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R E S E A R C H A R T I C L E

Experimental Evaluation of Anti-Epileptic Activity & In-vivo Antioxidant Activity of Aqueous Extract of Borassus Flabellifer Fruit Pulp in Albino Mice

N.Kalpana Devi[1]*, Chandrika Dontha[2], D.S.S.N. Neelima[3]

[1,2]Teegala Krishna Reddy College of Pharmacy, Hyderabad, Telangana State

[3]Vikas Institite of Pharmaceutical Sciences, Rajahmundary

A B S T R A C T

The aqueous extract of Borassus flabellifer fruit pulp was tested for its anti-epileptic action at 200 mg/kg and 500 mg/kg using the standard drugs phenytoin (for MES) and valproic acid (for PTZ) and the results were compared with those of control and standard groups. The important parameters studied were seizure latency (in PTZ method only), tonic hind limb flexion, tonic hind limb extension, clonus, post-ictal depression and recovery or death of the mice. The abolition of tonic hind limb extension was considered as a convenient end point (suggestive of protection against MES and PTZ seizures). In the present study, the test compound has shown protection against both MES and PTZ seizures at a dose of 500 mg/kg. Since tonic-clonic convulsions are the clinical correlates of MES seizures and absence seizures correlate with PTZ seizures, the aqueous extract of Borassus flabellifer fruit pulp is likely to be of use in the management of tonic-clonic and absence seizures.

Keywords: Borassus flabellifer, anti-epileptic, Phenytoin, valproic acid.

A R T I C L E I N F O

Corresponding Author

N. Kalpana Devi

Teegala Krishna Reddy College of Pharmacy,

Hyderabad, Telangana State

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C O N T E N T S

1. Introduction

Epilepsy is the second most common neurological disorder (after stroke), affecting ~ 1% of the world's population. The epilepsy is a chronic disorder of the brain characterized by spontaneous and recurrent unpredictable seizure activity, which is triggered by abnormal discharge of neurons and has neurobiological, cognitive, psychological, and social consequences for the patient. Epilepsies can be caused by a variety of pathologic conditions however, most epilepsies are idiopathic with a higher incidence among young children and the elderly. In most cases (70%), patients suffering from epilepsy will have their seizures controlled

or suppressed by the chronic administration of antiepileptic drugs (AED), if the diagnosis is well conducted. Although AED present clear efficacy against the many types of epilepsies, they often exert strong side effects, which vary in frequency and severity. Among the most common are gastric discomfort, sedation, diplopy, ataxia, nystagmus, gingival hypertrophy, hirsutism, cognitive impairment, behavior disturbances, as well as idiosyncratic reactions, such as rash, agranulocytosis, leucopenia, thrombocytopenia, aplastic anemia, and hepatic failure. Furthermore, it is commonly accepted that a significant proportion of patients with epilepsy suffers from intractable, that is, drug-resistant, types of seizures. In these cases, physical and intellectual damage may emerge due to the frequent and uncontrolled seizures. In the light of these facts, there is still a need for novel and better tolerated AED that could represent a therapeutic alternative, either for drug-resistant patients or for respondent patients who suffer from the side effects that often interfere with their quality of life.

2. Materials and Methods

Animals:

Male albino mice weighing 18 – 30 g were used for the study. The animals were housed in groups of six and maintained under standard conditions (27±2ºC, relative humidity 44 - 56% and light and dark cycles of 10 and 14 hours respectively) and fed with standard rat diet and purified drinking water ad libitum for 1 week before and during the experiments.

All experiments and protocols described in present study were approved by the Institutional Animal Ethical Committee (IAEC) of Samskruti College Of Pharmacy (1016/a/06/CPCSEA/010/2010). The animals are randomly selected and they are divided into 8 groups, each group consisting of 8 animals.

Chemicals and solutions

- Phenytoin sodium (Ciron pharmaceuticals) – standard drug for MES method
- Valproic acid (sun pharmaceuticals) – standard drug for PTZ method
- Pentylene tetrazole (PTZ) [Himedia labs] – chemoconvulsant
- Aqueous extract of Borassus flabellifer fruit linn – test compound
- Distilled water – vehicle.

Preparation of solutions of standard and test drugs

1. Phenytoin sodium:

The standard solution of phenytoin sodium was prepared by dissolving 400 mg of phenytoin sodium powder in 100 ml of distilled water at room temperature. The solution was freshly prepared everytime. It was protected from light. This solution had a concentration of 4 mg/ml.

2. Sodium valproate:

The standard solution of sodium valproate was prepared by diluting 2g of sodium valproate in 100ml of distilled water at room temperature. The solution was freshly prepared every time. This solution had a concentration of 20 mg/ml.

3. Pentylenetetrazole (PTZ):

The standard solution of pentylenetetrazole was prepared by dissolving 100 mg of pentylenetetrazole powder in 20 ml of distilled water at room temperature. This solution had a concentration of 5 mg/ml.

4. Preparation of aqueous extract of Borassus flabellifer fruit pulp (BFFP): Aqueous extract of Borassus flabellifer was extracted from fruits manually; using water in the ratio of 2: I (v/w) with respect to fruit pulp. The pulp was stored in polythene bags at -2°C.

Acute toxicity studies

The median lethal dose (LD50) determination was performed using the method of Lorke (1983) for intraperitoneal (IP) and oral routes in mice. The method consisted of two phases. In the first stage, three groups of three mice each were injected with the Borassus flabellifer fruit pulp extract at doses of 10, 100, 500 and 1000 mg/kg bodyweight orally and observed for signs of toxicity and death within 24 h. In the second stage, four groups of one mouse each were treated with four more specific doses of the extract based on the result of the stage 1. The LD50 value was determined by calculating the geometric means of the lowest dose that caused death and the highest dose for which the animals survived.

Experimental Design

In the present study through both Maximal electroshock (MES) method & Pentylenetetrazole (PTZ) method studies were done. In PTZ method, Pentylenetetrazole was administered orally at a dose of 90mg/kg body weight to induce the epilepsy. Aqueous extract of Borassus flabellifer fruit at a dose of 200mg/kg and 500mg/kg body weight were administered orally. To assess the antiepileptic activity of aquesous extract of borassus flabellifer fruit pulp against the methods of MES & PTZ induced epilepsy, the animals were divided randomly into eight groups of eight animal each, which were treated as per the treatment schedule.

After assessing the anti-epileptic activity the behavioral parameter (morris water maze test) was assessed. On last day after assessing the above parameter the rats were sacrificed, and their brains were removed and was subjected for invivo antioxidant study.

Methods

- Maximal electroshock (MES) method
- Pentylenetetrazole (PTZ) method

Experimental methods

Male albino mice weighing 18 – 30 g were used for the study. All the test animals which were tested for standard convulsive responses with MES and PTZ stimuli were subjected to further experiments of this study after 24 hours.

The test animals were divided into 4 groups containing 8 animals in each group – two groups (T1 & T2) for MES method and two groups (T3 & T4) for PTZ method.

Maximal electroshock (MES) method:

The mice were subjected to maximal electroshock convulsions with a current of 50 mA for 0.2 second via ear electrodes. The electrodes were moistened with saline solution before application. The resultant seizure passes through various phases: phase of tonic limb flexion, tonic limb extension, clonus, post-ictal depression followed by recovery or death. The mouse was considered as protected if

the drug prevented the appearance of hindlimb tonic extensor component of the seizure. The animals were divided into 4 groups, each consisting of 8 animals.

Control group-1 (C1):

In group C1 for MES method, mice were administered 0.25 ml of distilled water orally. After one hour, they are subjected to maximal electroshock with an alternating current of intensity 50 mA for 0.2 second through ear clip electrodes. The duration of various parameters like tonic hind limb flexion, tonic hind limb extension, clonus, postictal depression and the incidence of recovery or death were noted.

Standard group-1 (S1):

In group S1, all mice received 50 mg/kg of phenytoin orally. After one hour, they are subjected to maximal electroshock with an alternating current of intensity 50 mA for 0.2 second through ear clip electrodes. The results obtained were recorded in a similar way as for group C1.

Test group-1 (T1):

In group T1, all mice received 200 mg/kg of aqueous extract of Borassus flabellifer fruit pulp orally. After one hour, they are subjected to maximal electroshock with an alternating current of intensity 50 mA for 0.2 second through ear clip electrodes. The results obtained were recorded in a similar way as for group C1.

Test group-2 (T2):

In group T2, all mice received 500 mg/kg of aqueous extract of Borassus flabellifer fruit pulp orally. After one hour, they are subjected to maximal electroshock with an alternating current of intensity 50 mA for 0.2 second through ear clip electrodes. The results obtained were recorded in a similar way as for group C1.

Pentylenetetrazole (PTZ) method:

In this method, the mice received 90 mg/kg of PTZ subcutaneously. Only those animals that exhibited convulsive response in the form of clonus, tonic fore and hind limb flexion, tonic limb extension, post-ictal depression followed by recovery were used for experiment. In this method abolition of tonic hind limb extension phase was considered as protection conferred by the drug. The mice were divided into 4 groups, each consisting of 8 animals.

Control group-2 (C2):

This is the control group for PTZ method. In group C2, all mice received 0.25 ml of distilled water orally. After one hour, PTZ (90 mg/kg) was administered subcutaneously. The duration of various phases of ensuing convulsions were noted and subsequent mortality recorded.

Standard group-2 (S2):

In group S2, all mice received 300 mg/kg of sodium valproate intraperitoneally. After one hour, PTZ (90 mg/kg) was administered subcutaneously. Results were recorded in a similar way as for group C2.

Test group-3 (T3):

In group T3, all mice received 200 mg/kg of aqueous extract of Borassus flabellifer fruit pulp orally. After one hour, PTZ (90 mg/kg) was administered subcutaneously. Results were recorded in a similar way as for group C2.

Test group-4 (T4):

In group T4, all mice received 500 mg/kg of aqueous extract of Borassus flabellifer fruit pulp orally. After one hour, PTZ (90 mg/kg) was administered subcutaneously. Results were recorded in a similar way as for group C2.

Morris Water Maze Test – Behavioural Parameter

This test was developed by Richard morris, the specific test for evaluating spatial learning memory in rats.

Apparatus:

Maze consists of a circular pool (1.2 m in diameter and 0.47 m high) made of white plastic. The pool was filled to a depth of 20 cm with water (24°C-25°C) that was made opaque by the addition of titanium dioxide. An escape platform (10 cm in diameter), made of white plastic with a grooved surface for a better grip, was submerged 0.5 cm under the water level.

Principle:

Rats are placed into the maze (large circular pool of water). The animal has to swim until it finds the hidden platform. The platform is hidden by its placement just below the water surface and by opaque water. Thus the platform offers no local cues to guide escape behavior. The animal generally uses cues outside the maze to develop a spatial map of the environment and guide its performance.

Evaluation of *Invivo* Antioxidant Parameters

Preparation of Brain Homogenate

The animals were sacrificed and excised brain was weighed, the homogenate was prepared as follows.

Procedure: Excised brain were cross chopped with surgical scalps into fine slices and were chilled in the cold 0.25 M sucrose, quickly blotted with filter paper. The tissue was minced and homogenized in ice cold 10 mM tris HCl buffer (to pH 7.4) at a concentration of 10% (w/v) with 25 stokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The prolonged homogenization under hypotonic condition was designed to disrupt as far as possible the ventricular structure of cells so as to release soluble protein and leave only membrane and non-vascular matter in sedimentation form. It was then centrifuged at 5000 rpm at 20^{o} C temperature and clear supernatant was separated and used to estimate superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT) and lipidperoxidation (LPO).

Super Oxide Dismutase (Sod)

SOD was estimated by the method of Misra and Fridovich (1972).

Principle: Rate of auto oxidation of epinephrine and the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8-10.2. O2 generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O2 introduced. The auto oxidation of epinephrine proceeds by at least two distinct pathways only one of which is free radical chain reaction involving O2 and hence inhabitable by SOD.

Procedure: 0.5 ml of sample was diluted with 0.5 ml of distilled water. To this 0.25 ml ethanol, 0.5 ml of chloroform (all reagents chilled) were added. The mixture was shaken for 1 min and centrifuged at 2000 rpm for 20 min. The enzymatic activity in supernatant was determined. To it 0.05 ml of carbonate buffer (0.05 M, pH 10.2) and 0.5 ml of EDTA (0.49 M) was added. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in optical density/min was measured at 480 nm. SOD activity was expressed as units/mg protein change in

optical density/min. 50% inhibition of epinephrine to adrenochrome transition by enzyme is taken the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

Catalase (CAT)

Principle: Catalase was estimated by Hugo E. Aebi method; Hydrogen peroxide: hydrogen-peroxido reductase method.

In U.V range H_2O_2 can be followed directly by the decrease in absorbance (O.D 240) per unit time. It is the measure of catalase activity.

Procedure: Dilute homogenate 20 times with phosphate buffer (pH 7.0).

Blank : 4ml diluted homogenate + 2ml Phosphate buffer pH 7.0.

Test : 2ml diluted homogenate + 1ml H_2O_2 [8.5 micro lit. in 2.5 ml phosphate buffer (50 mM/l; pH 7.0). Add H_2O_2 just before taking the absorbance at 254 nm for 3 min. with 30 sec. interval.

Reduced Glutathione (GSH)

Procedure: To 1ml of sample, 1ml of 10% TCA was added. The precipitated fraction was centrifuged and to it 0.5 ml supernatant, 2 ml of DTNB reagent was added. The final volume was made up to 3 ml with phosphate buffer. The colour developed was read at 412 nm. The amount of glutathione was expressed as µg of GSH/mg protein. Reduced glutathione was used as standard (100 µg/ml).

Lipid Peroxidation (Malondialdehyde Formation)

A stock solution containing 50 mM/ml of 1,1,3,3 tetra ethoxy propane in tris hydrochloride buffer at pH 7 was prepared. 10 ml of stock solution was diluted to 100 ml to get a working standard of 50 nM malondialdehyde/ml. This was used for preparation of calibration curve.

Procedure:2 ml of sample was mixed with 2 ml of 20% trichloroacetic acid and kept in ice for 15 min. The precipitate was separated by centrifugation and 2 ml samples of clear supernatant solution were mixed with 2 ml of aq. 0.67% thiobarbituric acid. This mixture was then heated on a boiling water bath for 10 min. It was then cooled in ice for 5 min and absorbance was read at 535 nm. The values expressed as nm of MDA formed/mg of protein. Values are normalized to protein content of tissue.

Statistical analysis: The results of this study are expressed as mean± standard error of mean (mean± SE). Results are analyzed by student 't' test. Significance is established when probability value (p value) is less than 0.05. P values are denoted as * $P < 0.05$ as significant, ** $P < 0.01$ as highly significant and *** $P < 0.001$ as very highly significant.

MES method

Tonic hind limb flexion:

A comparison of mean duration of tonic hind limb flexion of control group with other groups (Table 4) indicates that there is a decrease in mean time of tonic hind limb flexion in group T1 (200 mg/kg of aqueous extract of Borassus flabellifer fruit pulp (BFFP)), group T2 (500 mg/kg of aqueous extract of Borassus flabellifer fruit pulp (BFFP)) and S1 (Phenytoin 50 mg/kg).

The test compound has shown statistically significant protection ($p < 0.01$) in both group T1 and T2. In group S1, there is complete abolition of flexor phase, which is statistically significant ($p < 0.001$).

Tonic hind limb extension:

A comparison of mean duration of tonic hind limb extension of control group with test groups (Table 4) indicate that there is a decrease in mean duration of tonic hind limb extension in both groups T1 and T2 and it is statistically significant ($p < 0.001$). In group S1, there is complete abolition of tonic hind limb extension which is statistically significant ($p < 0.001$).

A comparison of test groups (T1 and T2) with group S1 (Table 4) indicate that there is significant difference between S1 and T1 ($p < 0.001$) while no significant difference between S1 and T2.The abolition of tonic hind limb extension has occurred in 3 out of 6 mice in T1 and 5 out of 6 mice in T2. Since abolition of tonic hind limb extension is considered suggestive of protection against MES convulsions, the aqueous extract of Borassus flabellifer fruit pulp (BFFP) has anticonvulsant effect against MES convulsions at a dose of 500 mg/kg. This effect is comparable to that of phenytoin in this study.

Clonus:

Analysis of results when compared with control suggest that there is a decrease in mean duration of clonus in groups S1 and T1 while a slight increase in group T2. However these values are not statistically significant. Analysis of results when compared with standard (Table 4) suggest that there is no significant difference between groups S1 and test groups T1 and T2.

Post-ictal depression:

A comparison of mean duration of post-ictal depression with control indicates that there is a decrease in the mean duration in groups T1 and T2 which is statistically significant ($p < 0.001$). Group S1 has shown no post-ictal depression at all which is statistically significant ($p < 0.001$). A comparison of mean duration of post-ictal depression with standard indicates that there is no significant difference between the groups S1, T1 and T2.

PTZ method

Seizure latency:

Analysis of results compared with control group suggest that there is an increase in the mean duration of seizure latency in group T3 (200 mg/kg of aqueous extract of Borassus flabellifer fruit pulp (BFFP) and it is statistically significant ($p < 0.001$). In groups S2 (sodium valproate 300mg/kg) and T5 (500 mg/kg of aqueous extract of Borassus flabellifer fruit pulp), there is no seizure and hence no seizure latency and it is statistically significant ($p < 0.001$).

Tonic hind limb flexion:

Comparison of mean duration of tonic hind limb flexion with control group (Table 8) indicates that there is no significant difference between control group and group T3 while in groups S2 and T4, there is abolition of tonic hind limb flexion which is statistically significant ($p < 0.001$). Comparison of mean duration of tonic hind limb flexion of standard group (Table 8) with test groups T3 and T4 indicates that there is significant difference between group S2 and group T3 ($p < 0.01$), while there is no significant difference between group S2 and group T4.

Tonic hind limb extension:

Comparison of mean duration of tonic hind limb extension of control group with test groups and standard indicates that there is no significant difference between group C2 and group T3 while in groups S2 and T4, there is abolition of tonic extensor phase which is statistically significant ($p < 0.001$).

Comparison of mean duration of tonic hind limb extension of standard group S2 with test groups T3 and T4 (Table 8) indicates that there is significant difference between group S2 and group T3 ($p < 0.001$), while no significant difference between group S2 and group T4.

Post-ictal depression:

Comparison of mean duration of post-ictal depression of control group C2 with test and standard groups (Table 8) indicates that there is decrease in the mean duration of post-ictal depression in group T3 ($p < 0.001$) while there is no post-ictal depression in groups S2 and T4 which is statistically significant ($p < 0.001$).

Comparison of mean duration of post-ictal depression of standard group S2 with test groups (Table 8) indicates that there is significant difference between groups S2 and T3 ($p < 0.001$), while no significant difference between S2 and T4. The most significant effect of phenytoin is its ability to modify the pattern of MES. This seizure-modifying action is observed with many other antiseizure drugs that are effective against generalized tonic-clonic seizures. By contrast, phenytoin does not inhibit clonic seizures evoked by pentylenetetrazole.

3. Conclusion

The test compound Borassus flabellifer fruit pulp aqueous extract at a dose of 200 mg/kg has abolished tonic hind limb extension in albino mice in both the MES and PTZ methods. At a dose of 500 mg/kg body weight, the aqueous extract of has shown statistically significant anti-epileptic effect against both MES and PTZ convulsions. Thus the aqueous extract of Borassus flabellifer fruit pulp has shown efficacy in both MES and PTZ convulsions in mice in the present study. Since the clinical correlates of MES seizures are tonic clonic convulsions and correlates of PTZ seizures are absence seizures, the aqueous extract of Borassus flabellifer fruit pulp is likely to be useful in the treatment of tonic clonic and absence seizures. Further detailed study of the active principle/s of this plant are worth pursuing in this regard.

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