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CEREBRO PROTECTIVE EFFECT OF FLAVONOID OF *EVOLVULUS ALSINOIDES* IN BILATERAL COMMON CAROTID ARTERY (BCCAO) INDUCED CEREBRAL ISCHEMIA

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ABSTRACT

The present study was conducted to evaluate the cerebro protective activity of the flavonoid of methanolic leaf extract of *Evolvulus alsinoides* against cerebral ischemic reperfusion – induced cerebral infarction in rats. The leaves of *Evolvulus alsinoides* were extracted using soxhelet extraction. The extract was subjected to column chromatograph. The chemical tests were carried out toconform the flavonoid. The structural elucidation was done by different spectral analysis. This pure compound was used for further studies. Male Wistar rats weighing 250-280gms were used in the present study. The animals were anesthetized with thiopentone sodium (45mg/kg) by i.p. earlier published method was used for induction of ischemia followed by ketamine (100mg/kg) i.p which is skeletal muscle relaxant. Prior to the incision Lidocain administrated (4mg/kg) subcutaneously. Ischemic condition was achieved by BCCAO for 45 min, followed by a reperfusion period of 48h. The Allopurinol (10 mg/kg) (XO inhibitor), L-NAME (10 mg/kg) (NO inhibitor) and Nimesulide (20 mg/kg) (COX inhibitor) were administered 10min before ischemia and EA (200mg/kg) was administered for 7 days p.o. After the reperfusion period the animals were sacrificed immediately and the brain was decapitated, homogenized, supernatant was collected then when treated various enzymatic estimations were carried out. Histopathological examination showed that *Evolvulus alsinoides* conformed the cerebro protective effect. The biochemical estimation showed that there was a significant decrease in the oxidant levels of MDA and MPO and significant increase in the antioxidant levels of CAT and SOD. The Flavonoid of *Evolvulus alsinoides* significantly inhibit the percentage of damage to the brain. Therefore the present study exhibited cerebroprotective effect when administered alone. A synergistic effect was observed when *Evolvulus alsinoides* was administered along with Nimesulide.

Key words: Cerebral ischemia, Flavonoid, *Evolvulus alsinoides*, BCCAO, Oxidative stress, Reperfusion injury.

INTRODUCTION

Stroke is the second most common cause of death worldwide (Murray CJ, Lopez AD, 1997) and 1/6 of all human beings will suffer at least one stroke in their lives (Seshadri S *et al*., 2006). Ischemic stroke accounts for approximately 80% of all strokes and results from a thrombotic or embolic occlusion of a major cerebral artery (Aysan D, Turgut T, 2007).

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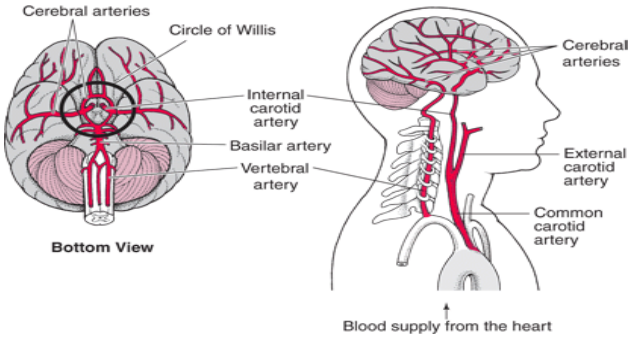


Carotid arteries are the blood vessels that deliver blood through the neck to the brain. There is one carotid artery on each side of the neck, where its pulsation can be felt with a finger below the jaw bone. Blockages in the carotid

artery decrease blood flow to the brain, causing a medical condition known as carotid artery disease. Interruptions in blood flow to the brain (commonly known as stroke) can cause permanent injury. The carotid arteries carry blood to the brain; like the blood vessels that supply blood to the heart, these arteries can become narrowed or blocked. The blockages are deposits of cholesterol, or atherosclerosis, that narrow the blood flow channel in the carotid arteries If these cholesterol deposits or blockages break or rupture, small blood clots and cholesterol fragments break off from the plaque, enter the blood flow to the brain, and can get caught in a smaller blood vessel in the brain, thus stopping blood flow to that area of the brain. The larger the cholesterol plaque and more severe the blockage of the carotid artery, the higher the risk of stroke (Piotr Sobieszczyk MD, Joshua B, 2006). So the stroke occurs due to sudden interruption of blood supply (normally caused by a thrombus or embolus occlusion or hemorrhage due to rupture of blood vessel) to a part of brain results in disruption of neurologic functioning. Cerebral ischemic stroke is a neurological disease where neuronal cell death is caused by a serial pathophysiological events, so called 'ischemic cascade' like energy failure, excitotoxicity, oxidative stress, inflammation, apoptosis etc. These all damaging factors are triggered by decreased/blocked blood flow (Mehta SL *et al*., 2007; Zhang F *et al*., 2010). Each of the above pathophysiological processes has a distinct time frame, some occurring over minutes, others over hours and days. These processes share overlapping and redundant features and cause injury to neurons, glia and endothelial cells. Within the core of the ischemic territory, where blood flow is most severely restricted, excitotoxicity and necrotic cell death occurs within minutes.

Therefore the Ischemia occurs due to the blockage of the carotid artery. So artificially Cerebral ischemia was induced in the male Wister rats by occluding the Bilateral common carotid artery (BCCAO). There were 10 groups namely SHAM group, Vehicle group, Ischemic reperfusion group, *Evolvulus alsinoides* group(EA), COX inhibitor group, COX inhibitor + EA group, XO inhibitor group, XO inhibitor+ EA group, NO inhibitor group and NO inhibitor+ EA group. In all these groups the rats of BCCAO are occluded except in SHAM group. In the SHAM group incision is done but not the occlusion. This group was selected to check whether the incision causes any brain damage. In the ischemic reperfusion group BCCAO are occluded for 45min and allowed for reperfusion, this was to check whether reperfusion causes further brain damage. COX, NO, XO are the main pathways through which cerebral ischemia occur. So the flavonoid of the plant *Evolvulus alsinoides* was simultaneously administered along with the COX inhibitor, NO inhibitor and XO inhibitor to find out the pathway of drug through which it acts. The histopathological and biochemical estimation was done to calculate the percentage of brain damage.

MATERIALS AND METHODS

Over centuries societies around the world developed their own tradition to make sense of medicinal plants and their uses. One such plant which claims various medicinal properties is *Evolvulus alsinoides* one of the popular and important medicinal plant in India. All parts of *Evolvulus alsinoides* possess valuable medicinal properties. This plant is widely used in Ayurveda. In Ayurvedic literature *Evolvulus alsinoides* is known as Vishnukrantha, which has been told to have '*Medhya*' '*Smritivardhaka*'and '*Buddhivardhaka*' (Memory enhancer) action and is categorized in the '*Samjnasthapana dravyas*' (Indhumol VG *et al*., 2013).
Plant : *Evolvulus alsinoides*
Family: Convolvulaceae

Preparation of Extract

The plant material obtained was subjected to grinding using a mechanical blender to get fine powder of uniform size. About 250gm of the powder was treated with methanol in a soxhelet apparatus for 2h. The liquid extract thus obtained was subjected for evaporation to remove the excess of solvent under shade, which will give a solid mass of the drug extract. The yield of the extract was calculated and stored for further use. The solid methanolic drug extract was further subjected to column chromatography for isolation of flavonoid, the flavonoid presence in the drug was confirmed with the help of chemical tests for flavonoid.

ACUTE ORAL TOXICITY STUDIES

Acute oral toxicity studies were carried out as per OECD guidelines no. 420 on Swiss albino. Flavonoid of Evolvulus alsinoides was dissolved in the water and diluted with 0.9% saline. Acute oral toxicity was assessed on healthy Swiss albino mice of either sex(8-10 weeks old) weighing 20-25gm.The mice were observed continuously for 1 hr for any gross behavioral changes and deaths intermittently for next 6 hr and then again 24 hr after dosing. LD_{50} of the compound was calculated according to OECD guidelines the behavior parameters observed are convulsion, hyperactivity, sedition, grooming loss of righting reflexes, increased or decreased respiration, food, water intake etc. The dose of the drug was fixed according to the results obtained by conducting the test.

Experimental animals

Albino male Wistar rats (8-10 weeks old) weighing 250-280g, were used for the experiment. They were acclimatized for one week prior to experiment. Animals were caged in fully ventilated room, were

maintained in 12:12 h light and dark cycle and were housed at temperature of 25 ± 2°C. They had free access to a standard chow diet and water ad libitum. All the experiments conducted on the animals were in accordance with the standards set for the use of the laboratory animal use and the experimental protocols were duly approved by the IAEC (Institutional Animal Ethical Committee). Occlusion: Surgical technique for the induction of cerebral ischemia was adopted from the earlier published method of Anshuman Trigunayat 2009. Under anesthesia midline incision was given. Common carotid arteries were identified and isolated carefully from Vago-sympathetic nerve. Ischemia was induced by occluding bi- lateral common carotid arteries (BCCAO) with thread for 30 min and reperfusion was allowed for 48 hr by removing the thread. Neck incision area was closed with thread using suture. All animals were given pre-treatment for 7 days p.o. with a dose calculated by the help of acute toxicity studies. All the inhibitors were given 10 min before occlusion (Nandagopal M *et al*., 2010)**.**

Surgery procedure and bilateral carotid artery occlusion induced cerebral ischemia in rats

After 1 week of pre-treatment with MEAR p.o., the stroke was induced using bilateral carotid artery occlusion (BCAO) method in male healthy Wistar rats. Anaesthetized rat with thiopentone sodium at a dose of 45mg/kg body weight. Rats were transferred to the surgery table. Checked anesthesia level intermittently (e.g. toe pinch) and level adjusted accordingly. Ventral neck region was shaved. Area was washed with 70% ethanol to cleanse. All loose fur were removed and treated with betadine solution. Temperature measurement was carried out. Started recording temperature and controlled with help of following method. Body temperature was easily controlled (37.0°C) with a heating blanket. An infrared lamp was also effective in maintaining temperature, but it must not be too close to cause burns. A small midline skin incision was made in neck (~ 2 cm long). The thyroid gland was gently separated with non-traumatic forceps. Both common carotid arteries was isolated. Care was taken to avoid damaging the vagal nerves and separated with the help of curved forceps. Silk suture was looped under each artery for each access to vessels. Vessels were made free enough to allow easy and rapid placement of clamps. Non-traumatic vessel clamps was applied to each artery for a defined period (10 reperfusion. Area was infiltrated with warm saline to prevent drying out of tissue during occlusion. After the end of defined period clamps were removed and checked arteries for good reflow. The order of clamp application and removal should be the same and within 10 sec of each other minimize asymmetrical injury. Silk suture was gently removed around each vessel. Incision was infiltrated with a few drops of lignocaine. Incision was sutured and surgical care was given to the animal and they were reoperated control animals underwent all the surgical procedure except occlusion of BCAO (Tiwari M *et al*., 2010).

Experimental design

Rats were randomly divided into 10 groups, 6 animals per each group.

Group 1: Ischemic reperfusion (I/R)
Group 2: Sham (Surgical incision)
Group 3: Vehicle
Group 4: *Evolvulus alsinoides*
Group 5: L-NAME (10mg/kg)
Group 6: L-NAME + EA
Group 7: Nimesulide (20mg/kg)
Group 8: Nimesulide +EA
Group 9: Allopurinol (10mg/kg)
Group 10: Allopurinol + EA

BIOCHEMICAL ESTIMATIONS

Superoxide dismutase (SOD)

Procedure

Superoxide dismutase (SOD) activity was determined by the method developed by Kakkar *et al*, 1984. Brain was homogenized with ice-cold phosphate buffer. It was centrifuged at 10,000 rpm for 15 min at 4°C. Aliquot of supernatant 0.1 ml was added to 1.2ml of 0.052 M sodium pyrophosphate buffer(pH 8.3) followed by addition of 0.1ml of 186µM phenazonium methosulphate, 0.3 ml of 300 µM nitroblue tetrazolium, 0.2 ml of 780µM NADH. Reaction mixture was incubated for 90 sec at 30°C, and the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml of *n*-butanol and centrifuged at 4000 rpm for 10 min. the absorbance of organic layer was measured at 560 nm. A control was prepared using 0.1 ml of distilled water devoid of 0.1 ml of homogenate. One unit of the enzyme activity is defined, as enzyme concentration required inhibiting the absorbance of chromogen production by 50% in control sample under the assay conditions (Komatsu T *et al*., 2002).

Formula for estimation of SOD

$$\frac{\text{Control O.D - Experimental O.D}}{\text{Control O.D/2}} \times \frac{1}{\text{protein in m}} = \text{units/mg protein}$$

Catalase (CAT)

Procedure

Catalase activity was measured by the method of Aebi *et al*, 1974. Brain was removed and homogenized with a homogenizer in a volume of 10 % of brain weight with ice-cold phosphate buffer. The homogenate was centrifuged at 10,000 rpm at 4°C for 15 min. Supernatant

0.1 ml was added to cuvette containing 1.9 ml of 50 mM phosphate buffer. To this mixture, 1.0 ml of freshly prepared 30 mM H_2O_2 was added and changes in absorbance for 3 min at 240 nm at an interval of 30 sec was measured. A control was prepared using 0.1 ml of distilled water devoid of 0.1 ml of homogenate. One unit of the enzyme activity is defined as enzyme concentration required inhibiting the change in the absorbance by 50% in one min in the control sample (Kristofikova Z *et al*., 1995).

Formula for the estimation of Catalase

$$\frac{\Delta A \times \text{vol. of R.M}}{43.6 \times \text{vol. of E}} \times \frac{1}{\text{protein in mg}} = \mu \text{ moles of } H_2O_2 \text{ metabolized/mg protein/min}$$

Estimation of MDA level

Procedure

The lipid peroxidation end product Malondialdehyde (MDA) was measured by the method of Okhawa *et al*, 1979. Brain was removed and homogenized with a homogenizer in a volume of 10 % of brain weight with ice-cold phosphate buffer. 0.2 ml of brain homogenate was treated with 0.2 ml sodium dodecyl sulphate (8.1%), 20% of 1.5 ml of acetic acid (pH 3.5), 1.5 ml thio-barbituric acid (0.8%). The mixture was made up to 5 ml with distilled water and then heated at 95°C in oil bath for 60 min. The mixture was cooled and 5 ml of n-butanol and pyridine mixture (15:1 v/v) was added. The mixture was shaken vigorously. After centrifugation of the mixture at 4000 rpm for 10 min, the organic layer was taken and the absorbance was measured at 532 nm. The concentration of MDA formed is expressed as nmol/gm wet tissue (Shi-Jye C *et al*., 2005).

Formula for the estimation of MDA

$$\frac{\text{O.D} - 0.002 \times 50}{0.0666} = \text{nmol/gm wet tissue}$$

Estimation of MPO level

Procedure

Myeloperoxidase (MPO) was measured by the method of Mullane *et al*, 1985. Brain was homogenized with a homogenizer in a volume of one-tenth of brain weight with ice-cold Phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide. The homogenate was centrifuged at 15000 rpm for 30 min at 4°C. 40 µl supernatant was added to 960µl of phosphate buffer containing O-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (0.0005%) solution and shaken vigorously. The change in the absorbance was measured at 460 nm for 3 min at an interval of 60 sec. One unit of enzyme activity was defined as the amount of MPO that causes a change in absorbance measured at 460 nm for 3 min. MPO activity data are presented as units/ml (Rudolph U Mohler H, 1999).

Formula for the estimation of MPO

$$\text{U/ml} = (\Delta A \times V_t \times 4) / (E \times \Delta t \times V_s)$$

SIGNIFICANCE OF CHOOSING

In existing research *Evolvulus alsinoides* plant has been used for many activities except for treating cerebral ischemia activity. The plant contains many chemical constituents like alkaloids, flavonoids, steroids and glycosides which were helpful mainly in increasing the blood flow. The claimed usefulness of herbs in several disorders might be due to their antioxidant activity. It also reported that herbal drugs protect lipids, blood and body fluids from the attack of free radicals, reactive oxygen species and superoxide radicals. So based on these activities the plant was selected for treating cerebral ischemia.

RESULTS

The collected plant material was undergone for different chemical tests. Phytochemical analysis of successive extract of leaves of *Evolvulus alsinoides.*

HISTOPATHOLOGICAL EXAMIMATION

1. Group – 1 [Ischemic reperfusion]

Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Most of the neuronal cells appear shrunken with dark coloured cell bodies [Fig.1, Long-Arrow], while some show nuclear pyknosis [Fig.1, Short-Arrow] [Dying / Degenerating neuronal cells – 70%]. Surrounding these neuronal cells, spongiform [microvacuolated] changes are noted in the neuropil fibers [Fig.2, Short-Arrow]. Also intact neurons are seen [Fig.2, Long-Arrow].

Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Some of the neuronal cells appear shrunken with dark coloured cell bodies [Fig.1, Long-Arrow], while some show nuclear pyknosis [Fig.1, Short-Arrow] [Dying / Degenerating neuronal cells – 40%]. Surrounding these neuronal cells, spongiform [microvacuolated] changes are noted in the neuropil fibers [Fig.2, Short-Arrow]. Also intact neurons are seen [Fig.2, Long-Arrow].

Group – 3 [Vehicle]

Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Most of the neuronal cells appear intact with round light basophilic cell bodies [Fig.1, Arrow]. Surrounding these neuronal cells, compact eosinophilic intact neuropil fibers [Fig.2,

Long-Arrow] are noted. Also seen are few dark coloured neuronal bodies without shrinkage [probably Artefacts].

Group – 4 [*Evolvulus alsinoides*]
Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Most of the neuronal cells appear shrunken, dark coloured cell bodies [Fig.1, Long-Arrow], while some show nuclear pyknosis [Fig.2, Long-Arrow] [Dying / Degenerating neuronal cells – 40%]. Surrounding these neuronal cells, spongiform [microvacuolated] changes are noted in the neuropil fibers [Fig.2, Long-Arrow]. Also intact neurons are seen [Fig.1, Short-Arrow].

GROUP 5 (L- NAME)
Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Most of the neuronal cells appear shrunken, dark coloured cell bodies [Fig.1, Long-Arrow] [Dying / Degenerating neuronal cells – 50%] while some show nuclear pyknosis [Fig.2, Short-Arrow] intact neurons are seen

GROUP 6 (L- NAME+ EA)
Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Surrounding these neuronal cells, compact eosinophilic intact neuropil fibers [Fig.1, short-Arrow]. [Dying/ Degenerating neuronal cells – 45%]. Neuronal cells at few areas, spongiform. While some show nuclear pyknosis [Fig.2, long-Arrow].

Group – 7 [COX inhibitor].

Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Some of the neuronal cells appear shrunken, dark coloured cell bodies [Fig.1, Long-Arrow], while some show nuclear pyknosis [Fig.1, Short-Arrow] [Dying / Degenerating neuronal cells – 30%]. Surrounding these neuronal cells at focal areas, spongiform [microvacuolated] changes are noted in the neuropil fibers [Fig.2, Short-Arrow]. Also intact neurons are seen [Fig.2, Long-Arrow].

Group – 8 [COX + *Evolvulus alsinoides*]
Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Few of the neuronal cells appear shrunken, dark coloured cell bodies [Fig.1, Long-Arrow], while some show nuclear pyknosis [Fig.1, Short-Arrow] [Dying / Degenerating neuronal cells – 20%]. Surrounding these neuronal cells at few areas, spongiform [microvacuolated] changes are noted in the neuropil fibers [Fig.2, Short-Arrow]. Also intact neurons are seen [Fig.2, Long-Arrow].

Group – 9 [XO inhibitor]
Microscopy

Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Few of the neuronal cells show nuclear pyknosis [Fig.1, Short-Arrow] [Dying / Degenerating neuronal cells – 20%]. Surrounding these neuronal cells at focal areas, spongiform [microvacuolated] changes are noted in the neuropil fibers [Fig.1, Long-Arrow]. Also intact neurons are seen [Fig.2, Arrow].

Group – 10 [XO inhibitor + *Evolvulus alsinoides*]
Microscopy

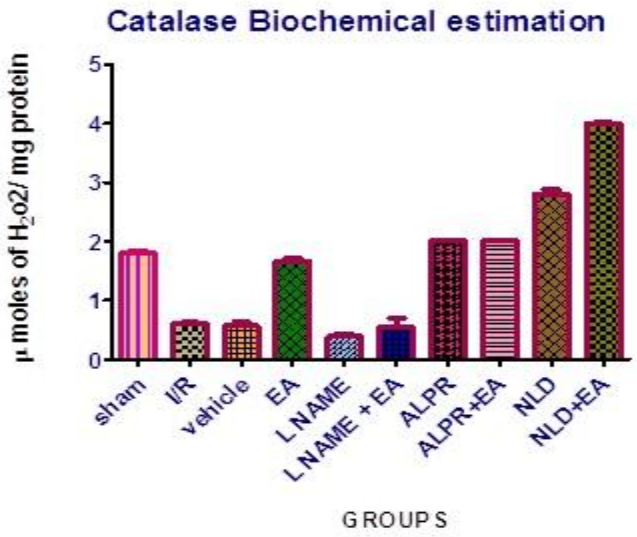
Section studied from the brain parenchyma consists of neuropil fibers with neuronal cells. Most of the neuronal cells appear intact with round light basophilic cell bodies [Fig.1, Arrow] [Dying / Degenerating neuronal cells < 05%]. Surrounding these neuronal cells, compact eosinophilic intact neuropil fibers [Fig.2, Long-Arrow] are noted. Also seen are few dark coloured neuronal bodies without shrinkage [probably Artefacts] [Fig.2, Short-Arrow].

Table 1. Bio Chemical Estimation Results

Groups	CAT	SOD	MDA	MPO
Sham^{r†}	1.808± 0.03^{r†}	39.10±0.07^{r†}	132.8±0.47^{r†}	1.892±0.01^{r†}
I/R†	0.6140± 0.01†	11.50± 0.79†	195.4±0.57†	3.038±2.93†
Vehicle $^{\Delta}$	0.6160± 0.05^{ns}	12.18±0.13	194.9±0.07	2.938±0.06
EA$^{\Delta}$	1.708± 0.05**	24.34±0.18**	128.2±0.43**	1.706±0.01**
L NAME$^{\Delta}$	0.3960± 0.04^{ns}	10.37±0.15^{ns}	186.8±3.88^{ns}	1.738±0.03**
L NAME+EA$^{\Delta}$	0.4120± 0.15^{ns}	13.31±0.45^{ns}	185.1±0.20^{ns}	1.554±0.20**
ALPR$^{\Delta}$	2.012± 0.03**	40.28±0.53**	111.61±0.85**	1.32±0.02**
ALPR+EA$^{\Delta}$	2.01± 0.05**	39.65±0.42**	111.61±0.52**	1.16±0.11**
NLD$^{\Delta}$	2.80± 0.07**	69.20±0.35**	98.30±0.09**	1.11±0.08**
NLD+EA$^{\Delta}$	4.00± 0.01***	88.55±0.46***	53.26±0.42***	0.43±0.03***

Values Are Expressed as Mean ± SD, n=5, P<0.001. IR† (Ischemic reperfusion) group was compared with the SHAM ^{r†} group. Remaining groups ($^{\Delta}$) were compared with the IR† group. EA- *Evolvulus alsinoides*. ns : non-significant. ***: Significant

Graph No. 1

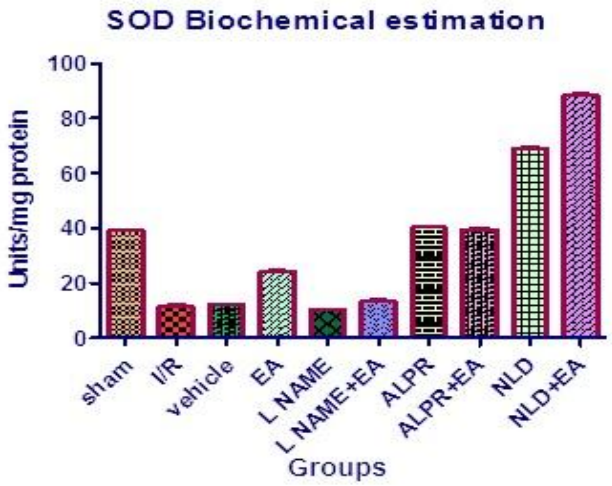


Values Are Expressed as Mean ± SD, n=5, P<0.001.

IR† (Ischemic reperfusion) group was compared with the SHAM †† group. Here IR† group was compared with remaining groups$^{\Delta}$. The level of CAT in the brain tissue were decreased to 29.75% when compared to the SHAM group (P<0.001). The administration of EA causes the increased CAT levels to 27.25%. Administration of different drugs LNAME, LNAME+EA, ALPR, ALPR+EA, NLD, NLD+EA, leads to increased CAT levels by 5%, 5%, 34.95%, 35.05%, 54.75% and 84.75%. The increased CAT levels in NLD, NLD+EA were significant. Histogram representing effect on cerebral CAT levels after treatment protocol.

I/R: ischemic reperfusion, Vehicle: DMSO (Dimethyl sulphoxide), EA: *Evolvulus alsinoides*, L- NAME: L-Nitro Arginine Methyl Ester, ALPR: Allopurinol, NLD: Nimesulide.

Graph No. 2

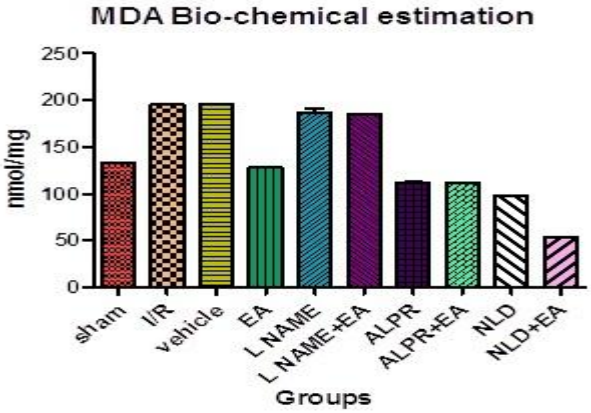


Values Are Expressed as Mean ± SD, n=5, P<0.001.

IR† group was compared with the SHAM †† group. IR† group was compared with remaining groups$^{\Delta}$. The level of SOD in IR group of the brain tissue were decreased to 31.37%% when compared to the SHAM group (P<0.001). The administration of EA causes the increased SOD levels to 14.59%.Administration of different drugs LNAME, LNAME+EA, ALPR, ALPR+EA, NLD, NLD+EA, leads to increased SOD levels by 2.06%,2.06%,32.66%,31.99%,65.57% and 86.94%. The increased levels of CAT in NLD, NLD+EA were significant. Histogram representing effect on cerebral SOD levels after treatment protocol.

Here I/R: ischemic reperfusion, Vehicle: DMSO(Dimethyl sulphoxide), EA: *Evolvulus alsinoides*, L- NAME: L-Nitro Arginine Methyl Ester, ALPR: Allopurinol, NLD: Nimesulide.

Garph No. 3

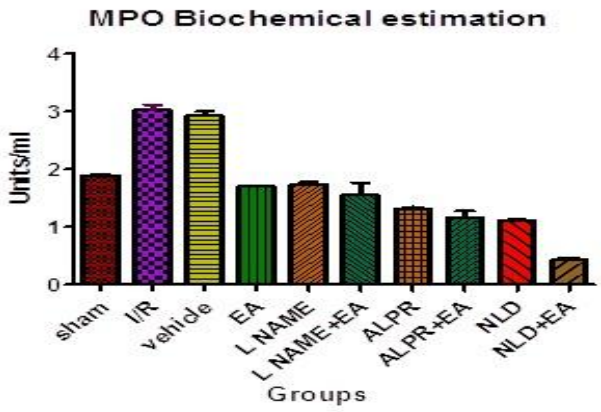


Values Are Expressed as Mean ± SD, n=5, P<0.001.

IR† (Ischemic reperfusion) group was compared with the SHAM †† group. IR† group was compared with remaining groups$^{\Delta}$. The level of MDA in IR group of the brain tissue were increased to 35.05% when compared to the SHAM group (P<0.001). The administration of EA causes the decreased MDA levels to 34.4%. Administration of different drugs LNAME, LNAME+EA, ALPR, ALPR+EA, NLD, NLD+EA, leads to decreased MDA levels by 4.41%, 5.28%, 42.89%, 42.89%, 49.7% and 72.75%. The decreased levels of MDA in NLD, NLD+EA were significant.

Here I/R: ischemic reperfusion, Vehicle: DMSO (Dimethyl sulphoxide), EA: *Evolvulus alsinoides*, L- NAME: L-Nitro Arginine Methyl Ester, ALPR: Allopurinol, NLD: Nimesulide.

Graph No. 4



Values Are Expressed as Mean ± SD, n=5, P<0.001.

IR† (Ischemic reperfusion) group was compared with the SHAM †† group. IR† group was compared with remaining groups$^{\Delta}$. The level of MPO in IR group of the brain tissue were increased to 37.73% when compared to the SHAM group (P<0.001).The administration of EA causes the decreased MPO levels to 43.85 %. Administration of the different drugs LNAME, LNAME+EA, ALPR, ALPR+EA, NLD,NLD+EA leads to decreased MPO levels by 42.8%, 48.85%, 56.56%, 61.69%, 63.4% and 85.79%. The decreased levels of MPO in NLD, NLD+EA were significant.

Here I/R: ischemic reperfusion, Vehicle: DMSO (Dimethyl sulphoxide), EA: *Evolvulus alsinoides*, L- NAME: L-Nitro Arginine Methyl Ester, ALPR: Allopurinol, NLD: Nimesulide.

Figure 1

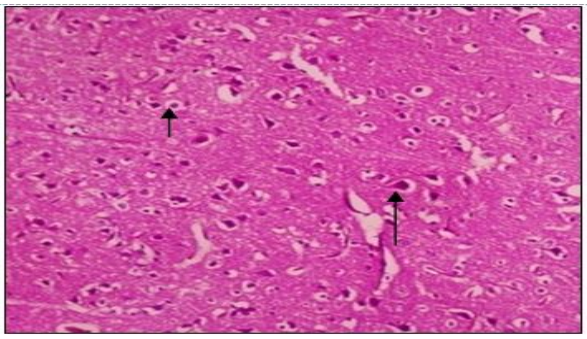


Fig 1a. [H&E, x200]

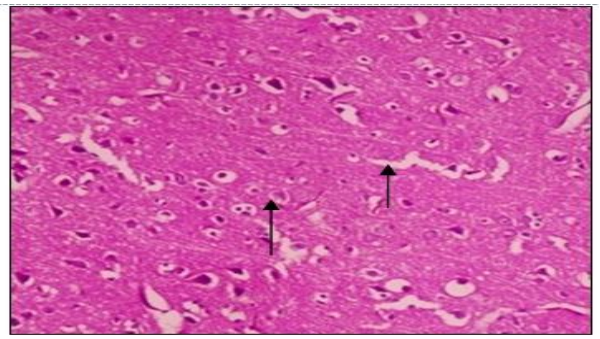


Fig 1b. [H&E, x200]

Figure 2

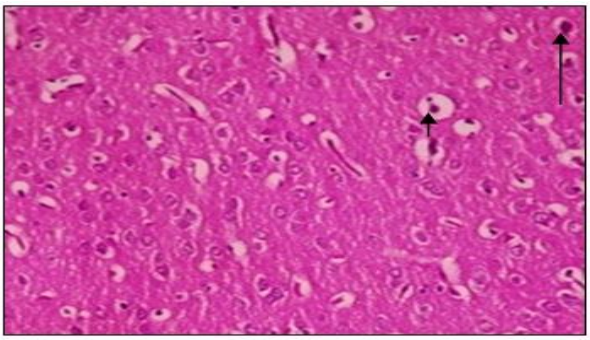


Fig 2a. [H&E, x200]

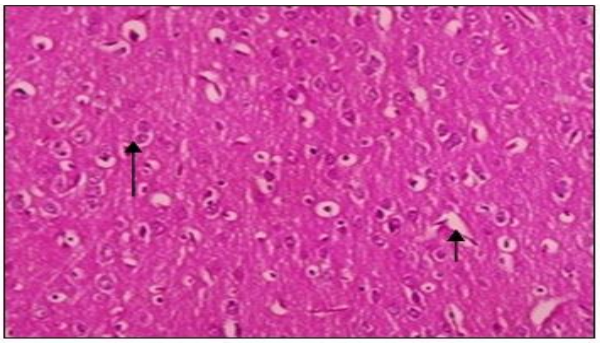


Fig 2b. [H&E, x200]

Figure 3

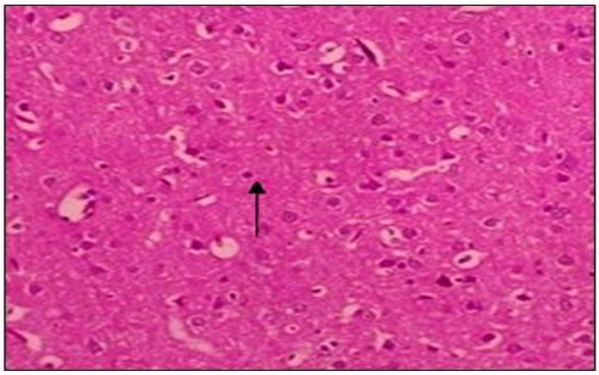


Fig 3a. [H&E, x200]

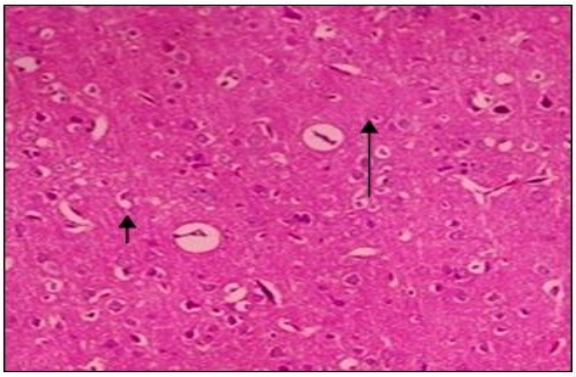


Fig 3b. [H&E, x200]

Figure 4

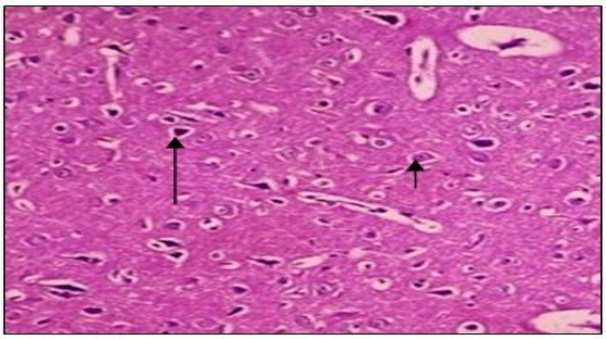


Fig 4a. [H&E, x200]

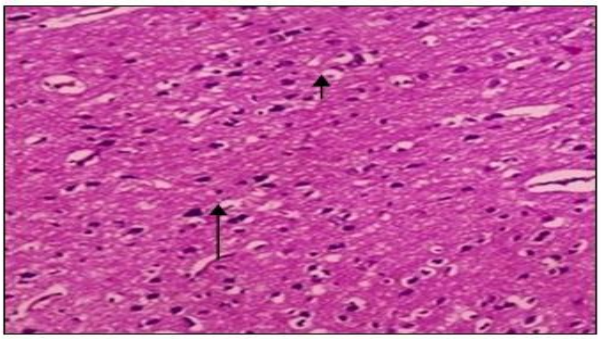


Fig 4b. [H&E, x200]

Figure 5

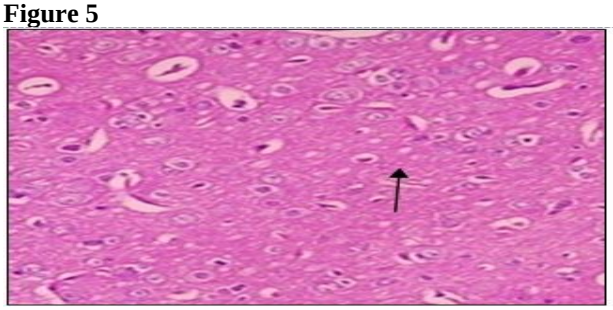


Fig 5a. [H&E, x200]

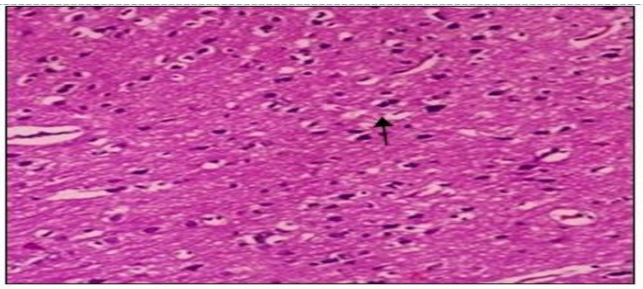


Fig 5b. [H&E, x200]

Figure 6

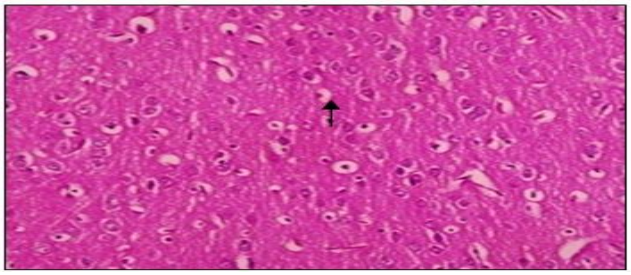


Fig 6a. [H&E, x200]

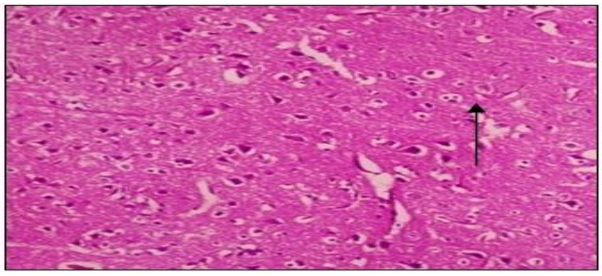


Fig 6b. [H&E, x200]

Figure 7

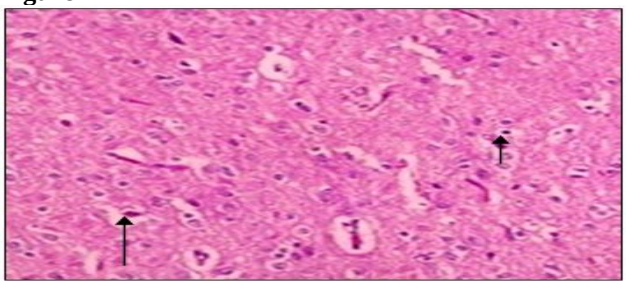


Fig 7a. [H&E, x200]

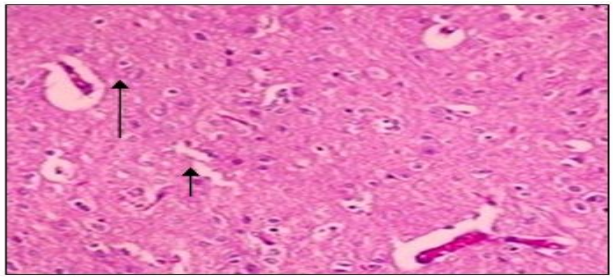


Fig 7b. [H&E, x200]

Figure 8

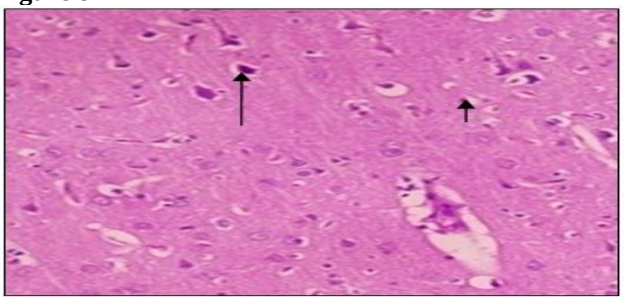


Fig 8a. [H&E, x200]

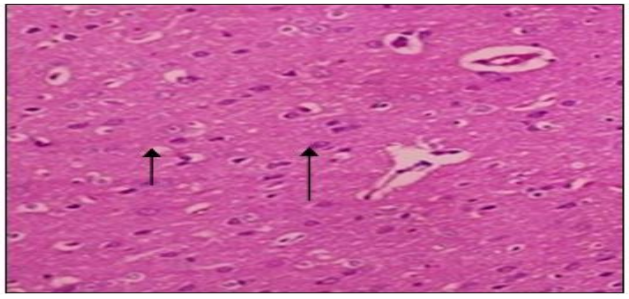


Fig 8b. [H&E, x200]

Figure 9

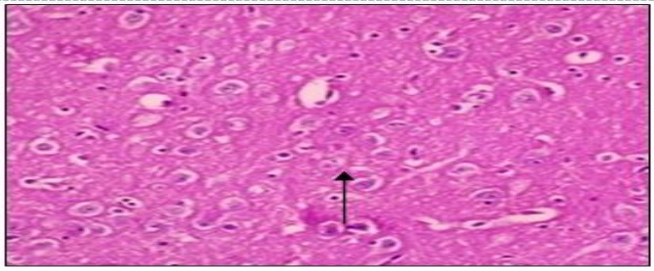


Fig 9a. [H&E, x400]

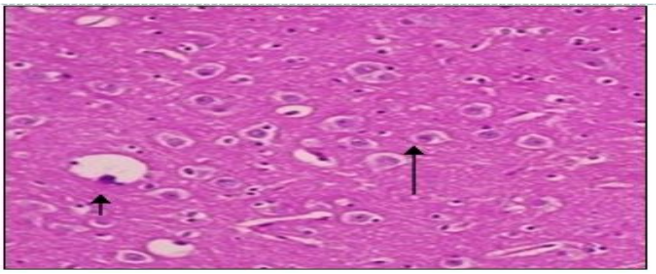


Fig 9b. [H&E, x400]

Figure 10

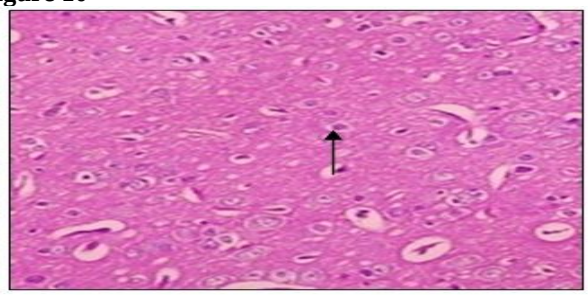


Fig 10a. [H&E, x400]

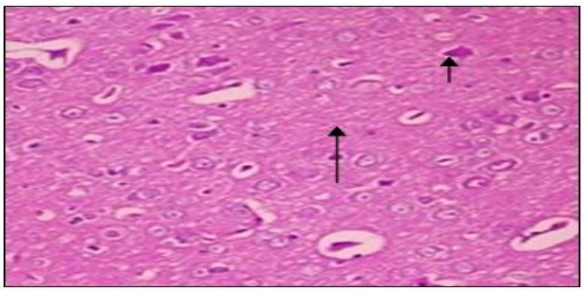


Fig 10b. [H&E, x400]

DISCUSSION

1. SHAM GROUP: In the biochemical estimation the antioxidants CAT and SOD and the oxidants MDA and MPO showed no neurological deficit (Graph 1-4). Histopathological studies also showed there was no neuronal degeneration in the tissues (Fig no.1)

2. ISCHEMIC REPERFUSION GROUP: The antioxidant levels of CAT and SOD showed decreased. The oxidant levels of MDA and MPO showed increased when compared to SHAM group (Graph 1-4).The histopathological studies showed neuronal shrunken, neuronal degeneration, hemorrhage, vacuolation and glial cell proliferation (Fig no. 2). This indicates there was severe damage in the brain

3. VEHICLE GROUP: In this group the antioxidants and oxidant levels of CAT, SOD and MDA, MPO were near to the values of Ischemic reperfusion group (Graph 1-4).The histopathological studies showed neuronal degeneration, neuronal shrunken and vacuolation (Fig no. 3). This showed there was severe damage which is equal to the ischemic reperfusion group and vehicle didn't affect the neuronal cells in adverse manner.

4. EA GROUP: Here the antioxidant levels of CAT and SOD levels became increased and the oxidant levels of MDA and MPO levels became decreased when compared with ischemic reperfusion group (Graph 1-4). The histopathological studies showed minimum levels of neuronal damage, mild vacuolation and relatively lower congestion (Fig no.4).This showed there an effective recovery in the damaged neuronal cells when compared with ischemic reperfusion group.

5. L NAME GROUP: It showed there was a decreased antioxidant levels of CAT and SOD. The oxidant levels of MDA and MPO also decreased in a very less manner when compared to the Ischemic reperfusion group which is non-significant (graph 1-4). The histopathological results showed there was a considerable amount of damage in the neuronal cells and vacuolation (Fig no.5). So the L NAME group was not significant when compare to the ischemic reperfusion group.

6. L NAME + EA GROUP: There was a decrease in the CAT and SOD antioxidants. The levels of MDA and MPO were non significantly decreased (Graf 1-4). The histopathological results showed there was a neuronal degeneration and vacuolation (Fig no. 6). This showed it was not significant.

7. ALLOPURINOL GROUP: The cerebral antioxidants levels of CAT and SOD were significantly increased and the oxidant levels of MDA and MPO levels significantly decreased (graph 1-4). The histopathological studies also showed minimum damage in the neuronal cells (Fig no. 7). This showed the group was significant.

8. ALLOPURINOL + EA: The cerebral antioxidant levels of CAT and SOD where increased and the oxidant levels of MDA and MPO were significantly decreased when compared to the ischemic reperfusion group (Graph 1-4). The results where almost similar to that of ALLOPURINOL group. The histopathological results showed there was a little bit damage in brain tissue (Fig no 8). This showed the group was significant as that of Allopurinol group.

9. NIMESULIDE GROUP: The cerebral antioxidant levels of SOD and CAT were significantly increased and the oxidant levels of MDA and MPO levels were significantly decreased when compared with the ischemic reperfusion group (graph 1-4). The histopathological studies showed there was no neuronal degeneration and cellular components were normal (Fig no 9).

10. NIMESULIDE + EA GROUP: The antioxidant levels of CAT and SOD were significantly increased and oxidant levels of MDA and MPO were significantly decreased (Graph 1-4). The histopathological studies showed not much considerable neuronal damage (Fig no 10).

The oxidative stress is believed to be a major source for generation of post cerebral ischemic injury. Various experimental models cerebral ischemic reperfusion injury shows significant neuroprotection and treated with anti oxidants (Collini M *et al.*, 2006). Reactive oxygen species have been denoted as one of the earliest and most important components of tissue injury after reperfusion of ischemic organ and extent of brain injury appears to depend on the experimental pattern of ischemia/reperfusion. Free radical production continuous during ischemia, while during reperfusion it is primarily confined to the early stage when fresh oxygen is supplied

to ischemic region. The brain is very susceptible to the damage caused by oxidative stress, due to the high rate of oxidative metabolic activity, high polyunsaturated fatty acid contents. Relatively low anti oxidant capacity and inadequate neuronal cell repair activity. Oxidative damage includes the excessive production of reactive oxygen species, including lipid peroxidation. Protein oxidation and DNA damage, which can lead to cell death. Furthermore, reactive oxygen species can activate diverse downstream signaling pathways, such as Xanthain oxidase(XO) pathway, over expression of cyclooxygenase 2(COX 2) and of inducible nitric oxide synthase (iNOS) have recently emergent as important determents of post ischemic inflammation, which contributes to the progression of brain damage (Margaill *et al*., 2005). Lipid peroxidation has been established as a major mechanism of cerebral injury. the mechanism involves a process whereby unsaturated lipids are oxidized to form additional radical species as well as toxic byproducts that can be harmful to the host system (Adibhatla, 2003). Polysaturated lipids are especially susceptible to this type of damage when in an oxidizing environment and they can react to form lipid peroxidase (Kinuta Y *et al*., 1989). Lipid peroxidase are themselves undergo additional decomposition to form a complex series of compounds including reactive carbonyl compounds (Horiguchi T, 2006). Polyunsaturated fatty acids peroxides further react to form malonaldehyde (MDA) Akondi RB *et al*., 2011). In the present study we observed increase in the tissue MDA activity in ischemic reperfused brain when compared with the sham groups and the results were in agreement with previous studies. Treatment with *Evolvulus alsinoides* (250mg/kg p.o) alone provided cerebroprotection but still more significant cerebroprotection was observed when EA was administered along with the Nimesulide (20mg/kg i.p) treatment of EA+ L NAME (10mg/kg i.p) showed no cerebroprotective activity and EA+ALLOPURINOL (10 mg/kg i.p) also showed some cerebroprotective activity indicating involvement of some Xanthine oxidase property of the EA.

Reactive species can be decreased or eliminated by a number of enzymatic and non enzymatic antioxidant mechanisms. SOD, which catalyzes the dismutation of the superoxide anion (o^{-2}) into the hydrogen peroxide and molecular oxygen, is one of the most important anti oxidative enzymes. In the present study SOD and CAT activity decreased in the ischemic reperfused group compared to the sham group and the results were in agreement with previous studies (Panickar KS, Anderson RA, 2011). This may due to the excessive formation of superoxide anions. A decrease in the SOD activity results in the decreased removal of superoxide anions, which can be harmful to the brain. The decline in the enzyme level may be explained by the fact that excessive superoxide anion may be in activate SOD, thus resulting in an inactivation of the H_2O_2 scavenging enzyme. The reduced SOD and CAT activity were increased by the administration of the EA alone but the activity was significantly increased when the EA was administered along with Nimesulide (20 mg/kg). It is less effective when given along with the L-NAME but slightly effective when administered along with Allopurinol (10mg/kg). Inflammation is an important component of cerebral ischemia. Pro inflammatory molecules such as NO synthase -2 (NOS 2), cyclooxygenase (COX-2), chemokines, and adhesion molecule have been implicated in the development of cerebral ischemic injury. Myeloperoxidase (MPO) is a highly cationic glycosylated heme enzyme secreted by activated phagocytes at site of inflammation.MPO is an enzyme that is involved in the production of free radical. Indeed MPO, uses H_2O_2 and NO^-_2 to generate reactive nitrogen species. The activities of enzymes MPO was also increased in ischemic reperfused group compared to the sham group. Administration of the EA alone showed inhibition if inflammatory mediators but they were significantly inhibited when EA along with the Nimesulide (20mg/kg i.p). Whereas L- NAME showed no cerebral activity but the Allopurinol and Allopurinol+EA groups showed same cerebroprotective activity when compared to the ischemic reperfusion group.

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