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EFFECT OF *Enicostema axillare w.p* EXTRACT ON NEUROTRANSMITTERS CONCENTRATIONS IN RAT BRAIN AFTER INDUCTION OF SEIZURES

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ABSTRACT

The whole plant of *Enicostema axillare* is traditionally used for the treatment of epilepsy, Diabetes mellitus, Rheumatism, Cancer and Hepatic disorders. Previous studies have demonstrated that extracts of these plants was subjected to acute toxicity and then screened for antiepileptic activity on Maximal Electroshock (MES) and Pentylenetetrazole (PTZ) induced seizures models in albino wistar rats. The purpose of the present study is to investigate the effect of Chloroform extract of *Enicostema axillare* (CEEA) on neurotransmitters concentrations in rat brain after induction of seizures by MES and PTZ. Our aim of study was relationship between seizure activities and altered the monoamines such as noradrenaline (NA), dopamine (DA), serotonin (5-HT) and Gamma amino butyric acid (GABA) in forebrain of rats in MES and PTZ seizure models. In MES model, CEEA (200 & 400 mg/kg) significantly restored the decreased levels of brain monoamines such as NA, DA, 5-HT and GABA. Similarly in PTZ model, CEEA significantly increased the monoamines in forebrain of rats. Thus, this study suggests that Chloroform extract of *Enicostema axillare* increased the monoamines on rat brain, which may be decreased the susceptibility to MES and PTZ induced seizure in rats.

Keywords: Antiepileptic activity, Traditional Medicine, *Enicostema axillare*, neurotransmitters,NA, DA, 5-HT and GABA

INTRODUCTION

The role of dopamine and serotonin in epilepsy remains controversial, but both have convincingly been implicated in the pathophysiology of seizures [1, 2]. All the currently available antiepileptic drugs are synthetic molecules. Medicinal plants used for the therapy of epilepsy in traditional medicine have been shown to possess promising anticonvulsant activities in animal models of anticonvulsant screening can be an invaluable source for search of new antiepileptic compounds. In previous study, the Chloroform extract of whole plant of *Enicostema axillare* (CEEA) was subjected to acute toxicity and then screened for antiepileptic activity on Maximal Electroshock (MES) and Pentylenetetrazole (PTZ) induced seizures models in albino Wistar rats was reported. Therefore, the present study was performed to examine the effect of *Enicostema axillare* on neurotransmitters concentrations in rat brain after induction of seizures by MES & PTZ model.

***Enicostema axillare* w.p** (Family: Gentianaceae) is native to tropical Africa, India, Southeast Asia and Malaysia. It is a perennial herb found throughout India and is common in coastal areas**.** The plant is used in folk medicine to treat epilepsy, Diabetes mellitus, Rheumatism, Cancer and Hepatic disorders [3]. Therefore, the present study was performed to verify the effect of *Enicostema axillare* on neurotransmitters levels in rat brain after induction of seizure by MES and PTZ model.

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MATERIALS AND METHODS

Plant collection

The whole plant materials of Enicostema axillare were collected in month of January from Tirupati, Chittoor

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(Dist), Andhra Pradesh, India. The taxonomical identification of the plant was done by Dr. K. Madhava chetty, Assistant Professor, Department of Botany, Sri Venkateshwara University (SVU), Tirupati. The voucher specimen of the plant was deposited at the college for further reference.

Preparation of extracts

The whole plants were dried in shade, separated and made into dry powder. It was then passed through 40 mesh sieve. A weighed quantity (80gm) of the powder was subjected to continuous hot extraction in Soxhlet Apparatus. The extract was evaporated under reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample. Percentage yield of Chloroform extract of *Enicostema axillare* was found to be 14.5 % w/w.

Animals used

Albino Wistar rats (150-200g) of either sex were obtained from the animal house in Sree Vidyanikethan College of Pharmacy, Tirupati. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages. The animals were fed with standard pellet feed (Hindustan Lever Limited., Bangalore) and water was given *ad libitum*. Ethical committee clearance was obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA. (Ref No. **IAEC / XIII / 05 / SVCP / 2010).**

Experimental design

Albino Wistar rats were divided into four groups of six animals each. Group I animals treated with normal saline (0.9% NaCl), 1 ml/100 gm, whereas Group-II received standard drug (Phenytoin, 25mg/kg) *i.p*, Group-III and IV, received Chloroform extract of the whole plant of *Enicostema axillare* w.p (200 and 400 mg/kg b.w) *p.o* respectively for 7 days. On the 7th day, seizures are induced to all the groups by using an Electro convulsiometer. The duration of various phases of epilepsy were observed.
Pentylenetetrazole (90mg/kg b.w, *s.c*) was administered to other groups to induce clonic convulsions after above respective treatment. Animals were observed for a period of 30mins post– PTZ administration.

A fluorimetric micromethod for the simultaneous determination of serotonin, noradrenaline and dopamine

On the 7th day after observation of convulsion all groups' rats were sacrificed, whole brain was dissected out and the forebrain was separated. Weighed quantity of brain tissue was homogenized in 0.1 mL hydrochloric acid - butanol, (0.85 ml of 37% hydrochloric acid in one liter *n*-butanol for spectroscopy) for 1 min in a cool environment. The sample was then centrifuged for 10 min at 2,000 rpm. 0.08 mL of supernatant phase was removed and added to an Eppendorf reagent tube containing 0.2 mL of heptane (for spectroscopy) and 0.025 mL 0.1 M hydrochloric acid. After 10 min of vigorous shaking, the tube was centrifuged under same conditions to separate two phases. Upper organic phase was discarded and the aqueous phase (0.02 mL) was used for estimation of Serotonin, Nor Adrenaline and Dopamine assay [4].

Nor-Adrenaline and Dopamine Assay

The assay represents a miniaturization of the trihydroxide method. To 0.02ml of HCl phase, 0.05ml 0.4M and 0.01ml EDTA/Sodium acetate buffer (pH 6.9) were added, followed by 0.01ml iodine solution (0.1M in Chloroform) for oxidation. The reaction was stored after two minutes by addition of 0.01ml Na_2SO_3 in 5m NaOH. Acetic acid was added 1.5 minutes later. The solution was then heated to 100 for 6 minutes. When the sample again reached room temperature, excitation and emission spectra were read in the microcuvette as with 5-HT: in some cases, the readings were limited to the excitation maxima. 395-485nm for NA and 330-375nm for DA uncorrected instrument values [4].

Serotonin Assay

As mentioned earlier, some modifications in reagent concentration became necessary together with changes in the proportions of the solvent, in order to obtain in a good fluorescence yield with reduced volume for 5-HT determination, the O-pthalaldehyde (OPT) method was employed. From the OPT reagent, 0.025ml were added to 0.02ml of the HCl extract. The fluorophore was developed by heating at 100°C for 10 min. After the samples reached equilibrium with the ambient temperature, excitation / estimation spectra or intensity reading at 360-470 nm were taken in the micro cuvette [4].

Estimation of brain GABA content

The brain amino butyric acid (GABA) content was estimated according to the method of **Lowe et al., (1958)** [5]. Animals were sacrificed by decapitation and brains were rapidly removed, and forebrain region was separated. It was blotted, weighed and placed in 5ml of ice-cold trichloroaceticacid (10% w/v), then homogenized and centrifuged at 10,000rpm for 10min at 0°C. A sample (0.1ml) of tissue extract was placed in 0.2ml of 0.14 M ninhydrin solution in 0.5M carbonate-bicarbonate 1 buffer (pH9.95), kept in a water bath at 60°C for 30min, then cooled and treated with 5ml of copper tartrate reagent (0.16% disodium carbonate, 0.03% copper sulphate and 0.0329% tartaric acid). After 10min fluorescence at 377/455nm in a spectofluorimeter was recorded.

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Statistical Analysis

The data were expressed as mean ± standard error mean (S.E.M).The Significance of differences among the group was assessed using one way and multiple way analyses of variance (ANOVA). The test followed by Dunnet's test p values less than 0.05 were considered as significance.

RESULTS

Effect of CEEA on monoamines levels in seizure induced rats by MES and PTZ:

Noradrenaline

In MES and PTZ models, Noradrenaline levels significantly ($p<0.01$) decreased in forebrain of epileptic control animals. CEEA at the doses of 200 & 400mg/kg, standard drugs phenytoin and diazepam treated animals have shown a significant ($p<0.05$ & $p<0.01$) increase in Nor adrenaline levels in forebrain of rats. **Table 1 and 2**

Dopamine

In MES and PTZ models, Dopamine levels was significantly ($p<0.01$) decreased in forebrain of epileptic control animals. CEEA at the doses of 200 & 400mg/kg, standard drugs phenytoin and diazepam treated animals have shown a significant ($p<0.05$ & $p<0.01$) increase in Dopamine levels in forebrain of rats. **Table 1 and 2**

Serotonin

In MES and PTZ models, Serotonin levels have been significantly ($p<0.01$) decreased in forebrain of epileptic control animals were observed. CEEA at the doses of 200&400mg/kg, standard drugs phenytoin and diazepam treated animals showed a significant ($p<0.05$ & $p<0.01$) increase in Serotonin levels in forebrain of rats. **Table 1 and 2**

Gamma amino butyric acid

In MES and PTZ models, GABA levels was significantly ($p<0.01$) decreased in forebrain of epileptic control animals were observed. CEEA at the doses of 200 & 400mg/kg, standard drugs phenytoin and diazepam treated animals showed a significant ($p<0.05$ & $p<0.01$) increase in GABA levels in forebrain of rats. **Table 1 and 2**

Table: 1. Effect of CEEA on neurotransmitters levels in rat brain after MES induced epilepsy

Group	Design of Treatment	Noradrenaline	Dopamine	Serotonin	GABA
I	Vehicle Control(0.9% normal saline, 1ml/100gm)	746±5.22	648.40±3.24	189.26±2.41	294.24±1.72
II	MES (0.9% normal saline, 1ml/100gm)	407.62±2.12 ^{a**}	474.62±2.14 ^{a**}	85±1.80 ^{a**}	242.26±2.64 ^{a**}
III	Phenytoin 25mg/kg,*i.p*	594.06±2.24 ^{b**}	652.56±3.19 ^{b**}	152.26±2.14 ^{b**}	272.46±1.42 ^{b**}
IV	CEEA 400 mg/kg,*p.o*	564.16±2.24^{b**}	642±1.62^{b**}	124.33±1.88^{b**}	267.45±1.54^{b**}
V	CEEA 200 mg/kg,*p.o*	397.12±2.16^{b*}	578.14±2.43^{b*}	92.52±1.82^{b*}	238.54±2.32^{b**}

Values are expressed as mean ± SEM of six observations. Comparison between: **a**- Group I Vs Group II, **b**- Group III Vs Group IV and Group V. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test *p<0.05;** p<0.01; Units = pg/mg of wet tissue.

Table: 2. Effect of CEEA on neurotransmitters levels in rat brain after PTZ induced epilepsy

Group	Design of Treatment	Noradrenaline	Dopamine	Serotonin	GABA
I	Vehicle Control(0.9% normal saline, 1ml/100gm)	774±3.52	847.50±3.24	189±2.12	298.13±1.34
II	MES (0.9% normal saline, 1ml/100gm)	547.16±2.37 ^{a**}	564.83±2.12 ^{a**}	97.5±3.24 ^{a**}	212.14±1.54^{a**}
III	Phenytoin 25mg/kg,*i.p*	628±2.3 ^{b**}	874.16±2.43 ^{b**}	138.42±2.57 ^{b**}	284.13±1.12^{b**}
IV	CEEA 400 mg/kg,*p.o*	596.13±2.64^{b*}	847.12±2.42^{b**}	119.52±1.28^{b**}	276±1.15^{b**}
V	CEEA 200 mg/kg,*p.o*	547.16±3.22^{b*}	764.42±2.35^{b**}	104.13±1.54 ^{b*}	257.16±1.42^{b**}

Values are expressed as mean ± SEM of six observations. Comparison between: **a** - Group I Vs Group II, **b**- Group III Vs Group IV and Group V. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test *p<0.05;** p<0.01;
Units = pg/mg of wet tissue.

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DISCUSSIONS AND CONCLUSION

The role of neurotransmitters in epileptogenesis and in recurrent seizure activity is well-documented. Spontaneous and experimentally induced deficiencies in gamma amino butyric acid (GABA), noradrenaline (NA), dopamine (DA) and/or serotonin (5-hydroxy- tryptamine or 5-HT). It has been implicated in the onset and perpetuation of many seizure disorders many experimental procedures designed to increase monoaminergic activity have proven antiepileptic properties [6-9].

In present study, the established antiepileptic drugs such as phenytoin and diazepam restored the monoamine levels on brain [10]. Similarly CEEA significantly ($p<0.05$ & $p<0.01$) increased monoamines levels in forebrain of rats. Many drugs that increase the brain contents of GABA have exhibited anticonvulsant activity against seizures induced by MES and PTZ [11]. MES is probably the best validated method for assessment of anti-epileptic drugs in generalized tonic-clonic seizures [12]**.**

GABA is a major inhibitory neurotransmitter of CNS and increase in its level in brain has variety of CNS dependent effects including anticonvulsant effect [13]. In addition to the GABA binding site, the $GABA_A$ receptor complex appears to have distinct allosteric binding sites for benzodiazepines, barbiturates, Chloroform etc [14]. We therefore studied the effect of *Enicostema axillare* extract on brain GABA content. *Enicostema axillare* extract showed significant ($p<0.05$ & $p<0.01$) increased GABA content in brain dose dependently. This suggests that the anticonvulsant activity of *Enicostema axillare* extract is probably through elevation of brain GABA content.

In Norepinephrine-lesioned rats showed a greater susceptibility to seizures induced by the chemoconvulsant PTZ and electroconvulsive shock [15]. The antiepileptic role of endogenous Norepinephrine was inferred from studies that showed harmful effects of a damage of Norepinephrine system on seizures induced by electrical stimulation or systemic administration of chemoconvulsants [16, 17]. In present study, CEEA significantly ($p<0.05$ & $p<0.01$) increased the NA in forebrain of rats and proves the antiepileptic activity of *Enicostema axillare* extract.

Chen et al. [18] demonstrated that pre-treatment with the monoamine-depleting agent reserpine decreased the epileptic threshold to PTZ and caffeine in mice. Reserpine lacks specificity, since this drug also depletes serotonin (5-HT) and DA, in addition to NE. Therefore, increased seizure susceptibility could be due to a multiple deficit of monoamines [19]. Subsequent the present studies confirmed and extended these results. It became clear that CEEA significantly increased the serotonin (5-HT) and DA and NA. It produces significantly decreased the susceptibility to various epileptic stimuli.

In conclusion neurotransmitters participate in the control of Maximal electroshock and pentylenetetrazole induced seizure in rat models. Our findings support the hypothesis that decreased the monoamines levels in rat brain after induction of seizure. In *Enicostema axillare* extract treated rats, monoamines such as NA, DA, 5-HT and GABA levels significantly restored on forebrain. Thus CEEA increases the seizure threshold and decreased the susceptibility to MES and PTZ induced seizure in rats. Hence we suggest that Chloroform extract of whole plant of *Enicostema axillare w.p* possess antiepileptic properties that may be due to restore the neurotransmitters in rat brain. These results support the ethnomedical uses of the plant in the treatment of epilepsy. However more experimentation, detailed phytochemical and experimental analysis are required for a definitive conclusion.

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