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A study of antioxidant enzymes on experimental epileptic rat models induced by kainic acid Excitotixicity

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***Abstract*---**The word neurodegeneration rightly refers to the degenerative changes in nerve cell structure and so in its functions too. The main characteristics of these neurodegenerative disorders are relentless progression and decline in cognition. Epilepsy is one of the neurodegenerative disorders that affects a major portion of people around the world and the common form of focal epilepsy is Temporal lobe epilepsy (TLE). According to researchers the major cause for nerve cell degeneration is the excess production of free radicals. Preventing their action or quenching them will make a big deal in preventing neurodegeneration. As research work in relation to neurodegeneration is very much limited in India, we aimed one as an initiative. As herbs are always well known for their antioxidant activity with negligible side effects, we planned to apply one of the well-known herb, Acorus calamus and studied the potency of it in maintaining the antioxidant enzyme levels. The aim of this study in particular is to analyze the preventive role of Acorus calamus and its active ingredient Beta asarone in neurodegeneration than treating neurodegeneration in epileptic rat models. For the same, we selected eight groups of animals. They were Control, Lesion control, Ethanolic extract of Acorus calamus 15, 25, 35mg and Beta asarone 10, 15 and 20mg/kg

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body weight. Though both drugs, Ethanolic extract of Acorus calamus and its active principle Beta asarone played roles in preventing the formation of free radicals, the pure active principle Beta asarone had an upper hand both in prevention of free radicals and so protecting the nervous system of experimental rats.

Keywords---Epilepsy, Neurodegenerative disorders, Temporal lobe epilepsy, Acorus calamus and Beta asarone.

Introduction

Epilepsy is a fatal neurodegenerative disorder caused duo to the degeneration of nerve cells mostly by hyperexcitation. Degenerative changes increase by time as the free radicals produced by the degenerating cells will damage the nearby cells too. As a concept, by any means if we can increase the level of the antioxidant enzymes that quenches the free radicals produced, we can prevent the degeneration of the surrounding cells at least, as the affected nerve cells cannot be regenerated, concept wise till now. This will prevent the prognosis of the disease further.

Kainic acid (KA) is a potent exitotoxin to the cells of the nervous system. According to researchers when kainic acid is administered intracerebrally it causes seizure repeatedly for a maximum of 2 days and also shows local lesion or brain damage. Cavalheiro et al., 1982 observed 4 distinct phases of epilepsy after administration with 0.1 to 3.0 micrograms of KA in to the right-side hippocampus of Wistar albino rats[1]. Intraventricular injection of kainic acid has effect on hippocampal neurons and was unusually sensitive to CA3 region and also affects the CA1 pyramidal cell. Lesion with kainic acid was always considered as an equal model of TLE in human (Nadler *et al.,* 1978)[2], as it is following the exact path of excitotoxicity in the creation of epilepsy in animal models.

The free radicals or reactive oxygen species (ROS) that are produced inside or outside the cell during various metabolic activities of the cells are the major factor for risk that initiate nerve cell degeneration (Praba *et al.,* 2014[3]). Hazra *et al.*, 2007[4], suggested that a*corus calamus* can be effectively developed as a drug against epilepsy as it prevented the $FeCl_3$-induced epilepsy by exhibiting modulations in the antioxidant enzyme system.

There are various methods of creation of epilepsy in rats like ferric chloride induced model by Hazra et al.,[4] and electrical induced model by Gopalakrishna et al.,[5] but they are not following the original human epileptogenesis model of excitotoxicity.

Longo BM and Mello LE[6], created an epileptic model with intrahippocampal injections of 0.5 microgram of KA. They also placed electrodes over the same stereotaxic positions, recorded the EEG and concluded that intra-hippocampal KA model is an effective and equivalent model that mimicking the TLE in patients with a large area of lesion with in the temporal lobe.

The main aim of this study is to analyse and to prove the protective role of Acorus calamus in neurodegeneration by quantifying the level of antioxidant enzymes in the brain tissues of the experimental animals and to analyse the dosage efficacy by comparing the level of antioxidant enzymes between different animal groups used in this study.

We designed the study on the light of literature so as to get a novel and equivalent model to mimic human epilepsy by using an exitotoxin Kainic acid and to employ Acorus calamus, which is a potent herb with antioxidant activity in Indian scenario. The model was produced by injecting 0.5 μg KA directly into the hippocampus of rats by using stereotaxic frame. The rats were pretreated with different dosage of the herb and its active principle 10 days prior to lesion surgery and the level of antioxidant enzymes, with or without treatment were analysed to study the effect of *Acorus calamus* and its active principle in preventing neurodegeneration.

Methodology

Animals

We used male adult SD rats between 200–250gm for this study. The total study design was approved by the CPCSEA (IAEC/XIII/10/CLBMCP/2008-09).

S.No	Groups of animals	Lesion with KA	Pre and post treatment of Acorus calamus	Pre and post treatment of beta asarone
1	CO (Control)	Not	Not	Not
2	LC (Lesion control)	Done	Not	Not
3	AC 15 (AC15mg)	Done	Done	Not
4	AC 25 (AC25mg)	Done	Done	Not
5	AC 35 (AC35mg)	Done	Done	Not
6	BA 10 (BA10mg)	Done	Not	Done
7	BA 15 (BA15mg)	Done	Not	Done
8	BA 20 (BA20mg)S	Done	Not	Done

Table showing animal groups used for enzyme study

Materials

Ethanolic extract of *Acorus calamus* - Elayaraja *et al.,* 2010[7].

Vector stain ABC elite kit – Vector laboratories - Japan.

Beta Asarone - from Fluka – Sigma USA.

Kainic acid – Caymen chemicals – USA, dissolved in 0.9% NaCl (Duigou *et al.,* 2008)[8], in order to get a concentration of 1 microgram in 1 microliter.

Equipments

1. Stereotaxic frame – to perform lesion surgery in hippocampus
2. Micromotor hand drill – to make a purr hole in the rat skull
3. Hamilton syringe – to kainic acid into the hippocampus in micro volumes
4. pH meter – to maintain the pH of the buffers
5. Tissue homogenizer – to homogenize the brain tissue for biochemical work
6. Centrifuge – to centrifuge the homogenate
7. Freezer -20ºC– storing chemicals and tissues
8. Spectrophotometer – to find the OD value

Lesion Surgery

Methodology

Hippocampal lesion was made by injecting 0.5 micrograms in 0.5µl of kainic locally into the right hippocampus with the help of steriotaxic frame. The frame was purchased from INCO Ambala, Hariyana.

Towards the end of the study, animals were sacrificed, brain was dissected out and the hippocampus was isolated to analyze the level of antioxidant enzymes. The results were tabulated and charts were drawn. Statistical study was done by using Anova single factor with the $P<0.05$.

Parameters Studied

1. Superoxide dismutase – following the methodology of Marklund and Marklund, 1974[9].
2. Glutathione peroxidase - following the methodology of Rotruck *et al.,* 1973[10].
3. Catalase (Sinha, 1972) [11].

Specimen preparation for Free Radical Scavenging Enzymes

Brain samples were quickly removed and were kept in cold saline.

Dissection of Hippocampus in Rat Brain

The cerebellum was separated from the cerebrum. With fine scissors the cerebral cortices were gently opened from the midline so as to expose the underlying hippocampus. The hippocampi on the right and left are joined in the midline and looked like a pair of banana. An incision was made in the midline region so as to separate them as we only need the right side of the hippocampus. The weight of the hippocampus was measured and was kept in the deep freezer until further use.

Fig-I

Figure showing the preservation of right hippocampus of different groups

Homogenization

A 10% homogenate was prepared by using ice cold 0.1M Tris-Hcl buffer in a homogenizer. Centrifugations of the homogenate was carried out at 2000rpm at 4°C for around15 minutes. The supernatant was collected and used for the enzyme assay.

Analysis of Superoxide Dismutase (SOD)

A mixture of ethanol chloroform 0.5 ml and 0.5ml of homogenate were mixed and centrifuged for 15 minutes. With 0.5 ml supernatant 2 ml Trish buffer, 0.5 ml pyrogallol were added. The absorbance was taken with the spectrophotometer al 470 nm. The quantity of SOD was expressed in units/min./mg.of protein.

Analysis of Catalase

1ml reaction mixture (4 ml hydrogen peroxide, 5 ml phosphate buffer and 1 ml homogenate), was poured into a tube with 2 ml DAA reagent, heated around 10 minutes, cooled and OD was measured in 570 nm. The level of catalase was calculated as μmole of hydrogen peroxide /min./mg. protein.

Analysis of Glutathione Peroxidase (GPX)

0.2ml homogenate was added to the mixture with 0.4ml phosphate buffer, 0.1ml sodium azide, 0.2 ml GSH, 1 ml distilled water, 0.1ml H2O2 and the reaction then was arrested at 0-, 1.5- and 3-min interval with 10%TCA, 1ml. With 0.5ml of supernatant (centrifuged at 2000 rpm), 0.5ml DTNB and 4ml disodium hydrogen phosphate were added and OD was taken at 420 nm. GPx level was calculated as μmoles of glutathione oxidized/min./mg. of protein.

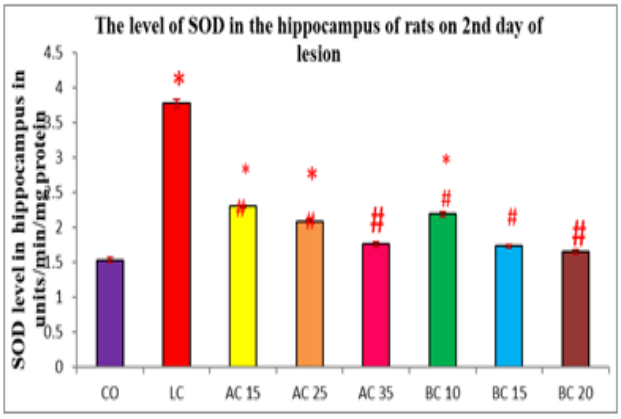
Results

SOD level on the 2nd day of lesion (CHART-I)

SOD is one of the major antioxidant enzymes that subsides the adverse effects of free radicals. Increase in SOD level says the formation of high amount of free radical than normal.

In this present study on the 2nd day the SOD level was significantly raised for the LC group animals while comparing CO animals. The groups AC15, AC25 and BA10 were showing high SOD in comparison with the CO groups and low in comparison with the LC group. But the animals groups AC 35 (df=3,20. F=132), BA 15 and BA 20 (df=3,20. F=70) had significantly low SOD level in comparison with the LC group and equal SOD level in comparison with the CO group shows good neuroprotection of the drug in the particular dose.

Chart-I



control Lesion control A.calamus 15mg A.calamus 25mg A.calamus 35 mg B.asarone 10mg B.asarone 15mg B.asarone 20mg

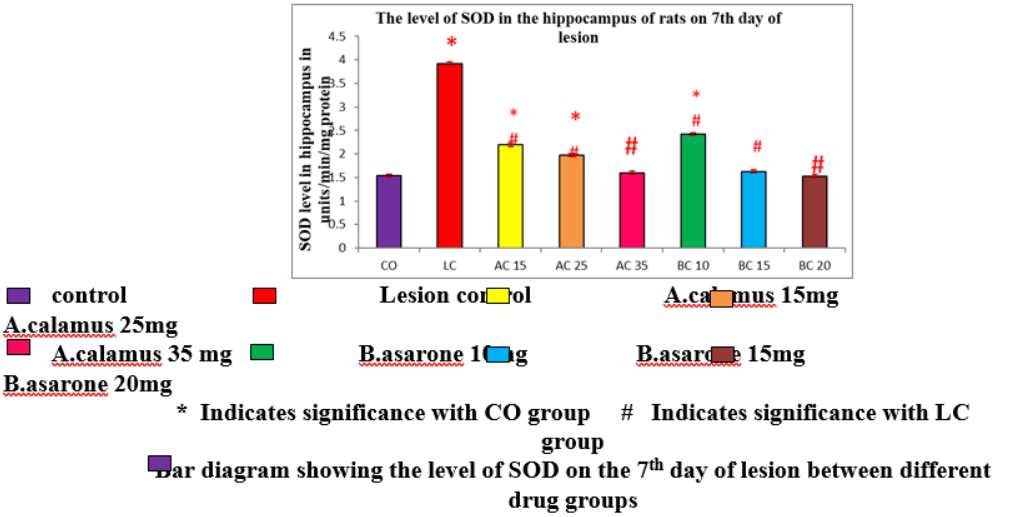
* Indicates significance with CO group # Indicates significance with LC group

Bar diagram showing the level of SOD on the 2nd day of lesion between different drug groups

SOD level on 7th day of lesion (CHART-II)

Analysis of SOD on the 7th day showed significantly high values in the LC group on comparison with CO animals. Animal belonging to AC15, AC25 and BA10 were not shown significant reduction in the SOD level in comparison with the SOD levels of the same groups on the 2nd day stating the dosage was low. But the other groups the AC 35 (df=3,20. F=67), BA 15 and BA 20 (df=3,20. F=137) were having significant reduction in the SOD levels in hippocampus in comparison with the same groups on the 2nd day, that shows these groups also have effect on treating the hippocampal region after lesion.

Chart-II



Bar diagram showing the level of SOD on the 7th day of lesion between different drug groups

The SOD levels of AC 35, BA 15 and BA 20 were equal in comparison with the CO group on the 7th day and significantly reduced when compared with the same groups on the 2nd day, that proved they both are effective in neuroprotection.

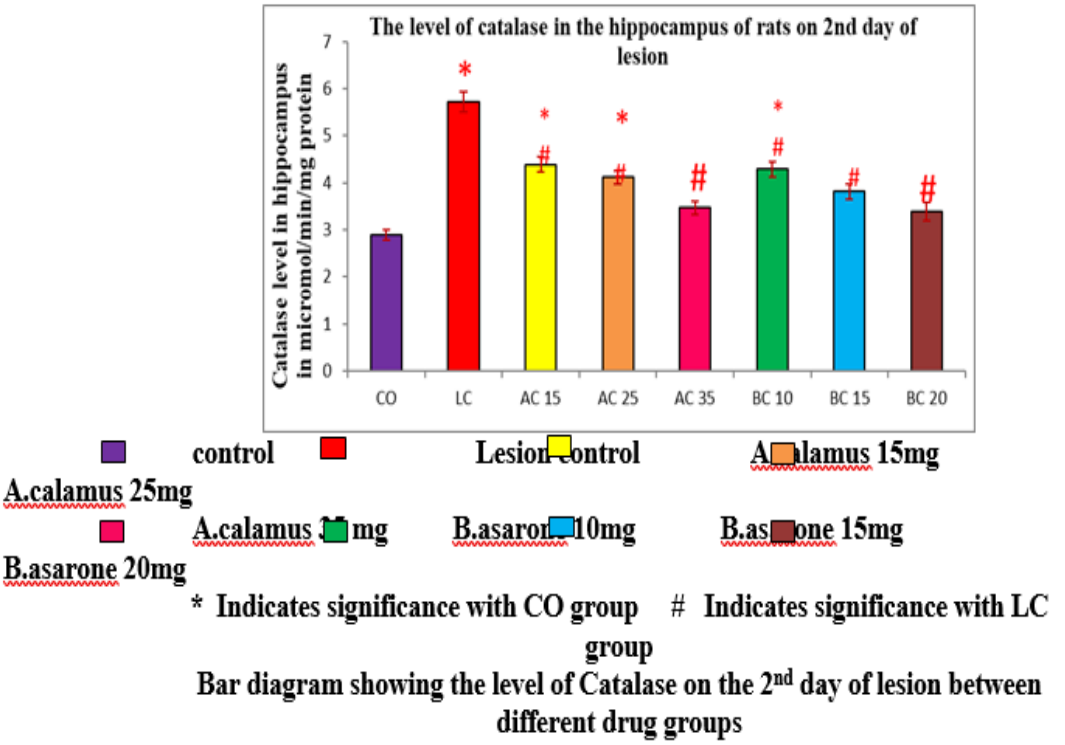
Catalase (Sinha, 1972)

Catalase is an antioxidant enzyme and is an important one. Normal nerve cells will produce certain amount of catalase and neutralise the deleterious effects of free radicals produced in the normal metabolic stages of the cells. The nerve cells will produce elevated levels of catalase during oxidative insult to neutralise the high level of free radicals produced. So elevated level of catalase is related to high nerve cell damage and functional deficit.

Analysis of catalase on the 2nd day of lesion (chart-III)

In this present study, on 2nd day catalase levels of LC group animals were significantly high and were saying the high free radical formation. The animals belong to the other groups like AC 15, AC 25, BC 10 and BC 15 were shown high catalase levels in comparison with the CO group and significantly low in comparison to the LC group animals of the 2nd day. The groups AC 35 (df=3,20. F=22) and BA 20 (df=3,20. F=13) the catalase levels were significantly low in comparison with the LC group and near equal when comparison with the CO group.

Chart-III



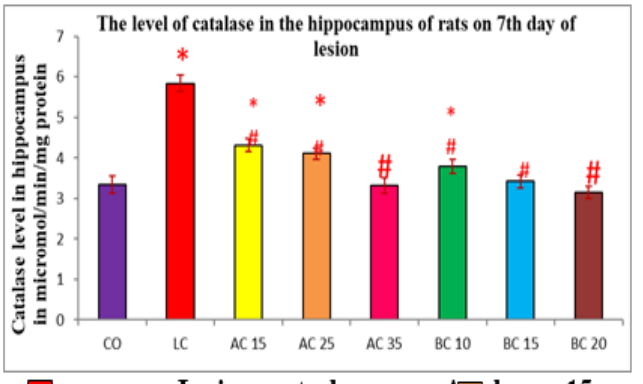
Bar diagram showing the level of Catalase on the 2nd day of lesion between different drug groups

Bar diagram showing the level of Catalase on the 2nd day of lesion between different drug groups

Analysis of Catalase on the 7th day of Lesion(chart-IV)

On the 7th day the LC animals exhibits significantly high catalase level while comparing with CO animals. The groups AC15, AC25 and BC10 were not shown significant decrease in the catalase level even on the 7th day when comparing with the same groups on the 2nd day. The drug group animals for AC 35 (df=3,20. F=8), BA 15 and BA 20 shown a decrease in catalase level in comparison with LC animals on the 7th day and the same groups of the 2nd day, stating they were good in preventing reactive oxygen species and also good in neuroprotection.

Chart-IV



control Lesion control A.calamus 15mg A.calamus 25mg A.calamus 35mg B.asarone 10mg B.asarone 15mg B.asarone 20mg

* Indicates significance with CO group # Indicates significance with LC group

Bar diagram showing the level of Catalase on the 7th day of lesion between different drug groups

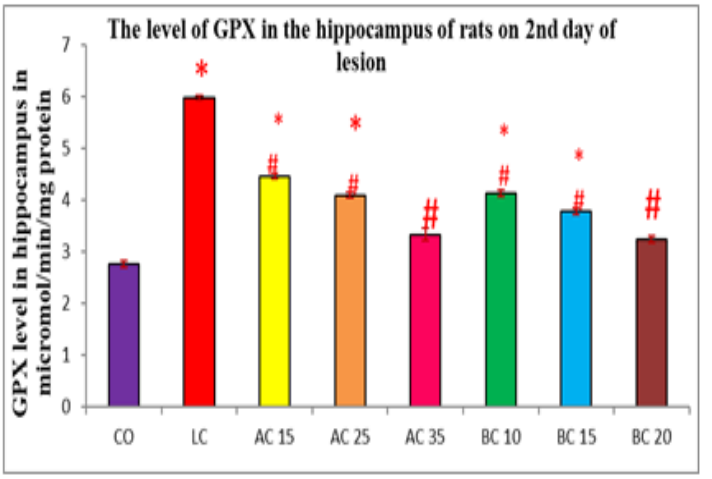
Glutathione Peroxidase (GPX) (Rotruck *et al.*, 1973)

When the production of free radicals is more, the neurons try to produce more amount of this antioxidant enzyme and that indirectly says the level of tissue damage too.

GPX level on the 2nd day of Lesion (chart-V)

The GPX levels were analysed on the 2nd day on the LC group that shown an increased GPX level while comparing with CO animals. The groups AC15, AC25, BA10 and BA15 also had an increase in GPX level in comparison with the CO group but significantly low when comparing with the LC group states poor protection. But the AC 35 (df=3,20. F=84) and BA 20 (df=3,20. F=81) animals were showing nearly equal in the GPX level in comparison with the CO group.

Chart-V



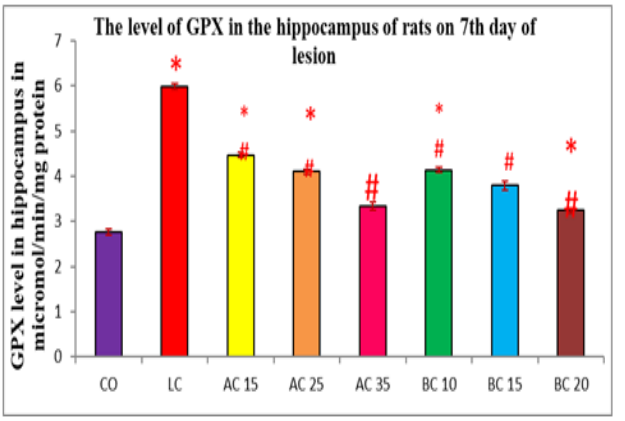
* Indicates significance with CO group # Indicates significance with LC group

Bar diagram showing the level of GPX on the 2nd day of lesion between different drug groups

GPX Level On The 7th Day Of Lesion (Chart-VI)

On the 7th day the LC animals exhibited a rise in the GPX level when comparison with the CO group and also with the LC group of the 2nd day. The animal groups AC 15, AC 25 (df=3,20. F=88), BA 10 and BC 15 were exhibited a decrease in the GPX Level in comparison with the CO group and GPX levels on the 2nd day animals of the same group.

Chart-VI



control Lesion control A.calamus 15mg A.calamus 25mg A.calamus 35mg B.asarone 10mg B.asarone 15mg B.asarone 20mg

* Indicates significance with CO group # Indicates significance with LC group

Bar diagram showing the level of GPX on the 7th day of lesion between different drug groups

The groups AC 35 and BA 20 (df=3,20. F=69) had significant decrease in the GPX level in comparison with the LC group and also with the GPX levels of the same groups on the 2nd day and indicates good neuroprotection.

Discussion

Accoding to Nasir, 2021[11], the leaf extract of *Acorus calamus* has nephroprotective effect and also protective effect over hepatotoxicity. Djarkasi *et al.*, 2019[12], proved the antioxidant acitivity of bioactive compounds of *A. calamus* extracted from the leaf and rhizome using different solvents. Dinev *et al.,* 2021[13], concluded that *Acorus calamus* has high potency in antioxidant activity and the free radical scavenging activity of Acorus calamus rhizome was proved by Sharma *et al.,* 2020[14]. Esfandiari et al.,[15], proved that Acorus calamus treatment increases the oxidant enzymes in the brain tissues of neuroinflammation rat models.

With this present study,on 2nd and 7th days the antioxidant enzyme levels were very high with the LC group than the CO group, and can be conclude as less effective in neuroprotection. The drugs AC 15, AC 25 and BA 10 were not effective on both the days against oxidative stress as may be the dosage was not enough.

According to *Singh et al.,* 2019[16], oxidative stress is the major mechanism behind neurodegenerative disorders and excitotoxicity is the major molecular mechanism behind oxidative stress and so Nerve cell degeneration in various neurodegenerative disorders, Yalcin and Yalcin, 2018[17].

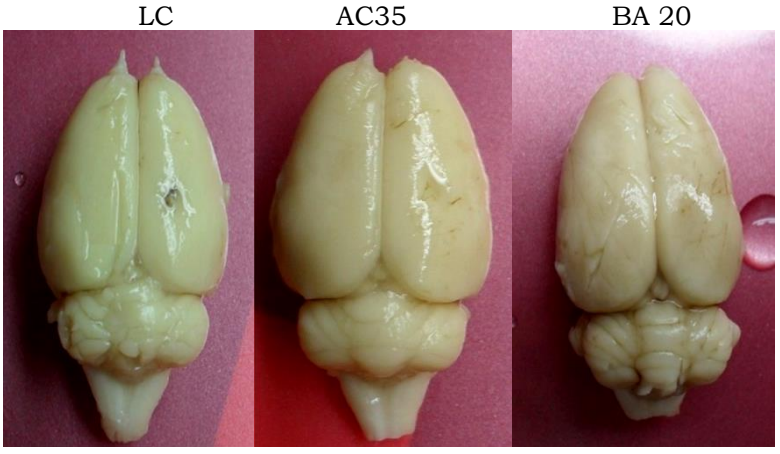
In this present study, the groups AC 35, BA 20 were showing only slight elevation in the level of antioxidant enzymes when compared with CO group on the 2nd day and was decreased on the 7th day telling the drug dosage was both protective against neurodegeneration by reducing the production of free radical and also can reverse the after effects of free radicals and so can apply as a neuroprotective drug and a drug to cure epilepsy.

Sharma et al.,[18] concluded that the phytoconstituents such as α-asarone, β-asarone, eugenol, and calamine have a big role in neurological disorders and claimed those can be used as phytomedicines in clinical research. Yang et al.,[19] concluded that β-asarone can prevent Alzheimer's Disorder by preventing the degeneration of astrocytes by downregulating AQP4 expression and Wei et al[20]., by their work proved that β-asarone prevent the apoptosis of nerve cells through CaMKII/CREB/Bcl-2 signaling pathway.

The neuroprotective activity of phytochemicals was also proved by Saki et al[21]., Naoi etal[22]., and Ma et al[23]., 2018. According to Balakrishnan et al.,[24] and Yang et al[25]., claimed that β-asarone prevent the hypoxic injury od PC12 cells by downregulating RPPH1/MiR-542-3p/ DEDD2 Axis.

In this current study, we have proved the efficacy of the herb anatomically by analyzing the level of lesion over the brain of different animals groups. The LC animals shown a large lesion both on the 2nd and 7th day,

Fig-II



Figures shows gross anatomical changes in the cortex of rat brain on the 2nd day of lesion

but with the groups AC 35, BA 20 the lesion was very small on the 2nd day and nearly closed on the 7thday, which proved the protective and treating nature of the drug employed.

Conclusion

Aging and social burden increasing the neuronal disorders and that can be prevented by the secondary metabolites of α- and β-asarone through their antioxidant or antiapoptotic activity according to a number of researchers including us.

So, in the light of the above reviews, we conclude that this herbal drug can be very well used as a regular supplement along with other regular food or drinks to prevent neurodegeneration as we all know that prevention is better than cure. As neurodegeneration is a threat to all the mankind after a certain age, we also have plans to elaborate this study to analyze the treatment part of this herb.

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