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EFFECT OF *ARECA CATECHU* EXTRACT ON MAXIMAL ELECTROCONVULSIVE SHOCK INDUCED SEIZURE IN RATS WITH SPECIAL EMPHASIS ON OXIDATIVE STRESS AND HISTOLOGICAL CHANGES IN THE BRAIN

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Abstract

In this study, we looked at the antiepileptic properties of an *Areca catechu* fruit hydroalcoholic extract in electrically induced seizures in Wistar albino rats, with a focus on oxidative stress and histopathological changes in different areas of the brain. The hydroalcoholic extract of *Areca catechu* fruit (1g/kg) and (2 g/kg) were tested for antiepileptic activity in Wistar albino rats during a maximal electroconvulsive shock-induced seizure using an electroconvulsiometer. The hydroalcoholic extract of *Areca catechu* significantly decreased the tonic hind limb extension (THLE) duration and protected rats from the seizures induced by maximal electroconvulsive shock. The study concluded that an alcoholic extract of the *Areca catechu* fruit has antiepileptic properties because it shortens the duration of the extensor phase of seizures.

Keywords: Antiepileptic activity, *Areca catechu*, Seizures, Tonic hind limb extension, Rats.

INTODUCTION

Epilepsy is a chronic neurodegenerative disorder of the brain affecting 30,000 people worldwide every year (1). Globally, a reduction in the mortality rate and severity of the disease has been seen from 1996 to 2016 (2). There are several classes of antiepileptic drugs available (3). The antiepileptic drug is chosen primarily based on the type and severity of the seizure (4).Anti-epileptic drugs used correctly provide control in 60-70% of patients (5).The currently used anti-epileptic drug does not prevent the progression of the pathological condition underlying epilepsy. Hence, herbal medicines provide an alternative research area for the scientist with fewer adverse and better treatment effects (6).Herbal medicines have been used as anti-epileptic agents since

ancient times, but their use is limited due to a lack of sufficient evidence of their efficacy and toxicity (7).Hence, the effect of *Areca catechu* fruit extract is studied for its antiepileptic activity.

The plants are rich sources of therapeutically potential chemical components that are easily available and considered to be safer than synthetic molecules. Severe oxidative stress, followed by neuroinflammations and degenerative changes to vital brain tissues, is the pathology underlying many neurodegenerative disorders (8).Several research groups have attempted to assess the effect of plant extracts and their isolated components for neuroprotective activity against many neurodegenerative conditions in recent decades and found some promising results; thus, the present study evaluate the preventive effect of Areca catechu plant extract against maximal electroconvulsive-induced seizure and neurodegenerative changes in Wistar albino rats. *Areca catechu* has variety of medicinal properties, including antioxidant (9,10), analgesic (10), anti-inflammatory(10), antidiabetic (11), hepatoprotective (12), wound healing(13) etc.

MATERIALS AND METHODS

Requirements

Surgical Scissors, Forceps, Electroconvulsiometer, Ear electrodes, Capillary tubes, Clot activator tubes, EDTA tubes, Eppendorf tubes, Sample containers, Ice cold saline phosphate buffer, Semi-autoanalyzer, Remi refrigerated centrifuge, Potter - Elvehjem homogenizer, UV-Visible spectrophotometer (Shimadzu –UV 1800), Micropipettes, Light microscope (Olympus microscope), Sony digital camera (for photomicrographs).Chemicals, reagents, drugs used in this study were purchased from authorized dealers.

Areca catechu fruit collection

Areca catechu fruit ranging in age from 6 to 7months was collected from the coastal region of Karnataka, and identified at the Pharmacognosy Department, SDM Centre for Research, Kuthpady, Udupi. The fruit was removed from the adulterants and crushed to open the nut. The nut was powdered after being sundried.(Figure 1)

Extraction Methodology and yield

About 100gm of the sample was accurately weighed in a stoppered glass flask. To this 200 ml of alcohol (approximately 95%) and water (1:1) was added. Occasionally shaken for 6 hours.Then it was allowed to stand for 18 hours. Rapidly filtered without the loss of any solvent. filtrate was allowed to dry on hot water bath after which filtratewas kept in an air oven at 105^0C for 6 hours, cooled in desiccator for 30 minutes and weighed. The extract was calculated for the percentage of yield and yield was 11.83% (14) (Figure2).

Figure 1: Areca catechu row fruit and dried fruit

Figure 2: Hydroalcoholic extract of *Areca catechu* (HAEAC)

Figure 3: Wistar albinorats

Animals

Male Wistar albino rats (15,16) were selected and housed under standard temperature, humidity, 12 hours light cycle and dark cycle. Standard pellet food and the water were supplied ad libitum. Animals had full access to the feed and water. The study was approved from Animal Ethical Committee, Saveetha Medical College, IAEC approval number SU/CLAR/RD/003/08/2020 dated August13, 2020 and conducted as per the CPCSEA guidelines, Government of India. (Figure 3)

Experimental design

The rats were pre-tested 24 hours prior to the administration of test drugs for sensitivity to electric shock, and those rats that exhibit full extensor phase were selected for further study. Five groups of rats, each consisting of six rats, were grouped in cages. The groupI served as normal control; the group II served as seizure control, which received 0.5 ml of distilled water; and the group III served as standard, which received the standard drug phenytoin sodium (20-25 mg/kg). group IV and group V were the test groups that received the HAEAC(1g/kg) and HAEAC(2g/kg) body weight respectively.Respective groups received control, standard and test drugs for 14 consecutive days.

Induction of seizure

One hour after treatment on the14th day, the rats were subjected to maximal electroconvulsive shock by using an electro convulsiometer (150 mA to 180 mA) through ear electrodes for 0.2sec. The behavioral convulsion parameters noted are the duration of tonic flexion,

tonic extension, clonus, tonic hind limb extension, stupor and recovery time for each animal. A reduction or decrease in the extensor phase of seizure was taken as index for measurement(17). (Figure 4)

Figure4: Applying Maximal Electroconvulsive Shock

Seizure score and percentage protection

Animals were placed individually in the glass cage and monitored convulsive parameters for 30 min. Seizure onset, duration, and severity score were all recorded. The severity of the seizure was graded using following scoring system,

Grade 0	No response for seizure activity
Grade 1	Facial movements - facial clonus, closing of eye, twitching of ear
Grade 2	Head nodding and increased facial clonus
Grade 3	Clonus of one or two fore limbs
Grade 4	Complete forelimb extension and partial hindlimb extension
Grade 5	Jumping and posture loss
Grade 6	Generalized Clonic/tonic seizures
Grade 7	Complete tonic
Grade 8	Death

The rats were observed for 30 minutes and the end point was animals not showing or decrease in the duration of THLE. The percentage protection was given by

$$\text{Percentage protection}(\%) = \frac{\text{Number of animals with THLE absent}}{\text{Total Number of animals}} \text{X } 100$$

(THLE- Total Hind Limb Extension)

The time span of seizure episode is recorded using a timer. The rats were monitored for 5 more minutes to make sure that they recovered from the seizures.

After the seizure episode, blood was collected from the retroorbital puncture. In a K3 EDTA and clot activator tube, 1 ml of sample was collected. K3 EDTA tube sample was used for complete blood count estimation and a clot activator tube was centrifuged at 3000 rpm for 10

minutes to obtain serum. Serum was collected in an Eppendorf tube and used to estimate C-reactive protein. The rats were sacrificed without anesthesia by cervical dislocation. Rats brain were quickly dissected and rinsed in ice-cold phosphate buffered saline. The weight of the brain was noted. Then an immediately isolated brain section was used for further investigations. (Figure 5).

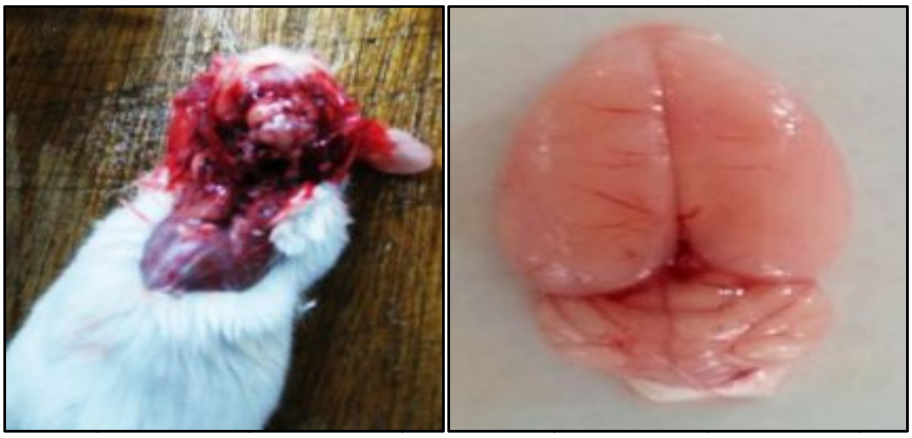

Figure 5: Dissected rat andthe brain

Estimation of complete blood count

Complete blood count was performed from the whole blood in an automated hematology analyser Biorad for estimating red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB), hematocrit (HCT), number of platelets.

Estimation of CRP

The serum sample taken in test tube and allowed to stand for 15-20 min. Centrifuged at 3000 rpm for 15 minutes. A quantitative turbidimetric method CRP-turbilatex was used to measure the CRP. Working reagent was prepared by adding 1ml of latex reagent in 9ml of buffer solution. CRP calibrator was reconstituted with 1ml of distilled water. The turbidimetry analyser was calibrated and reading was stored by using adding 1000µl of working reagent in 5µl of calibrator. The sample reading was taken similarly by adding 1000 µl of working reagent and 5µlof serum sample. The CRP reading was obtained in mg/L.

Preparation of Tissue homogenate

In Tris - HCl buffer (0.1 M, 4°C, pH 7.4), the weighed brain tissues were homogenized using a Potter - Elvehjem homogenizer with a Teflon pestle run for 3 minutes at 600 rpm. It was centrifuged at 3000 rpm at 4°C for 10 minutes with Remi refrigerated centrifuge and used for determine the antioxidant parameters. The protein levels of the tissue homogenate were measured bythe Bradford assay.

Assay oflipid peroxidation (LPO)

Lipid peroxidase estimation was done by detectionof thiobarbituric acid reactive substances (TBARS). The assay tubes contained 580µL of phosphate buffer (0.1 M, pH 7.4),20µL of $FeCl_3$, and 200µL of ascorbic acid (100 mM), 200µL of supernatant. In a water bath, the tubes were incubated at 37°C for 60minutes. 1ml of trichloroacetic acid (10%) and 1mL of thiobarbituric acid (0.66%) was added to the tubes to stop reaction. 20minutes tubes were kept

in a water bath.Tubes were cooled in an ice-cold water, and centrifuged for 10 min. The supernatant absorbancereading was taken at 535 nm using an UV-Visible spectrophotometer (Shimadzu –UV 1800) and expressed as TBARS- nmol/mg protein (18).

Assay of SOD

Superoxide dismutase (SOD) activity is estimated by the reaction of xanthine oxidase and xanthine producing superoxide radicals which later reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form red formazon dye. Mixed substrate (300μL) was added to tissue homogenate (200μL). The sample tubes are mixed properly and 75μL xanthine oxidase was added to the reaction tubes. Absorbance of the tubes was taken at 505 nm using an UV-Visible spectrophotometer and the SOD effect was then measured and expressed in U/mg protein.[19]

Assay of Catalase

About 10μL of tissue homogenate was taken in a test tube and 100μmol/ml of hydrogen peroxide in Tris-HCl buffer pH7 was added and incubated for 10mins. The reaction was ended by promptly adding 4% ammonium molybdate and the absorbance measured at 410 nm using an UV-Visible spectrophotometer. Catalase activity was expressed asμmole/H_2O_2/min/mg protein (20).

Estimation of glutathione content

About 15μL of tissue homogenate was added to 260μL assay buffer and 5 μL Ellman reagent. Sample tube were kept 15 minutes at room temperature and the yellow colorformed from the TNB^{2-} was estimated in anUV visible spectrophotometer at 412nm. The standard curve generated from known GSH standard. Absorbance values were associated with a standard curve.The values obtained were expressed in U/g protein (21).

Histopathological examination

Brain tissue was subjected to paraffin sectioning for histopathology and light microscopy. The hydration and dehydration of the tissues were done in sorted alcohol, which was then cleared using xylene and chloroform. Then, using a rotary microtome it was fixed in paraffin wax. After taking the sections of the tissues (10μm), they were placed overnight at room temperature. Following that, it was de-paraffinized and moistened with descending alcohol concentrations, followed by dist. H_2O. The brain sections were stained using hematoxylin and eosin stain and then subjected to increasing alcohol concentrations. The permanent slide was prepared using a DPX mount.The slides were observed under an Olympus light microscope, and photomicrographs were taken by a Sony digital camera

Statistical Analysis

Protection of the animals from the maximal electroconvulsive shock was expressed as percentage of protection. Other data were mentioned as mean ± Standard deviation. The statistical analysis of the obtained data was performed using one-way ANOVA followed by Dunnett's test. The p value of <0.05 is considered as significant.

RESULTS

In the maximal electroconvulsive model, the duration of THLE and recovery time after a seizure episode were reduced in drug treated groups HAEAC (1g/kg) and HAEAC (2g/kg) The

duration of THLE in seizure control group was 16.55±0.56, in standard group was 3.46±0.32 and in HAEAC (1g/kg) and HAEAC (2g/kg) was 13.45±0.61, 9.14±0.55 seconds respectively.The recovery time after seizure episode in seizure control group was 3.42±0.19, in standard group was 1.26±0.11 and in HAEAC (1g/kg) and HAEAC (2g/kg) was 2.49±0.07, 2.34±0.08 minutes respectively(Table 2).

Seizure scores of the groups HAEAC (1g/kg) and HAEAC (2g/kg) was compared with seizure control and standard group. The seizure scores of seizure control group was 8.0±0.0, standard group was 2.83±0.41 and drug treated group HAEAC (1g/kg) and HAEAC (2g/kg) was 7.0±1.10,5.66±1.21 respectively (Table 2). Weight of the excised brain in HAEAC (1g/kg) and HAEAC (2g/kg) is compared with normal control and standard group. Weight of the brain in HAEAC (2g/kg) group was almost equal with the brain of the standard group. (Table 2)

The seizure control group showed 0% protection, the standard group showed 100% protection, the test group HAEAC (1g/kg) and HAEAC (2g/kg) showed 33.2% and 66% protection respectively(Table 2).

The biochemical parameter and inflammatory markers of the drug treated groups was compared with standard and seizure control group. The inflammatory markers like WBC and CRP was levels were decreased in drug treated groups when compared to seizure control. The WBC of the seizure control group was 10.24±0.31 whereas in the HAEAC (1g/kg) and HAEAC (2g/kg) was 7.38±0.33 and 6.78±0.22 respectively. The CRP of the seizure control group was 0.52±0.05 whereas in the HAEAC (1g/kg) and HAEAC (2g/kg) was 0.42±0.02 and 0.39±0.02 respectively. The other hematological parameters were not changed significantly (Table 3).

The antioxidant parameterslipid peroxidation (LPO), catalase activity (CAT), superoxide dismutase activity (SOD), glutathione (GSH) content in the brain was measured by using UV-visible spectrophotometer (Shimadzu –UV 1800). The defensive antioxidant parameters like SOD, CAT and GSH contents in brain in the drug treated group HAEAC (1g/kg) and HAEAC (2g/kg) was significantly increased when compared with seizure control group, whereas lipid peroxidase (LPO) content in the group HAEAC (1g/kg) and HAEAC (2g/kg) was significantly decreased when compared along with seizure control group which will prevent the brain damage from the oxidative stress in the body (Table4).

Table 1: Effect of oral administration of HAEAC on body weight (g) of rats

Body weight	**Normal control**	**Seizure control**	**Standard**	**HAEAC (1g/kg)**	**HAEAC (2g/kg)**
Day 1st	179.2±2.43	178.4±3.62	176.8±2.87	178.0±1.70	174.9±2.26
Day 14th	184.8±2.61	186.2±1.81	183±1.80	185.1±2.49	178.1±4.59

HAEAC-hydro alcoholic extract of *Areca catechu,* Data were expressed as mean±STD.

Table 2: Effect of oral administration of HAEAC on MES induced seizure, seizure score and percentage protection,brain weight

Seizure parameter	Normal control	Seizure control	Standard	HAEAC (1g/kg)	HAEAC (2g/kg)
Tonic Flection(Sec)	0	5.29±0.12	2.31±0.07	4.47±0.28	3.67±0.39
Tonic extension(Sec)	0	7.88±0.09	1.44±0.17	7.15±0.06	6.64±0.25
Clonic Convulsion (Sec)	0	22.39±1.22	15.07±0.79	18.84±0.65	16.57±1.33
THLE(Sec)	0	16.55±0.56	3.46±0.32	13.45±0.61	9.14±0.55
Recovery time (Min)	0	3.42±0.19	1.26±0.11	2.49±0.07	2.34±0.08
Seizure score	0	8.0±0.0	2.83±0.41	7.0±1.10	5.66±1.21
Percentage protection	0	0	100%	33.2%	66%
Brain weight -14th day	1.56±0.06	1.33±0.04	1.45±0.05	1.37±0.03	1.45±0.02

HAEAC- Hydro alcoholic extract of *Areca catechu,* MES-Maximal Electro Convulsive Shock

The values are expressed as mean±STD,p value <0.05 when compared with the seizure control group.

Table 3: Effect of oral administration of HAEAC on hematological and biochemical parameters

Blood parameters	Normal control	Seizure control	Standard	HAEAC (1g/kg)	HAEAC (2g/kg)
RBC (10^6/µl)	5.43±0.21	4.97±0.34	5.19±0.25	5.38±0.22	5.35±0.27
WBC (10^3/µl)	5.99±0.28	10.24±0.31	6.10±0.22	7.38±0.33*	6.78±0.22*
Platelets (10^3/µl)	1140±7.01	1094±11.94	1138±6.61	1150±6.29	1158±4.48
HGB (g/dl)	13.15±0.35	12.07±0.20	13.66±0.47	13.54±0.25	13.30±0.32
PCV (%)	42.36±0.37	42.02±1.04	43.05±0.36	42.91±0.35	43.38±0.22
CRP(mg/L)	0.32±0.03	0.52±0.05	0.35±0.03	0.42±0.02*	0.39±0.02*

RBC-red blood cells, WBC–white blood cells, HGB-hemoglobin, PCV-packed cell volume, CRP–C reactive protein, HAEAC-hydro alcoholic extract of *Areca catechu*

The values are expressed as mean±STD,* p value <0.05 when compared with the seizure control group.

Table 4: Effect of oral administration of HAEAC on antioxidant parameters of brain tissues

Oxidative stress	Normal control	Seizure control	Standard	HAEAC (1g/kg)	HAEAC (2g/kg)
SOD(units /mg ptn)	15.12±0.49	5.73±0.40	13.18±0.53	7.97±0.54	9.05±0.51
CAT(µmole/H_2O_2/min/mg ptn)	28.72±0.50	10.18±0.45	22.08±1.44	16.6±0.77	20.17±1.04
GSH (µmole/mg ptn)	71.48±0.88	21.38±0.82	61.20±0.86	31.78±0.77	50.05±1.05
LPO (nmol/mg tissue)	2.55±0.23	8.05±0.24	3.36±0.17	6.03±0.10	4.24±0.11

ptn – protein, SOD-superoxide dismutase, CAT-catalase, GSH-glutathione, LPO-lipid peroxidase, HAEAC-hydro alcoholic extract of *Areca catechu*

Values are expressed as mean±STD, p value <0.05 when comparedwith the seizure control group.

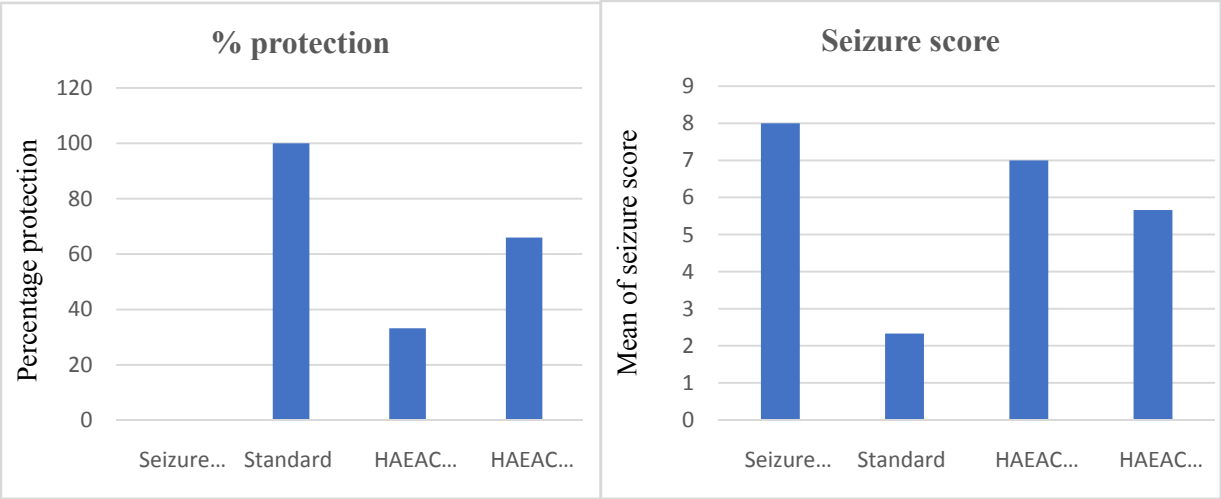

Figure 6: Effect of HAEAC on percentage protection and seizure score in MES

Histopathological study

Histopathological study of the hippocampus region and cerebral cortex of all the group of rats were studied at magnification 40X, scale bar 25µm on Olympus light microscope.Photomicrographs were taken by Sony digital camera. (Figure 7)

Interpretation:

Control (group I)

Image 1, 2,3 -In the normal control rats showed granular layer cells have large vesicular nuclei with well-defined nucleoli (black small arrow), while pyramidal cells are normal in size.

Image 4- In normal control rats hippocampus showed, CA3 area having molecular, pyramidal, and polymorphic layers. The pyramidal cells have large neurons with clear rounded vesicular nuclei (orange arrow), some are binucleated (green arrow).

Seizure control (Group II)

Image 5, 6 & 7 – In the seizure control group rat's cerebral cortex showed focal gliosis (green arrow) and showing congestion of cerebral blood vessel (blue arrow), hemorrhage in meninges, Glial cells are appeared in the molecular layer, binucleated cells (head arrow), shrunken pyramidal cells with pyknotic nuclei (red arrow).

Image 8 – Hippocampus showed evident signs of degeneration, pyknotic nuclei is seen in the small pyramidal cells (black broad arrow), perineuronal spaces are wide, karyolitic nuclei also seen in some pyramidal cells (orange arrow).

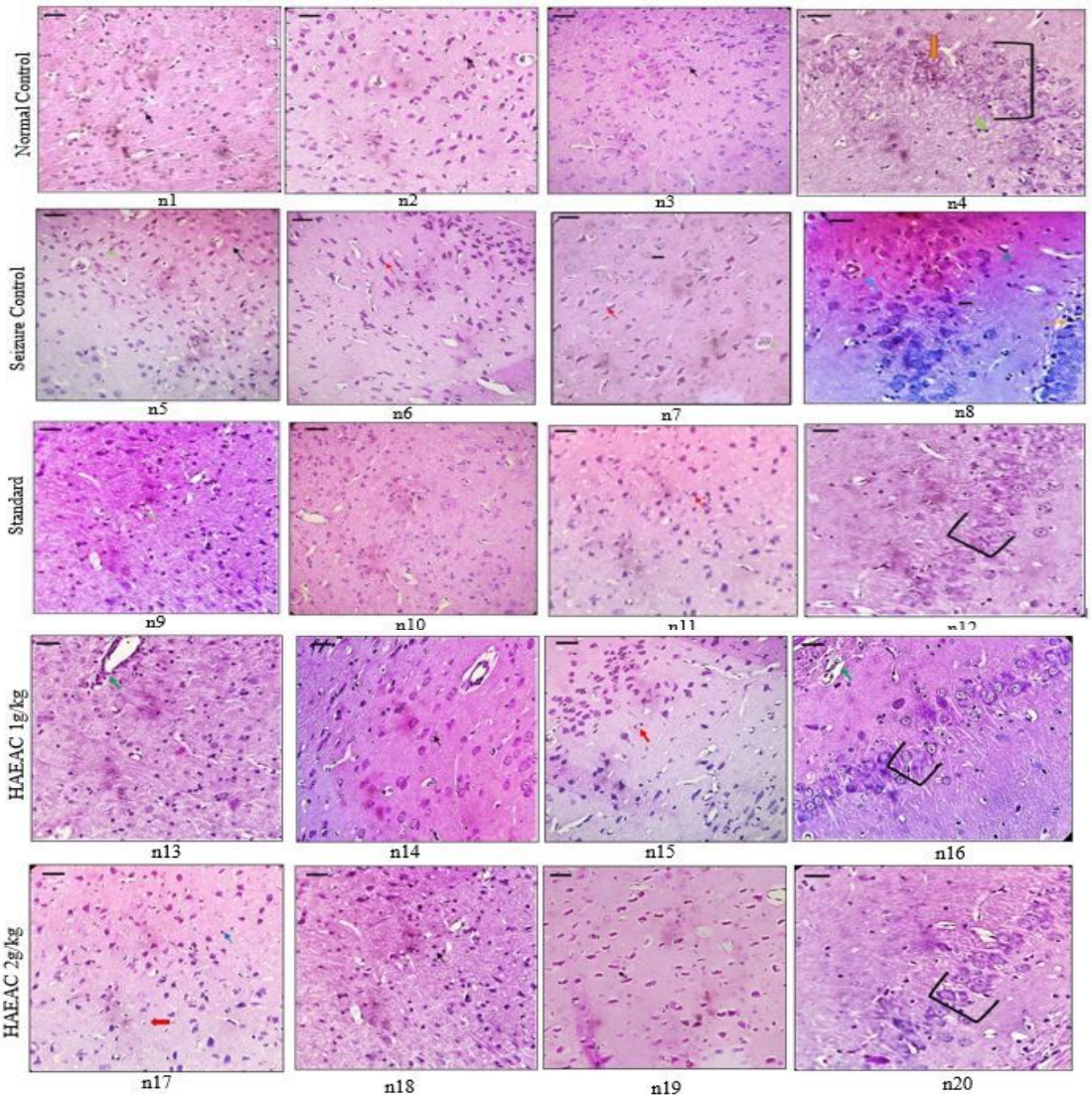

Figure 7: Histological study slides of cerebral cortex and hippocampus of rats

Standard (Group III)

Image 9, 10, 11- In standard phenytoin sodium group rat's tissues showed reduced gliosis, some pyramidal cells have karyotic nuclei.

Image 12- The hippocampus was histologically intact. The pyramidal cells have round vesicular nuclei with large neurons.

HAEAC 1g/kg (Group IV)

Image 13, 14, 15- In HAEAC (1g/kg) rats showed inflammatory cells (green arrow), and reduced gliosis (red arrow) normal nucleated pyramidal cells (black arrow), degeneration of some Purkinje cells (red arrow). (↑), some are binucleated.

Image 16- The hippocampus showed less neurodegeneration, less inflammation in molecular layer (green arrow) and improved number of cells.

HAEAC 2g/kg (Group V)

Image 17, 18 & 19 - In HAEAC (2g/kg) rats showed interneurons (blue arrow), glial cells (red broad arrow) and pyramidal cells with appearance of vesicular nuclei (black arrow).

Image 20- The hippocampus showed less neurodegeneration and normal cell density.

DISCUSSION

The development and deterioration of the pathological condition of epilepsy are significantly influenced by oxidative stress. Oxidative stress releases reactive oxygen species, which leads to the worsening of the epileptic condition. The percentage of protection and decrease in THLE in the maximal electroconvulsive shock model is considered an index for the study of Areca catechu extract's antiepileptic property. The present study protected 33.2 % and 66% of animals in groups HAEAC (1g/kg) and HAEAC (2g/kg) from the THLE, respectively(Figure 6). The duration of THLE and recovery time after a seizure episode were significantly reduced in drug treated group when compared with the seizure control group. The seizure score was decreased and the weight of the brain increased in the HAEAC (1g/kg) and HAEAC (2g/kg) groups when compared with the seizure control group (Table 2, Figure 6). In MES induced seizures, THLE is decreased or abolished by drugs like phenytoin and carbamazepine, which act by prolonging the voltage gated Na^+ channel inactivation and also decreasing the release of presynaptic excitatory neurotransmitters. The abolition or decrease of THLE, which prevents the spread of seizures in the brain and the increase in the percentage protection provided by the hydroalcoholic extract of *Areca catechu* suggest that plant fruit possesses antiepileptic properties.

The seizure episode causes inflammatory reactions in the brain and leading to an increase in the level of inflammatory markers in the blood. When compared to the seizure control, inflammatory markers such as WBC and CRP levels are significantly lower in the HAEAC (1 g/kg) and HAEAC (2 g/kg) groups. This suggests that plant fruit extracts can prevent the progression and deterioration of epilepsy caused by inflammatory reactions, implying antiepileptic properties.

The defensive anti-oxidant parameters superoxide dismutase, catalase, and glutathione were markedly increased, and the aggressive parameter lipid peroxidase content was decreased in HAEAC (1g/kg) and HAEAC (2g/kg) groups compared to the seizure control group. (Table 5). The oxidative stress caused by the seizure episode causes an increase in free radical concentration by releasing too much excitatory neurotransmitter glutamate into the synaptic cleft. Increased defensive and decreased aggressive antioxidant synthesis by interfering with the

release of excitatory neurotransmitters, which leads to free radical generation in the drug treated group, may contribute to the antiepileptic properties of the hydroalcoholic extract of *Areca catechu*.

Histopathological study of the cerebral cortex and hippocampal region of the brain also supports the antiepileptic properties of the hydroalcoholic extract of *Areca catechu.* The focal gliosis, congestion of cerebral vessels, brain hemorrhages, and shrunken pyramidal cells in the cerebral cortex and hippocampus showed significant neurodegeneration, and pyknotic nuclei were seen in the pyramidal cells, indicating the characteristic feature of apoptosis. Whereas in the drug treated HAEAC (1g/kg) and HAEAC (2g/kg) groups, the cerebral cortex showed reduced gliosis and inflammatory cells, pyramidal cells are normal with a vesicular nucleus. The hippocampus showed less inflammation in the molecular layers, improved cell number and density. This clearly indicates an improvement in the pathological condition of neurons in the brain (Figure 7).

CONCLUSION:

The current study revealed that the hydroalcoholic extract of *Areca catechu* possessed antiepileptic property by increasing the percentage protection and decreasing the THLE time. The reduction of the inflammatory markers WBC and CRP, as well as the increase of protective antioxidant markers such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), as well as the reduction of the lipid peroxidase (LPO), which is progressively involved in membrane rupture and cell death, Histopathologically, there was also improvement in the pathological condition. Hence, *Areca catechu* fruit could possibly be used as an antiepileptic agent; however, further research is warranted to confirm the exact mechanism of action.

CONFLICTS OF INTEREST:

The author declares no conflicts of interest.

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