-maze, Digital thermometer, thermally controlled operating table and surgical materials.

Experimental animals: Healthy adult male wistar rats weighing between 250-300gm were obtained from Central Animal House, Sri Krishna Teja Pharmacy College, Tirupati and used for the present study. The animals were housed in polypropylene cages at a controlled room temperature of 25± 2^0 C, under a 12 h light and 12 h dark cycle. After one week of acclimatization, the animals were used for experimentation. All the experiments and protocol described in the present study was

approved by the Institutional Animal Ethical Committee. The surgical procedure and all the behavioural experiments were carried out in a room adjacent to that in which the rats were housed under the same conditions of temperature, humidity and light cycle.

Preparation of extract: The collected leaves of *Walsura Psidia* Roxb were dried in shade for a period of 14 days, at an ambient temperature of 22˚C. The dried samples were grinded properly using a grinder, to obtain the powdered form. And then the weighed quantity of powder was passed through 40 mesh sieve. Extraction was done successfully by soxhlet apparatus with 95% of alcohol and distilled water. The plant material was extracted for 15 cycles with solvent. The solvent from extract was recovered by distillation apparatus under reduced pressure. A brownish green waxy residue was obtained. The dried extract thus obtained was kept in a dessicator and was used for further studies. The animals received this extract orally in dosages of 200 and 400 mg/k g d a y dissolved in distilled water. The particular dose was selected on the basis of preliminary and acute oral toxicity studies.

Qualitative phytochemical analysis: The ethanolic extract of *Walsura piscidia* Roxb [EEWP]leaveswas subjected to different chemical tests separately for the identification of various active constituents such as alkaloids, proteins, amino acids, glycosides, flavonoids, tannins and steroids.

Acute toxicity study: Acute toxicity study of EEWP was carried out in rats according to OECD 423 guidelines. Different doses of EEWP were administered up to 2000/kg b.w. (p.o.) and the rats were observed for a period of 72 hr for behavioural changes, toxic symptoms and mortality.

Induction of global cerebral ischemia: Global cerebral ischemia was induced by temporary bilateral carotid artery occlusion followed by reperfusion. Anaesthetizedrat with ketamine and xylazine at a dose of 80 mg/kg i.p. Rats were transferred to the surgery table. Ventral neck region was shaved. Area was washed with 70% ethanol. All loose fur were removedand treated with betadine solution. Temperature measurement was carried out and was maintained at 37.0°C. A small midline skin incision was made in neck. The thyroid gland was gently separated with non-traumatic forceps. Both common carotid arteries were isolated. Care was taken to avoid damaging the vagal nerves and separated with the help of curved forceps. Silk suture was loopedunder each artery for each access to vessels. Vessels were made free enough to allow easy and rapidplacement of clamps. Non-traumatic vessel clamps was applied to each artery for a defined period (10 min) and after that allowed for reperfusion.24 hours after reperfusion, behavioral tests and cognitive tests were performed.

Experiment procedure: The animals were randomly divided into 5 groups of 6 animals each and EEWP was freshly suspended in distilled water and administered to animals by oral feeding needles. Group 1 Animals (Positive control) with sham operation (without occlusion) and treated with control vehicle, normal saline only (p.o). Group 2 Animals (Negative control) with BCAO and treated with control vehicle (normal saline) only (p.o). Group 3 animals with BCAO and treated with 250 mg kg of Vitamin C (p.o). Group 4 animals with BCAO and treated with 200 mg /k g of EEWP (p.o). Group 5 Animals with BCAO and treated with 400 mg /k g of EEWP (p.o). The treatment was continued for 21 days. On 22nd day the animals were anaesthetized and stroke was induced by occlusion of bilateral carotid artery (BCAO) for definedperiod (10 min) with aneurism clamps placed on both arteries.

Table 1: Treatment schedule for assessment of ameliorative effect of EEWP against transient global ischemia in rats

S. No	Group	No. of animals	Treatment	No. of days
1	Normal	6	Vehicle (Normal saline, p.o) only	21
2	Control	6	BCAO + Vehicle (Normal saline, p.o) only	21
3	Standard	6	BCAO + Vitamin C (250mg/kg, p.o) only	21
4	Test-1	6	BCAO + EEWP (200mg/kg, p.o) only	21
5	Test-2	6	BCAO + EEWP (400mg/kg, p.o) only	21

Table 2:Phyto-chemical screening of aerial parts extract of *WalsurapiscidiaRoxb*

S.No	Chemical Constituents	EEWP
1	Alkaloids	-ve
2	Glycosides	+ve
3	Saponin glycosides	+ve
4	Flavonoids	+ve
5	Tannins	+ve
6	Steroids	+ve
7	Triterpenoids	+ve
8	Coumarine	+ve
9	Phenols	+ve
10	Protiens	-ve
11	Carbohydrates	+ve

+ve sign indicates presence; - ve sign indicates abscence;

Table 3: Effect of EEWPon Infarct volume (% Infarct volume) (Mean ± S.E.M)

S.No	Groups	Treatment	% Infarct volume
1	Normal	Received Normal saline 1ml/kg (p.o)	3.6 ± 0.81
2	Control	BCAO + Normal saline 1ml/kg (p.o)	74.45 ± 2.9$^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	27.03 ± 1.98***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	58.98 ± 2.94**
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	50.17 ± 3.01***

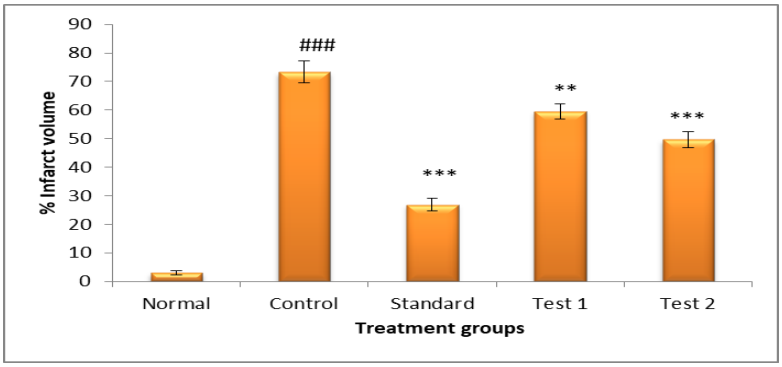


Fig 2:Effect of EEWP on % Infarct volume

Normal = vehicle treated; Control = BCAO + vehicle treated; Standard = BCAO + Vitamin C (250mg/kg, p.o); Test -1 = BCAO + EEWP (200mg/kg, p.o); Test -2 = BCAO + EEWP (400mg/kg, p.o)

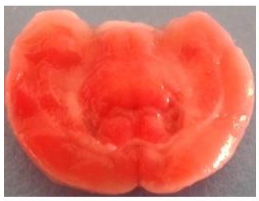


Photo no: 1 Infarct area in Normal

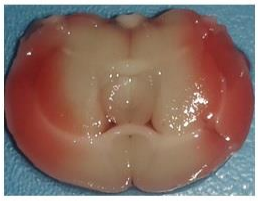


Photo no: 2 Infarct area in BCAO

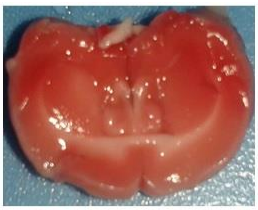


Photo no: 3 Infarct area in Standard (Vitamin C)

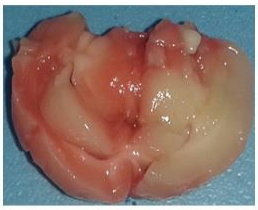


Photo no: 4 Infarct area in Test 1 (EEWP low dose)

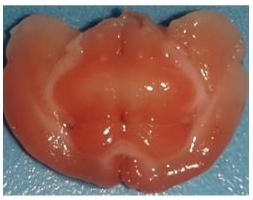


Photo no: 5 Infarct area in Test 2 (EEWP high dose)

Table 4:Effect of EEWPon Beam balance (Score) (Mean ± S.E.M)

S.No	Groups	Treatment	Score
1	Normal	Received Normal saline 1ml/kg (p.o)	1.39 ± 0.19
2	Control	BCAO + Normal saline 1ml/kg (p.o)	5.40 ± 0.63$^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	1.99 ± 0.35***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	3.74 ± 0.10**
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	2.49 ± 0.34***

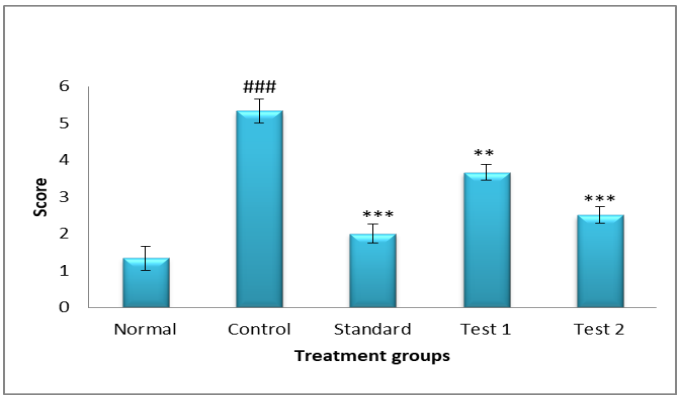


Fig 3: Effect of EEWP on beam balance test

Table 5: Effect of EEWPon Beam walk (Score) (Mean ± S.E.M)

S. No	Groups	Treatment	Score
1	Normal	Received Normal saline 1ml/kg (p.o)	5.99 ± 0.42
2	Control	BCAO + Normal saline 1ml/kg (p.o)	1.94 ± 0.28$^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	5.21 ± 0.57***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	3.98 ± 0.26**
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	4.56 ± 0.31***

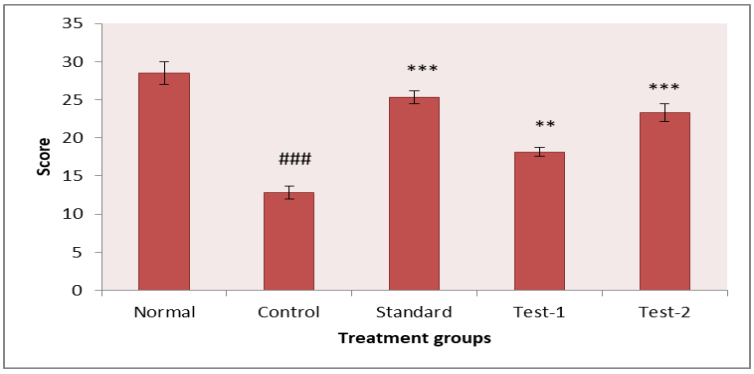


Fig 4:Effect of EEWP on beam walk test

Table 6: Effect of EEWPon Rota rod (time in sec) (Mean ± S.E.M)

S. No	Groups	Treatment	Time in sec
1	Normal	Received Normal saline 1ml/kg (p.o)	31.26 ± 1.93
2	Control	BCAO + Normal saline 1ml/kg (p.o)	13.01 ± 0.94$^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	25.18 ± 0.63***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	17.92 ± 0.83**
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	23.93 ± 0.72***

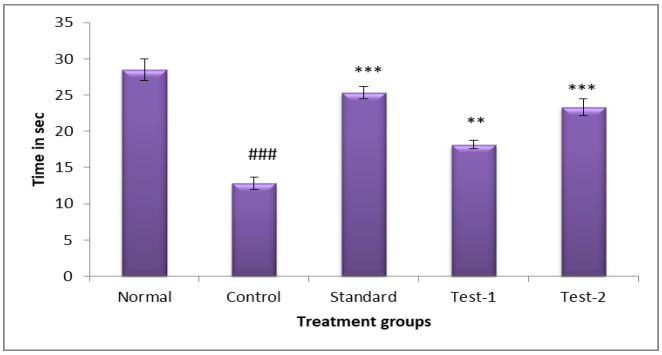


Fig 5:Effect of EEWP on Rota rod test

Table 7:Effect of EEWP on Y maze (% Spontaneous alterations) (Mean ± S.E.M)

S. No	Groups	Treatment	% Spontaneous Alterations
1	Normal	Received Normal saline 1ml/kg (p.o)	82.02 ± 1.19
2	Control	BCAO + Normal saline 1ml/kg (p.o)	50.12 ± 1.83$^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	77.04 ± 1.28***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	56.23 ± 1.17**
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	67.13 ± 1.93***

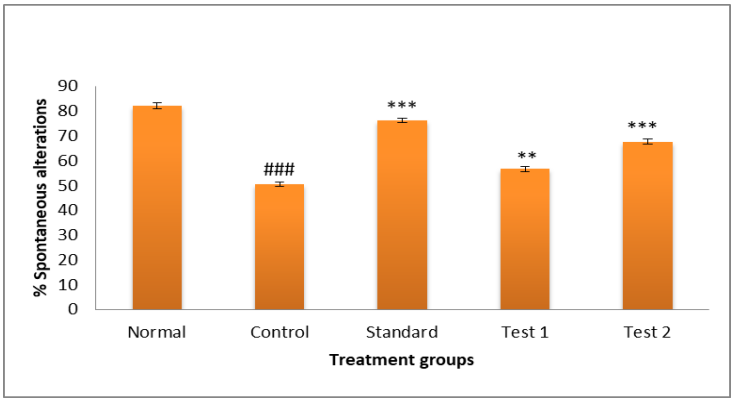


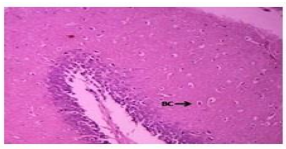
Fig 6: Effect of EEWP on Y maze test

Table 8: Effect of EEWP on Open Field Test (Mean ± S.E.M)

S.No	Groups	Treatment	Parameters				
			Ambulations	Rearings	Groomings	Immobility	Defecation
1	Normal	Received Normal saline 1ml/kg (p.o)	631.82 ±27.81	21.35 ± 0.42	11.14 ± 0.53	36.83 ± 2.05	3.82 ± 0.25
2	Control	BCAO + Normal saline 1ml/kg (p.o)	1085.00 ± 68.30$^{###}$	7.63 ± 0.45$^{###}$	3.14 ± 0.05$^{###}$	120.16 ± 4.07$^{###}$	1.24 ± 0.61 $^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	771.20 ± 17.35***	18.97 ± 0.59***	8.45 ± 0.74***	60.21 ± 2.43***	3.53 ± 0.27***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	859.23 ± 21.93**	12.13 ± 0.30**	7.12 ± 0.35**	102.98 ± 3.08**	2.41 ± 0.29*
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	820.62 ± 36.21***	16.94 ± 0.92***	8.14 ± 0.97***	48.73 ± 3.92***	3.12 ± 0.53***

Table 9:Effect of EEWPon Brain tissue antioxidant Levels (Mean ± S.E.M)

S.No	Groups	Treatment	LPO (nM /mg protein)	SOD (Units/mg protein)	GSH (µg of GSH/mg protein)	CAT (µ moles of H_2O_2 consumed/min/mg)
1	Normal	Received Normal saline 1ml/kg (p.o)	5.04 ± 0.31	19.02 ± 0.47	146.5 ± 3.05	1.65 ± 0.05
2	Control	BCAO + Normal saline 1ml/kg (p.o)	11.06 ± 0.39$^{###}$	10.73 ± 0.58$^{###}$	73.86 ± 1.49$^{###}$	0.62 ± 0.02$^{###}$
3	Standard	BCAO + Vitamin C 250mg/kg (p.o)	5.69 ± 0.43 ***	18.20 ± 0.83***	140.1 ± 2.30***	1.45 ± 0.01***
4	Test 1	BCAO + EEWP 200mg/kg (p.o)	7.94 ± 0.29 **	15.82 ± 0.85*	114.2 ± 4.27**	0.85 ± 0.02**
5	Test 2	BCAO + EEWP 400mg/kg (p.o)	5.58 ± 0.85***	16.32 ± 0.73***	135.38 ± 1.85***	1.14 ± 0.03***



no 6: Photomicrograph of the hippocampus of normal group

BC = Brain cells

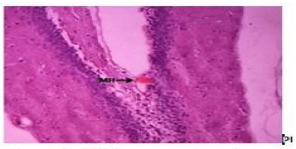


Photo no 7: Photomicrograph of the hippocampus of control group

MH = Mild hemorrhage

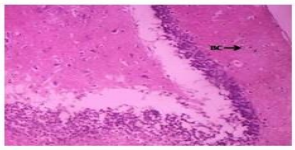


Photo no 8: Photomicrograph of the hippocampus of standard group

BC = Brain cells

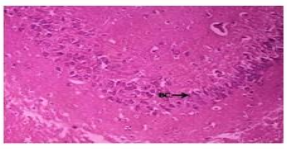


Photo no 9: Photomicrograph of the hippocampus of low dose group

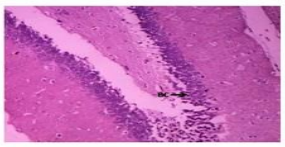


Photo no 10: Photomicrograph of the hippocampus of high dose group

Later clamps were removed to allow reperfusion and animals were then returned to their cages. 24 hours after reperfusion behavioural tests and cognitive tests were performed. And the animals were sacrificed. Brain were sliced to 2mm and stained with 2, 3, 5-triphenyltetrazoliumchloride for measuring infract volume. The remaining brain homogenized content wasused for the estimation of anti-oxidant levels. Histopathology of hippocampal CA1 region was carried out.

Statistical analysis: In the present study, all the data was expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one way ANOVA followed by the Tukey test using computer based fitting program (Prism graph pad 5.0). Statistical significance was set accordingly.

RESULTS AND DISCUSSION

Preliminary phytochemical screening:

The data corresponding to table 2 describes the preliminary phytochemical investigation report of ethanolic extract of leaves of Walsurapiscidia. Phenols and flavonoids, glycosides, saponins, steroids, tannins, coumarins, carbohydrates and triterpenoids are present in ethanolic extract.

Acute oral toxicity study:

The leaves of Walsurapisidia was found to be safe since no animal died at the maximum tested single dose of 2000 mg/kg when administered orally and the animals did not show any gross behavioral changes.

Selection of dose:

Based on the literature survey, acute and sub-acute toxicity studies on *Walsura Psidia* revealed that both aqueous and alcoholic extracts are practically nontoxic at single dose of 2000mg/kg and did not shown any behavioral changes, no morbidity and mortality. Hence, 1/10th and 1/5th of LD_{50} dose were taken for this study.

Effect of EEWP on infarct volume:

Infarct volume is the ischemic part in the brain. TTC converts into red formazone pigment by nicotinamide adenine dinucleotide (NAD) and dehydrogenase present in living cells. Hence viable cells were stained deep red. The ischemic area where the cells are live are unstained and it is measured and expressed in percentage infarct volume. The results were shown in Table 3 Graph no: 1 and Photos 1-5.

Effect on beam balance:

The beam balance test assesses motor and vestibular function by quantifying the ability to balance on a

narrow wooden beam. The animal's performance was rated with the scale of Clifton which ranges from 1 to 6, with a score of 1 being normal and a score of 6 indicating that the animal was unable to support itself on the beam.

Effect of EEWP on beam walk:

The beam walking test was used to assess deficits in coordination and integration of motor movement, especially in the hind limb. The animal's performance was rated with the scale of Feeney which ranges from 1 to 7. A score of 7 indicates normal beam walking with less than 2 foot slips, and a score of 1 indicates that the rat is unable to traverse the beam.

Effect of EEWP on rota rod:

Motor integrity and coordination were assessed by the time latency from the placement of the animal on the rotating drum until it felt.Rats of group – II receiving vehicle shown momentous decline in time latency (13.01 ± 0.94; $p<0.001$) compared to group – I (31.26 ± 1.93) that indicates shortfall of motor integration and coordination in control than normal group.

Effect of EEWP on Y maze: Short term memory was assessed by spontaneous alternation behavior in the Y maze test. Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short term memory.

Effect of EEWP in open field test:

In this test scoring the behaviour of a rodent that has been forced into a novel arena. The stress procedure produced an initial state of behavioral arousal, as seen on psychomotor measures, and a reduction in emotionality as seen in a lowered defecation score. Between groups comparisons for all measures are presented in Table no: 8.

***In vivo* Antioxidant Parameters:**

In the present study, various antioxidant parameters were assessed in the brain at the end of the study on 23rd day. BCAO induced transient global ischemia shrunken the antioxidant levels in brain. The results are shown in the Table no: 9.

Histopathological examination:

Histopathological examination was monitored by haemotoxylin eosin staining revealed that hippocampal CA1 region in BCAO control group showed decreased in neuronal density than normal group and also mild hemorrhage was observed. It means swelling of nucleus, cellular shrinkage and neuronal cell death in hippocampus.In standard Vitamin C group the hippocampus shows dense neuronal cells and similar to normal cytoarchitecture. Pretreatment of EEWP low dose (200mg/kg) shown dense neuronal density and interestingly in high dose high density and similar to normal cytoarchitecture is observed. These results of Histological examination were reported in Photo 6- 10.

CONCLUSION

The present research work concluded that evaluation of neuroprotective effects of *Walsurapisidia* leaves against transient global cerebral ischemia in rats.The neuroprotective effect of EEWP in cerebral ischemia and reperfusion induced by BCAO in rats can be confirmed by decrease in percentage of infarct volume. 21 days pretreatment with the high dose of 400mg/kg EEWP was efficient against apoptosis caused cerebral ischemia.The neurobehavioural tests i.e, sensorimotor and cognitive parameters revealed that EEWP has significant beneficial effects in BCAO induced global ischemic neuronal injury. It is observed that effect is dose dependent. This effect might be due to reduced post ischemic damage in CA1 region of hippocampus. EEWP is expected to act as scavenger of free radicals contributing to mitigation of BCAO induced global ischemic neuronal injury. Furthermore we found that the expression level of lipid peroxidation, SOD, reduced glutathione and catalase which might contribute to the recruitment of EEWP into the brain and its

cerebroprotective effects. EEWP offered improvement in neuronal aggregation in CA1 region of hippocampus.In summary, this study showed those 21 days pretreatment with EEWPnot only caused pathological and physiological improvement on braindamage which had been subjected to hypoxiaischemia but also produced improvements in cognitive function.The neuroprotectiveeffects of EEWPhave been attributable almost entirely toits antioxidant actions.However, the precise underlyingmechanism and possible active ingredient are stillrequired further study.

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