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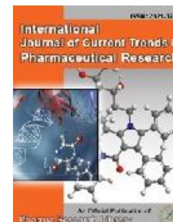

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# Neuroprotective Effects of Ethonolic Extract of Aeral parts of Clerodandrum serratum against Bilateral Carotid Artery Occulsion Induced Trasient Gliobal Cerebral Ischemia in Rats

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**A B S T R A C T**

**Objectives:** The objective of this study is to evaluate the neuroprotective effect Global cerebral ischemia was induced by temporary bilateral carotid artery occlusion followed by reperfusion (Oxidative stress). **Materials and Methods:** Albino wister rats of wither gender were used in the study. Evaluation of neuroprotective activity of flavonoid *Clerodandrum serratum* (in200 and 400 mg/kg oral doses) was carried out by using the global cerebral I/R model by bilateral carotid artery occlusion for 10 min, followed by 24 h reperfusion. The antioxidant enzymatic and non-enzymatic levels were estimated along with histopathological studies. **Result:** *Clerodandrum serratum* showed dose-dependent neuroprotective activity by significant decrease in lipid peroxidation ($P < 0.001$) and increase in the superoxide dismutase, catalase, glutathione and total thiol levels in gossypin treated groups when compared to control group. Cerebral infarction area was markedly reduced in *Clerodandrum serratum* treated groups when compared to control group. **Conclusion:** *Clerodandrum serratum* showed potent neuroprotective activity against global cerebral I/R injury- induced by temporary bilateral carotid artery occlusion followed by reperfusion (oxidative stress) in rats.

**Keywords:** Neuroprotective, flavonoids, Clerodandrum serratum, ischemia/reperfusion, natural products, oxidative stress.

**A R T I C L E I N F O**

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# 1. Introduction

Cerebrovascular diseases include some of the common disorders such as ischemic stroke, hemorrhagic stroke, cerebrovascular anomalies etc. They cause 2 million deaths each year and are a major cause of disability.(1) Stroke has been ranked third most common cause of death world-wide and cerebrovascular diseases are considered to be the second most frequent cause of projected deaths in the year 2020.(2) Cerebral infarction induced brain damage is closely associated with inflammatory responses especially infiltration of circulating neutrophils to ischemic tissue,(3)which are potential sources of reactive oxygen species (ROS) when activated. (4) In the global ischemia model in rats, blood supply to the brain is reduced by occluding the common carotid arteries with a reduction in blood pressure.(5)The restriction of blood flow causes mitochondrial failure and adenosine triphosphate (ATP) depletion, resulting in excessive glutamate release into the synapse. Excitotoxicity is associated with a massive increase in both cytoplasmic Ca 2+ and ROS production. The electrochemical gradient produced by this chain is used by the ATPase to synthesize ATP. The continuation of anaerobic glycolytic metabolism leads to lactate formation and cellular acidification.. In spite of great number of clinical trials, no neuroprotective agent has yet been shown to be effective in the treatment of stroke. (6,7) Flavonoids from plants are reported to have anti-oxidant activity, but their protection against ischemia/reperfusion (I/R) induced oxidative stress is less explored.(8)

Herbal medicine may be beneficial for the treatment and prevention of stroke. Herbal therapies used by millions of peoples now days with prescription and non prescription medications.(9) *Clerodendrum Serratum (L.)* was growing wild in the ravinea of Sivaa River near Sanoti village. Locally this species is called furedetu. Root is pungent, bitter, acrid, dry, heating, anti-inflammatory, digestive, carminative, depurative, expectorant, antispasmodic, stimulant, appetizer and anti-helmintic. It is used clinically in treatment of bronchitis, asthma, fevers, blood disease, tumours and inflammations, burning sensation, epilepsy, malaria, ulcer and wounds. Leaves are used in fever and hiccough. Its boiled leaves are used in cephalgia and opthalmia where as its boiled seeds in butter milk is used as aperients, in dropsy and in catarrhal affection of lungs. Root bark contains mainly sapogenins, while leaves contain flavonoids and phenolic acids. (10)

# 2. Materials and Methods

**Collection of plant and authentification:**

The aerial parts of *clerodendrum serratum* was collected from venkatchalam forest, Tirumala, Chitoor Dt, Andhra Pradesh,India. The plant was authentified by Dr. Madhavachetty, Associate Professor, Department of Botany, Sri Venkateswara .University, Tirupati, A.P, India.

**Preparation of extract**

The collected aerial parts of *clerodendrum serratum* were washed well using tap water. Then by cutting them into small pieces and it was 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 weighed quantity of powder 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 black waxy residue was obtained. The dried extract thus obtained was kept in a dessicator and was used for further studies.

**Experimental animals**

Healthy adult male wistar rats weighing between 250-300gm were obtained from Raghavendra enterprises (Bangalore) used for the present study. The animals were housed in stainless steel 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.

**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,Y-maze, Digital thermometer, thermally controlled operating table and surgical materials.

**Induction of global cerebral ischemia**

Global cerebral ischemia was induced by temporary bilateral carotid artery occlusion followed by reperfusion. Anaesthetized rat 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 removed and treated with betadine solution.[11] 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.[12] Both common carotid arteries were isolated. Care was taken to avoid damaging the vagal nerves and separated with the help of curved forceps. Non-traumatic vessel clamps was applied to each artery for a defined period (10 min) and after that allowed for reperfusion. Sham-operated control animals underwent all the surgical procedure except bilateral carotid artery

occlusion [BCAO]. 24 hours after reperfusion, behavioral tests and cognitive tests were performed.(13)

**Experiment procedure**

The animals were randomly divided into 5 groups of 6 animals each and EECS was freshly suspended in distilled water and administered to animals by oral feeding needles.

**Pharmacological study:**

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 defined period (10 min) with aneurism clamps placed on both arteries and later clamps were removed to allow reperfusion and animals were then returned to their cages. 24 hours after reperfusion behavioral tests and cognitive tests were performed. And the animals were sacrificed. Brain were sliced to 2mm and stained with 2, 3, 5-triphenyl tetrazoliumchloride for measuring infract volume. The remaining brain homogenized content was used for the estimation of anti-oxidant levels. Histopathology of hippocampal CA1 region was carried out.(14)

**Infract Volume**

The animals were sacrificed and brains were removed. The brains were cooled immediately after saline perfusion in phosphate-buffered saline and sectioned into 2-mm coronal sections.(15) The infarcted regions of each of the sections were visualized by 30-minute staining at room temperature in 2% 2, 3, 5 – triphenyltetrazolium chloride (TTC) in phosphate-buffered saline. TTC is an indicator of mitochondrial respiratory enzymes and is considered to be a rapid and convenient stain for detection of infarcted tissue.The infarct volume is expressed in percentage infarct formed of total volume of brain section (Aronowski *et al.,* 1994).

**Cognitive parameter**

**Elevated plus maze** Elevated plus maze is an established task to evaluate spatial memory functions in rats (Ukai *et al.,* 1993). The apparatus consisted of two open arms (50×10 cm each), two enclosed arms (50 × 10 × 20 cm each) and a central platform (10×10 cm), arranged in such a way that the two arms are opposite to each other. The elevation of the maze was 100 cm above the floor. Rat was placed on the edge of the open arm and trained to escape into the central compartment. Cut off time 90 seconds was maintained. Later 5 minutes of this experiment each animal was placed at the centre of the maze facing one of the enclosed arms. (16,17)

**Biochemical parameters**

**SOD**

SOD activity was determined based on its ability to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH. (18) Briefly, 25 µl of the supernatant obtained from the centrifuged brain homogenate was added to a mixture of 0.1 mM epinephrine in carbonate buffer (pH 10.2) in a total volume of 1 ml and the formation of adrenochrome was measured at 295 nm. The SOD activity (U/mg of protein) was calculated by using the standard plot.

**CAT**

CAT activity was assayed by the method of Calibore. (19)Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 0.05 ml homogenate (10%, w/v) in a total volume of 3 ml. Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of nM $H_2O_2$ consumed /min/mg protein.

**GSH**

GSH was estimated in various tissues by the method of Sedlak and Lindsay. (20) Briefly, 5% tissue homogenate was prepared in 20 mM ethylenediaminetetraacetic acid (EDTA), pH 4.7 and 100 µl of the homogenate or pure GSH was added to 0.2 M tris-EDTA buffer (1.0 ml, pH 8.2) and 20 mM EDTA, pH 4.7 (0.9 ml) followed by 20 µl of Ellman s reagent (10 mmol/l DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. Samples were centrifuged before the absorbance of the supernatants was measured. (21)

**LPO**

Thiobarbituric acid reactive substances (TBARS) in the homogenate were estimated by using standard protocol. Briefly, the 0.5 ml of 10% homogenate was incubated with 15% 2, 4, 6-trichloroanisaole, 0.375% 2, 4, 6-tribromoanisole and 5 N HCl at 95°C for 15 min, the mixture was cooled, centrifuged and absorbance of the supernatant measured at 512 nm against appropriate blank. The amount of LPO was determined by using $=1.56 \times 10^5$ /M/cm and expressed as TBARS nmoles/mg of protein.(22)

**Histopathological studies**

After completion of sensorimotor and behavioural tests rats were immediately euthanized and transcardially perfused with buffered formalin. Brains were removed, and kept in the same fixative for 3 days. Serial coronal sections (10 µm) through the dorsal hippocampus (between 1.5 and 2.0 mm posterior to the bregma) were obtained using a cryostat and stained with hematoxylin and Eosin. The neuronal density for a given animal represents the average of both the right and left neuronal cell densities. Neuronal density values are expressed as mean ± SEM.

**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.

## 3. Results and Discussions

Based on the literature survey, acute and sub acute toxicity studies on *clerodendrum serratum* 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 (Sarath *et al.*, 2014).

**Table 1:** Treatment schedule for assessment of Neuroprotective effect of EECS 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 + EECS (200mg/kg, p.o) only | 21 |
| 5 | Test-2 | 6 | BCAO + EECS (400mg/kg, p.o) only | 21 |

**Table 2:** Effect of EECS on Infarct volume (% Infarct volume) (Mean ± S.E.M)

| S.No | Groups | Treatment | % Infarct volume |
|---|---|---|---|
| 1 | Normal | Received Normal saline 1ml/kg (p.o) | 3.0 ± 0.73 |
| 2 | Control | BCAO + Normal saline 1ml/kg (p.o) | $73.33 \pm 3.7^{###}$ |
| 3 | Standard | BCAO + Vitamin C 250mg/kg (p.o) | $26.83 \pm 2.13^{***}$ |
| 4 | Test 1 | BCAO + EECS 200mg/kg (p.o) | $59.5 \pm 2.74^{**}$ |
| 5 | Test 2 | BCAO + EECS 400mg/kg (p.o) | $49.67 \pm 2.72^{***}$ |

**EECS:** Ethanolic extract of *Clerodendrum serratum,* BCAO: Bilateral common carotid artery occlusion, n=6 animals in each group, values are expressed as mean ± S.E.M, $^{###}$p<0.001; $^{##}$p<0.01 vs. normal ,***p<0.001; **p<0.01; *p<0.05 vs. control.

**Table 3:** Effect of EECS on No. of entries in closed and open arms (Mean ± S.E.M)

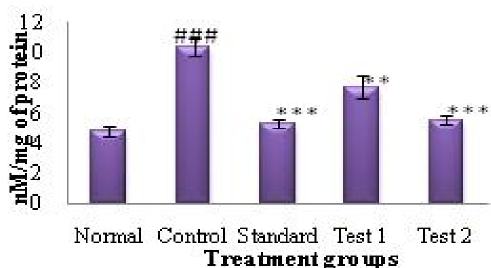
| S.No | Groups | Treatment | No. of Entries | |
|---|---|---|---|---|
| | | | Closed | Open |
| 1 | Normal | Received Normal saline 1ml/kg (p.o) | 1.84 ± 0.32 | 6.49 ± 0.41 |
| 2 | Control | BCAO + Normal saline 1ml/kg (p.o) | $6.04 \pm 0.53^{###}$ | $2.03 \pm 0.26^{###}$ |
| 3 | Standard | BCAO + Vitamin C 250mg/kg (p.o) | $3.10 \pm 0.26^{***}$ | $6.49 \pm 0.43^{***}$ |
| 4 | Test 1 | BCAO + EECS 200mg/kg (p.o) | $3.64 \pm 0.21^{**}$ | $4.05 \pm 0.26^{**}$ |
| 5 | Test 2 | BCAO + EECS 400mg/kg (p.o) | $2.54 \pm 0.49^{***}$ | $5.30 \pm 0.20^{***}$ |

n=6 animals in each group, values are expressed as mean ± S.E.M, $^{###}$p<0.001; $^{##}$p<0.01 vs. normal,***p<0.001; **p<0.01; * p<0.05 vs. control.

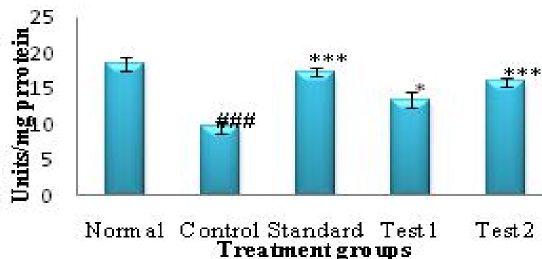
**Table 4:** Effect of EECS on Brain tissue antioxidant Levels (Mean ± S.E.M)

| 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) | 4.82 ± 0.21 | 18.72 ± 0.49 | 144.3 ± 2.74 | 1.62 ± 0.02 |
| 2 | Control | BCAO + Normal saline 1ml/kg (p.o) | $10.43 \pm 0.45^{###}$ | $9.83 \pm 0.59^{###}$ | $69.38 \pm 2.94^{###}$ | $0.59 \pm 0.01^{###}$ |
| 3 | Standard | BCAO + Vitamin C 250mg/kg (p.o) | $5.50 \pm 0.21^{***}$ | $17.56 \pm 0.45^{***}$ | $138.64 \pm 3.71^{***}$ | $1.42 \pm 0.03^{***}$ |
| 4 | Test 1 | BCAO + EECS 200mg/kg (p.o) | $7.62 \pm 0.43^{**}$ | $14.03 \pm 0.98^{*}$ | $112.3 \pm 4.32^{**}$ | $0.83 \pm 0.01^{**}$ |
| 5 | Test 2 | BCAO + EECS 400mg/kg (p.o) | $5.48 \pm 0.26^{***}$ | $16.01 \pm 0.42^{***}$ | $134.53 \pm 2.46^{***}$ | $1.11 \pm 0.03^{***}$ |

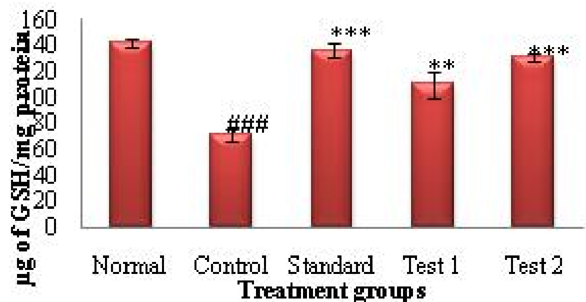
n=6 animals in each group,values are expressed as mean ± S.E.M, $^{###}$p<0.001; $^{##}$p<0.01 vs. normal,***p<0.001; **p<0.01; * p<0.05 vs. control



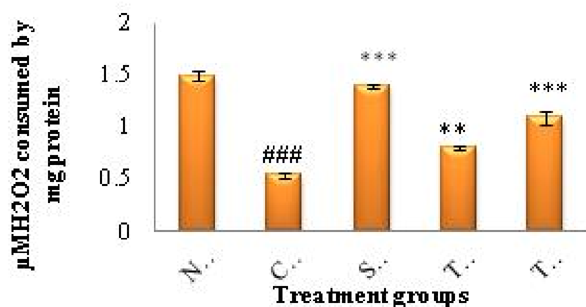

**Graph no. 1:** Effect of EECS on LPO levels




**Graph no. 2:** Effect of EECS on SOD levels




**Graph no. 3:** Effect of EECS on GSH levels




**Graph no. 4:** Effect of EECS on CAT levels

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

**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 EECS 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 **Figure.** 1-5

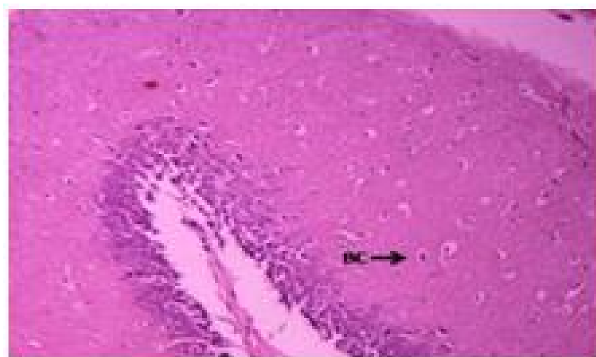



**Figure 1:** Hippocampus of Normal Group, BC=Brain cell

**Figure 2:** Hippocampus Control Group, MH=Mild hemorrhage

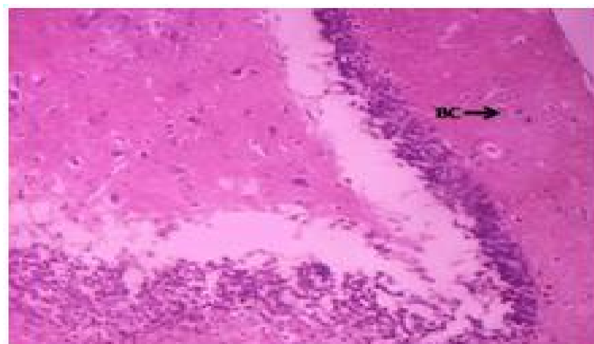



**Figure 3:** Hippocampus of Standard Group

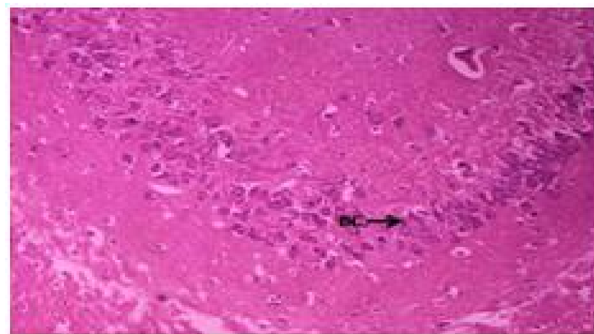



**Figure 4:** Hippocampus of Low Dose Group

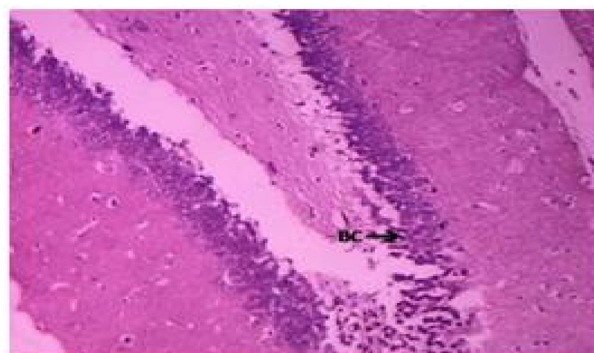



**Figure 5:** Hippocampus of High Dose Group

**Discussion:** Cerebrovascular diseases are conditions that develop as a result of problems with the blood vessels that supply the brain The study revealed that there is significant neuroprotection by the treatment group to the natural defensive mechanism by raise in superoxide dismutase, glutathione and catalase levels. These kinds of results resemble with the earlier reports on potent antioxidants ascorbic acid and Vitamin E results. Hence EECS should

consider having antioxidant potential in global cerebral ischemic conditions. Sarath *et al*., established enough evidence on antioxidant potential of *Clerodendrum serratum* (Sarath *et al.,* 2014).Histopathological examination revealed that hippocampal CA1 region in control group shows decreased in neuronal density than normal group and also mild haemorrhage is observed. Here clearly cellular shrinkage and neuronal apoptosis is observed. In vitamin C treated groups there is marked increase in neuronal density. Both EECS treated shows increase in neuronal density. There is similarity of treatment groups with the normal group in neuronal aggregation. This indicates the neuroprotective effect of EECS in global ischemic conditions (Fig:4&5). The present study indicates that the aerial parts ethanolic extract of *Clerodendrum serratum* may be considered for neuroprotection. However prophylactic treatment with EECS could mitigate memory impairment following ischemic stroke. Both low and high doses show dose dependent effects.

## 4. Conclusion

The neuroprotective effect of EECS 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 EECS was efficient against apoptosis caused cerebral ischemia. The neurobehavioural tests i.e, sensorimotor and cognitive parameters revealed that EECS 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.

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