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RESEARCH ARTICLE

Evaluation of anticonvulsant activity of hydroalcoholic extract of *Aegle marmelos* (Linn. Correa, stem bark)

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Anticonvulsant, *Aegle marmelos*, ϒ – aminobutyric acid (GABA), antiepileptic drugs (AEDs)**,** Seizure, Pentylene tetrazole (PTZ), Maximal electroshock (MES)

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Abstract

A prehistoric plant, *Aegle marmelos* commonly known as "Bael" wildly growing across the country and contribute wide range of pharmacological actions which were proven by scientific data published over the past year. Bael is well acknowledged with its ethno-medicinal uses and therefore be more magnetism promote to use of. Present study primarily design to assess an anticonvulsant activity in *vivo* followed by Pentylene tetrazole (PTZ), Maximal electroshock (MES) induced convulsion which will initiate seizure with in animal. An assessment of percentage protection against seizure and mortality- a key important evaluation parameter and estimation of ϒ-aminobutyric acid (GABA) level- inhibitory neurotransmitter belief to provokes seizure in rodent was compare with treatment group of hydro-alcoholic extract (*Aegle marmelos*, stem bark) at different dose level (200 & 400 mg/kg, *i.p.*). In rodent higher dose of *Aegle marmelos* suggests, significant (*$p<0.05$) reduction in the duration HLTE (hind limb tonic extensor) facilitated by MES model. In addition, markedly decrease in percentage mortality (66.6 %) against PTZ model. There were increases with the GABA level at higher doses as compared to lower dose & normal control treatment group. This would be the next direction for the estimation of GABA level with isolated phytochemical compound.

INTRODUCTION

Epilepsy is a chronic neurological disorder that affects people of all ages. Around 50 million people worldwide have epilepsy. Nearly 80% of the people with epilepsy are found in developing regions.[1] Population-based neuroepidemiologic studies in different regions of India have shown that epilepsy constitutes nearly a third to a fifth of all neurologic disorders. The prevalence of epilepsy varies from 2.5 to 11.9 per 1,000 populations. In the Bangalore Urban Rural Neuroepidemiological Survey (BURN Survey; supported by Indian Council of Medical Research) covering a population 102,557 were observed a prevalence rate of 8.8 per 1,000 population, with the rate in rural community being twice that of urban area. Epilepsy was found to be the second leading neurologic problem in both urban and rural populations, next to vascular headache.[2, 3, 4] Goal of pharmacological therapy in management of epilepsy is complete control of seizures with minimal adverse effect. Monotherapy with conventional antiepileptic agent is effective and well tolerated by majority of epileptics.[5] Despite of drug management in epileptic patient founds to continue with seizure episodes either due to tolerance with single drug or polypharamcy has a more incidence to contributing drug interaction and adverse effect. Most of the standard AEDs like phenobarbital, carbamazapine, benzodiazepine, ethosuuximide, phenytoin and valproate have been found effectively in limited spectrum of activity and that limits their usage in complication patients management. However, newer molecule of AEDs is coupled with standard AEDs to optimal a seizure-free condition over period. Nature is always blessing to human kind for their needs and from ancient times human being is using herbal plant in form of extract or raw material to cure or for prophylaxis of disease. Presently authors have decided

to design a study to evaluate antiepileptic activity of our Indian traditional medicinal plant- *Aegle marmelos* is commonly known as "Bael" having vast pharmacological activities like anti-diabetic,[6,7,8] hepatoprotective,[9] antimicrobial,[10] analgesic, anti-inflammatory, antipyretic,[11] radio protective [12] were reported in past few years.

MATERIALS AND METHODS

Drugs and chemicals

Diazepam (Calmpose-Ranbaxy), phenytoin (Eptoin –Abbott), pentylenetetrazole (PTZ- Sigma chemicals, USA) and gamma- aminobutyric acid (Loba chemi, Mumbai) were used for experimentation.

Procurement and identification of plant materials

The stem bark was collected in April-May from the hilly areas of Mangalore region, Karnataka state, India and authenticated by Dr. U Srinivasa (Professor, Head of Pharmacognosy and Phytochemistry department).

Preparation of extract

A Shade dried stem barks of *A. marmelos* was coarsely fined powdered. The powder was loaded into Soxhlet extractor in batches of 200 grams of each and subjected to extraction with distilled water (1 part): ethanol (1 part) at 40-45°C. The percolate was cooled, filtered and concentrated under reduced pressure on a water bath at a temperature below 50°C till syrup consistency obtained. Then HAEAM (Hydroalcoholic extract *Aegle marmelos* stem bark) was dried in a desiccator and stored in refrigerator (2-8°C) temperature for further use.

Animals

Swiss albino mice (20-25 g) of either sex were used for the experiment. The animals were maintained under standard conditions (temperature 24±2°C, relative humidity 50±5% and 12 h light/dark cycle) and have free access to standard pellet diet supplied by Pranav Agro Industries Ltd. Sangli (protein 10%, arachis oil 4%, fibers 1%, calcium 1%, vitamin A 1000 IU/gm and vitamin D 500 IU/g) and water *ad libitum*. Animals were acclimatized for a period of 7 days before the study. Experimental protocol was reviewed and approved by the Institutional Animal Ethical Committee (SCP/CPCSEA/P04/F150/2012) and the care of the laboratory animals were taken as per the IAEC standards.

Acute toxicity

Acute toxicity of hydro-alcoholic extract of *Aegle marmelos* was done as per OECD guideline.[13]

Preliminary phytochemical screening

Phytochemical screening was performed by standard procedure.[14, 15]

Experimental design

Animals were divided into four main groups and each group contains 6 animals. Each groups were categorised and received: Group I- normal control (1% w/v of gum acacia, *i.p.*), Group II- standard drug (diazepam- 5 mg/kg, *i.p* and phenytoin- 25 mg/kg, *i.p.*), Group III- lower dose (HAEAM 200 mg/kg, *i.p.*) and Group IV- higher dose (HAEAM 400 mg/kg, *i.p.*). Each group was received respective treatment for 15 days and seizure was induced by different method upon receiving of last day of dosing.

Experimental models

Pentylenetetrazole (PTZ) induced seizure

Swiss albino mice (20-25 g) were used for experiment and standard convulsive agent PTZ (80 mg/kg, *i.p*) was administered 30 min. after administration of vehicle (1% gum acacia, *i.p.*), standard drug (diazepam 5 mg/kg, *i.p.*), lower and higher doses of HAEAM (200 mg/kg, 400 mg/kg, *i.p.*-respectively). The animals were placed individually in plastic case and observed immediately upon injection of PTZ for a period of 30 minutes. The onset of clonic-tonic convulsion and percentage protection against mortality were recorded. [16]

Maximal electroshock induced seizure

Swiss albino mice (18-30 g) were selected for experiment. Group I- received vehicle (1% gum acacia, *i.p.*), Group II- pheytoin (25 mg/kg, *i.p.*), Group III- HAEAM (200 mg/ kg, *i.p.*) and Group IV- HAEAM (400 mg/kg, *i.p.*). Test started by an apparatus with corneal or ear electrodes were used to deliver electric stimuli (at 50 mA; 50 Hz; 0.2- sec duration) after 30 min *intra peritoneal* (*i.p.*) injection of vehicle, phenytoin and test compound (HAEAM 200 & 400 mg/kg, *i.p*). Up on electrical shock induction the animals were observed closely for 2 min. Disappearance or delay of the hind limb tonic extensor (HLTE) convulsion was used as positive outcome. Percent of inhibition of seizures relative to controls was calculated.[17]

Estimation of GABA level in mice

The animals were randomly divided into 4 groups (n=6 mice per group). The different groups were assigned as described below. Group I was treated with vehicle (1% gum acacia, *i.p.*), Group II- diazepam (5 mg/kg, *i.p.*), Group III- HAEAM (200 mg/ kg, *i.p.*) and Group IV- HAEAM (400 mg/kg, *i.p.*). After 45 min. the animals were sacrificed and brain was isolated immediately and transferred to homogenization tube containing 5 ml of 0.01N HCl and homogenized.

Brain homogenate was then transferred to a bottle containing 8 ml of ice cold absolute alcohol and was kept for 1 hour at 0°C. The content was centrifuged for 10 min. at 16000 rpm. The supernatant was collected in Petri dish. Precipitates were washed with 3-5 ml of 75% alcohol for three times and washes combined with supernatant. Content in Petri-dish was evaporated to dryness at 70-90°C on water bath under stream of air. To the dry mass, 1 ml of water and 2 ml of chloroform was added and centrifuged at 2000 rpm. Upper phase consisted of GABA was separated and 10 µl of it was applied as a spot on ascending chromatographic whatman paper (NO. 41). The mobile phase consisted of n-butanol (50 ml): acetic acid (12 ml): water (60 ml). The chamber was allowed to saturate for half hour with mobile phase. The paper chromatogram was developed with ascending technique. After the paper was developed, it was dried in hot air and then spread with 0.5% ninhydrin solution in 95% ethanol. The paper was dried for 1 hr at 90°C. Blue colour spot developed on paper was cut and heated with 2 ml ninhydrin solution on water bath for 5 min. water (5 ml) was added to solution and was kept aside for 1h. Later the Supernatant was decanted and absorbance was measured at 570 nm.

For the estimation of GABA level in mouse brain different concentration of standard GABA solutions were prepared and their concentrations were obtained by running the paper chromatography and finally measuring the absorbance by U.V spectroscopy at 570 nm. For this stock solution of standard GABA, 1 mg/ml was prepared in 0.01N HCl solution. Serial dilutions were done to get concentrations 1 ng/10 µl to 1000 ng/10 µl. To obtain a standard concentration curve for GABA same procedure will be followed replacing brain homogenate with standard GABA solutions. [18]

Statistical analysis

Using Statistical Package for the Social Sciences (SPSS version 16.0; SPSS Inc., Chicago, USA), normally distributed data were expressed as mean ± standard error of mean and analyzed by one way analysis of variance (ANOVA) followed by post hoc Dunnet- t test. $p<0.05$ is considered statistically significant when compared to control group.

RESULTS

Acute toxicity was done as per OECD guidelines and hydroalcoholic extract of *Aegle marmelos* (stem bark) was found to be safe up to 2000 mg/kg. Phytochemical screening reveals that extract posses glycoside, tannins and phenolic ring structure compound, flavanoids and triterpenoids.

PTZ induced convulsion model helps to identify a protection in normal control group (I) with respect of seizure and mortality, (Table 1, figure 1) while standard drug (Diazepam) treatment group(II) effectively affects on seizure and diminished seizure event. More ever the protection against seizure is highest amongs all other groups. In case of HAEAM group (III & IV) - lower and higher dose of extract improves score against mortality and tonic-clonic convulsive event, delayed with respect to the dose wise. Though these groups (III & IV) were score lesser than the standard group but comparatively shown positive effect against normal control group.

TABLE 1: EFFECT OF HAEAM ON PTZ INDUCED CONVULSIONS

Group	Treatment	Onset (sec.) Mean± S.E.M.	
		Clonic	Tonic
I	Vehicle control	59.16 ± 6.97	403.00 ± 28.35
II	Diazepam (5 mg/kg)	0.00 ± 0.00	0.00 ± 0.00
III	HAEAM (200 mg/kg)	97.50 ± 9.95*	511.50 ± 38.47^{ns}
IV	HAEAM (400 mg/kg)	160.67 ± 12.26**	643.17 ± 48.48**

n=6 mice in each group. Data are expressed as mean ± SEM and different treatment groups were analyzed by one way ANOVA followed by post-hoc Dunnet- t test. *-$p<0.05$, **-$p<0.01$, compared to control.

TABLE 2: EFFECT OF HAEAM ON MES INDUCED CONVULSIONS

Group	Treatment	Onset HLTE time (Sec.) Mean± S.E.M.	Duration of HLTE (Sec.) Mean± S.E.M.
I	Vehicle control	3.6 ± 1.01	101.50 ± 8.36
II	Phenytoin (25 mg/kg)	14.06 ± 1.47**	8.70 ± 2.78**
III	HAEAM (200 mg/kg)	10.23 ± 0.89*	64.26 ± 13.99*
IV	HAEAM(400 mg/kg)	8.98 ± 2.87^{ns}	47.60 ± 8.48*

n=6 mice in each group. Data are expressed as mean ± SEM and different treatment groups were analyzed by one way ANOVA followed by post-hoc Dunnet- t test. *$p<0.05$, *- $p<0.01$, compared to control.

MES induced convulsion model shown that there was no % inhibition against hind limb tonic extension (HLTE) in normal control group (Table 2 & figure 2). Standard drug (Phenytoin) treatment group has a better control over the seizure event and thus shown a better percentage inhibition of HLTE. Lower dose of HAEAM shows a % inhibition of HLTE but which was lesser than that of higher dose of HAEAM when compared to normal control group.

Figure 1: % protection against mortality in PTZ induced convulsion

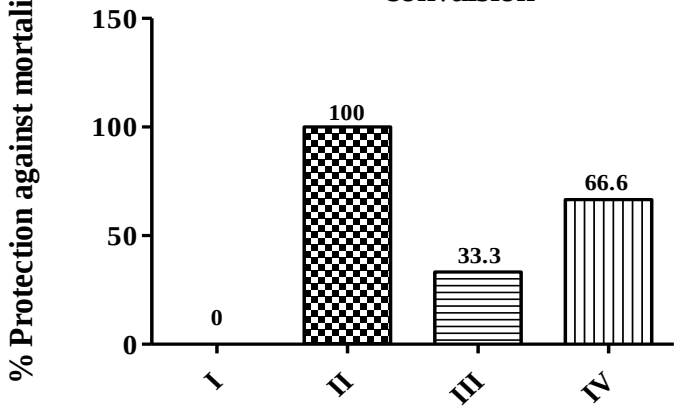
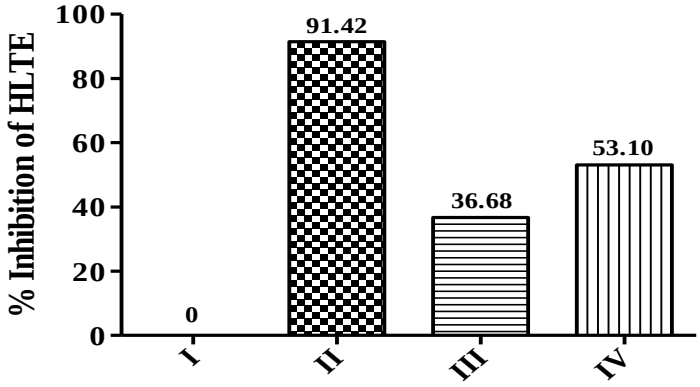


Figure 2: % Inhibition of HLTE in MES induced convulsion



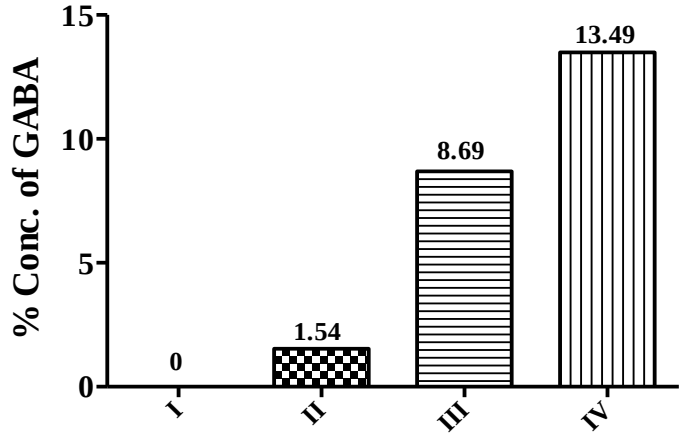
GABA estimation which is a key important parameter gives a clear cut idea about- any changes occurs during the drug treatment groups (II, III & IV) in animals revealed that there were increase in GABA level (Table 3 & figure 3) especially with group III & IV. This estimation gives a brief idea to authors about involvement of GABA and its level for preventing actions, involvements of drug mediated action on GABA.

TABLE 3: ESTIMATION OF GABA LEVEL IN MICE

Treatment	Volume applied on paper (µl)	GABA concentration (ng)
Control	10	70.76±4.63
Diazepam (5 mg/kg)	10	71.85±36
HAEAM (200 mg/kg)	10	76.91±4.01^{ns}
HAEAM (400 mg/kg)	10	80.31±4.96 ^{ns}

n=6 mice in each group. Data are expressed as mean ± SEM and different treatment groups were analyzed by one way ANOVA followed by post-hoc Dunnet- t test.
No data was statistically significant with when it was compare with control group.

Figure 3: % Increase in GABA level



DISCUSSION

Higher degree excitation of excitatory neurons or little inhibition of inhibitory neurons can leads to convulsions, anxiety, high blood pressure, restlessness and insomnia. Over expression of inhibitory neurons or lesser excitation of excitatory neurons may results in to depression, comma, low blood pressure, sedation.

ϒ - Aminobutyric acid a major inhibitory neurotransmitter thus plays an important role for excitatory and inhibitory neuronal pathways. Hence restoration of balance between excitation and inhibition mainly achieved by GABA mediated mechanisms. GABA receptor is mainly divided in to two: ligand gated ion channel ($GABA_A$ & $GABA_C$ receptors) and G- protein coupled receptor ($GABA_B$).[19, 20]

There are 16 different subunits comprising the $GABA_A$ receptor family: α1-6, β1-3, ϒ-3, δ, ε, π and θ.[21] The major disorders for which $GABA_A$ receptors represent important therapeutic targets include anxiety disorders, cognitive disorders, epilepsies, moods disorders, schizophrenia and sleep disorders. Inherited mutation in $GABA_A$ receptors leads to idiopathic generalised epilepsy. [22]

In PTZ induced convulsion model, PTZ antagonise the GABA a major inhibitory neurotransmitter actions and PTZ having the affinity towards the $GABA_A$ receptors. As a result, PTZ leads to the excitatory effects and aggravate the convulsive actions.

Known flavonoids were found to be acts on the gamma-aminobutyric acid type A ($GABA_A$) receptor in the central nervous system (CNS) which may be have works same as benzodiazepine-like molecules. This is supported by their behavioural effects in animal models of anxiety, sedation and convulsion.[23, 24] Primarily, the natural flavonoids are considered to be acts on the $GABA_A$ receptors would be a strong intuitive force to spare the neurogenic GABA level and enhance the level during an epileptic episode. Diazepam has no significant effects on GABA level, though benzodiazepine like drugs having the GABA facilitating or mimics action and hence unused GABA showing either increasing or no changes in level through up or down regulations.

MES-induced seizures can be prevented either by drugs that inhibit voltage dependent Na^+ channels such as phenytoin, sodium valproate, felbamate and lamotrigine; or by drugs that block glutamatergic receptor such as felbamate. On other hand, drugs that reduce T-type Ca^{2+} currents such as ethosuximide can prevent seizures induced by PTZ. Drugs that enhance gamma amino butyric acid type A ($GABA_A$) receptor mediated inhibitory neurotransmission such as benzodiazepines and phenobarbital and perhaps valproate and felbamate can prevent this type of seizure. [25]

The present study was limited with the parameters: onset tonic-clonic convulsion, onset HLTE and GABA levels. To understand the complete mechanism of action of *A. marmelos* with regards to the antiepileptic actions needs to be more exploration with other relevant parameter.

CONCLUSION

Screening of an antiepileptic activity for *Aegle Marmelos* reveals the protection against the mortality and delay or abolitions of seizures episodes was found in greater amount with the higher dose (400 mg/kg) for both PTZ and MES model. Low dose (200 mg/kg) shown preventive actions but was comparatively less effective than the higher dose.

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