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Anticonvulsant effect of *Phylanthus amarus* by modulation of endogenous biomarkers

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ABSTRACT

Epilepsy is a neurological disorder that affects a wide range of people throughout the world. It is a disorder of brain characterize by unpredictable and periodic occurrence of a transient alteration of behavior due to the disordered, synchronous and rhythmic firing of populations of brain neurons. Incidence of epilepsy in developed countries is approximately 50 per 100,000 while that of developing country is 100 per 100,000 (WHO, 2006). It has been observed that the presently available antiepileptic drugs are unable to control seizures effectively in as many as 25% of the patients.

The aim of present investigation was to evaluate the anticonvulsant activity of alcoholic extract of Phylanthus amarus (30, 60 and 120 mg/kg) against Pentylenetetrazole (PTZ), Picrotoxin (PTX) *and maximal electroshock (M.E.S.) induced convulsions in mice. Diazepam and Phenytoin were used as reference anticonvulsant drugs for comparison. Intraperitoneal administration of PTZ (80mg/kg) and subcutaneous administration of picrotoxin (3.5 mg/kg) resulted in tonic-clonic convulsion along with lethality in mice. It also significantly altered levels of brain Gamma amino butyric acid (GABA) along with nitric oxide (NO) and xanthine oxidase (XO) in mice. Treatment with p. amarus (60 and 120 mg/kg) delayed onset of convulsion along with duration of tonic-clonic convulsions as well as it significantly reduced PTZ and PTX-induced mortality in mice ($P < 0.05$ - $P < 0.001$). Mice treated with P. amarus (120 mg/kg) significantly increased level of brain GABA whereas it significantly decreased elevated level of brain NO and XO. In conclusion, the findings of present study provide pharmacological credence to anticonvulsant profile of P. amarus. The protection against the convulsions and restoration of endogenous enzyme level give an innuendo to its probable mechanism of action which may be mediated through the GABAergic pathway and inhibition of oxidative injury.*

Keywords: *Phylanthus amarus, Pentylenetetrazole, Picrotoxin, M.E.S.*

INTRODUCTION

Epilepsy is exemplified by a number of maladies of central nervous system which are the consequence of the severe dysfunction and discharge in brain regions, at a focal point, leaing to the conscription of nearby brain regions into an epileptic manifestation. [1,2] It has been well proven that over expression of excitatory amino acids like AMPA and NMDA receptors leads to seizures and neuronal death. [3,4] The existing antiepileptic drugs possess many unwanted adverse effects and toxicities. [5] Epilepsy continues to be a neurological disorder awaiting safer drugs with improved anticonvulsant and anti-epileptogenic effectiveness as currently available drugs fail to provide adequate control of epileptic seizures in about one third of patients and do not prevent progressive epileptogenic changes are not well understood. This fact has stimulated a considerable number of research of new antiepileptic drugs. In this regard, the medicinal plants have been an important source to the development of drugs with this biological activity. Additionally, numerous herbal medicines are recognized as active in the central nervous system (CNS), and they have at least a hypothetical potential to affect chronic conditions such as anxiety, depression, headache or epilepsy that do not respond well to conventional treatments. Hence, there is still need of further research to find out drugs with relatively less side effects.

Phyllanthus has been used in Ayurvedic medicine for over 2,000 years and has a wide number of traditional uses. This includes employing the whole plant for jaundice, gonorrhea, frequent menstruation and diabetes and using it topically as a poultice for skin ulcers, sores, swelling and itchiness. The plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge and antiseptic. It is useful in dropsy, jaundice, diarrhoea, dysentery, intermittent fevers, and diseases of urino-genital system, scabies ulcers and wounds.

The young shoots of the plant are administered in the form of an infusion for the treatment of chronic dysentery. Its efficacy in the field of gastro intestinal disorders like dyspepsia, colic, diarrhoea, constipation and dysentery is undisputed. In females it is used as a galactogogue, in leucorrhoea, menorrhagia and mammary abscess.

The powdered leaves of *P. amarus* (Bahupatra) were used in clinical studies evaluating its usefulness in patients suffering from chronic damage to the liver due to the protracted hepatitis B virus infection. This type of infection results in inability of the body's immune system to eliminate the virus from the liver cells. This condition is described as a carrier state, because a continuously harbors the virus. The powdered leaves of *P. amarus* were given in form of capsules to the patients with chronic viral hepatitis B in a dose of 200 mg three times a day for 30 days. *P. amarus* treated patients tested negative for the viral antigen 15-20 days after the end of the treatment. Due to its antiseptic, styptic, carminative, deobstruent, coolant, febrifugal, stomachic, astringent and diuretic properties of this plant it is very much utilized in traditional medicine. [6-8]

The aim of present investigation was to investigate the anticonvulsant activity of *P. amarus* in various animal models of convulsions by assessing various behavioral and biochemical parameters.

MATERIAL AND METHOD

2.1 Animals:

Adult male Swiss albino mice (18-22 g) were purchased from National Institute of Biosciences, Pune and kept in quarantine for one week in housed at the institute animal house in groups of six animals per cage at standard laboratory conditions at a temperature of $24^0C \pm 1^0C$, relative humidity of 45–55% and 12:12 h dark and light cycle. The experiments were carried out between 10:00 am to 5:00 pm. Animals had free access to food (standard chaw pellet, Pranav Agro industries Ltd., Sangli, India) and water ad libitum. Experimental protocols and procedures were approved by the Institutional Animal Ethics Committee (IAEC No. CPCSEA/02/03/11). Animals were brought to testing laboratory 1 h before the experimentation for adaptation purpose. The experimentation was carried out in noise free area.

2.2 Drugs and solutions:

Pentylenetetrazole (PTZ), Picrotoxin (PTX) (Sigma Aldrich, India), Phenytoin (PHY) (Eptoin®, Sun Pharma Ltd., India), Diazepam (DZP) (Calmpose®, Ranbaxy Ltd., India) All chemicals were dissolved in saline. Dosage forms of alcoholic extract of *P. amarus* was prepared with Tween 80 (2.5%) and CMC (0.5%) in glass mortar with gradual addition of WFI to make volume. All other reagents were purchased from S.D. Fine Chemicals, Mumbai, India.

2.3 Assessment of Anticonvulsant activity:

2.1.1 *Pentylenetetrazole (PTZ) induced convulsions:*

The mice were randomly divided into five groups containing six mice in each group as follows:

Group I: *P. amarus* (30 mg/kg, i.p.);
Group II: *P. amarus* (60 mg/kg, i.p.);
Group III: *P. amarus* (120 mg/kg, i.p.);
Group IV: Diazepam (5 mg/kg, i.p.);
Group V: Vehicle control (Tween 80 (2.5%) and CMC (0.5%)).

Swiss albino male mice (25 ± 2 g) were used. Vehicle, extract or the standard drug (diazepam 5 mg/kg) were administered by intraperitoneal route. PTZ 80 mg/kg was injected intraperitoneally to all mice after 45 minutes of vehicle or extract and 30 min after the standard drug. Immediately after PTZ administration mice were placed individually and observed for: [1] Latency to tonic convulsions (elapsed time from injection until convulsion occurred), [2] Latency to clonic convulsions [3] Incidence (no. of mice showing convulsions) and [4] Mortality for the duration of 30 min. [9]

2.1.2 Maximal electroshock (M.E.S.) induced convulsions:

The mice were randomly divided into five groups containing six mice in each group as follows:

Group I: P. amarus (30 mg/kg, i.p.);
Group II: P. amarus (60 mg/kg, i.p.);
Group III: P. amarus (120 mg/kg, i.p.);
Group IV: Diazepam (5 mg/kg, i.p.);
Group V: Vehicle control (Tween 80 (2.5%) and CMC (0.5%)).

Swiss albino mice (25 ± 2 g) of either sex were used. Test was started 45 min after intraperitoneal administration of vehicle or extract and 30 min after standard drug (phenytoin 20 mg/kg i.p). To start session a 60 Hz alternate current of 45 mA for 0.2 sec was applied to the animal through corneal electrodes. To enhance electro-conductivity two drops of 0.9% NaCl were applied on each eye before applying current. After electric stimuli, latency and incidence of tonic hind limb extension (THLE) and mortality was observed for duration of 15 min. [9]

2.1.3 Picrotoxin (PTX) induced convulsions:

The mice were randomly divided into five groups containing six mice in each group as follows:

Group I: *P. amarus* (30 mg/kg, i.p.);
Group II: *P. amarus* (60 mg/kg, i.p.);
Group III: *P. amarus* (120 mg/kg, i.p.);
Group IV: Diazepam (5 mg/kg, i.p.);
Group V: Vehicle control (Tween 80 (2.5%) and CMC (0.5%)).

Swiss albino mice (25 ± 2 g) of either sex were used. Vehicle, extract or the standard drug (diazepam 5 mg/kg) were administered by intraperitoneal route. Forty-five minutes after administration of vehicle or extract and 30 min after diazepam all mice were treated with 3.5 mg/kg picrotoxin by subcutaneous route. Immediately after picrotoxin injection mice were observed for following symptoms during next 45 min:

[1] Latency to tonic convulsions,
[2] Latency to clonic convulsions
[3] Incidence (no. of mice showing convulsions) and
[4] Mortality. [10]

2.4 Biochemical evaluation:

2.4.1 Brain GABA estimation:

Swiss male albino mice (25 ±3 g) were divided in 9 groups consisting 6 mice in each group. Treatment schedule was as follows:

Group		Dose (mg/kg)
1	Vehicle	5ml/kg
2	PTZ	80 mg/kg
3	PTX	3.5 mg/kg
4	PTZ + *P. amarus*	80 mg/kg + 60 mg/kg i.p
5	PTZ + *P. amarus*	80 mg/kg + 120 mg/kg i.p
6	PTX + *P. amarus*	3.5 mg/kg + 60 mg/kg i.p
7	PTX + *P. amarus*	3.5 mg/kg + 120 mg/kg i.p
8	PTZ + Diazepam	80 mg/kg + 5 mg/kg i.p
9	PTX + Diazepam	3.5 mg/kg + 5 mg/kg i.p

2.4.1.1 Sample preparation:

Forty-five min after vehicle (1 % DMSO) or *Phylanthus amarus* and 30 min after diazepam (50 mg/kg), Phenytoin (200 mg/kg) mice were sacrificed. PTZ (90 mg/kg), INH (300 mg/kg) and STR (3 mg/kg) treated animals were sacrificed as soon as onset of convulsions occurs. Animals

which received PTZ (90 mg/kg), INH (300 mg/kg) and STR (3 mg/kg) after 45 min of *Phylanthus amarus* (10 and 200 mg/kg) and sacrificed at the exact time of onset of convulsions. Brain was isolated immediately and transferred to homogenization tube containing 5 ml of 0.01N hydrochloric acid and homogenized. Brain homogenate was transferred to bottle containing 8 ml of ice cold absolute alcohol and kept for 1 hour at 0 ^{0}C. The content was centrifuged for 10 min at 16000 rpm, supernatant was collected in petridish. Precipitate was washed with 3-5 ml of 75% alcohol for three times and washes were combined with supernatant. Contents in petridish were evaporated to dryness at 70-90 ^{0}C on water bath under stream of air. To the dry mass 1 ml water and 2 ml chloroform were added and centrifuged at 2000 rpm. Upper phase containing GABA was separated and 10 µl of it was applied as spot on Whatman paper (No. 41).

2.4.1.2 Chromatographic conditions:

The mobile phase consisted of n-butanol (50 ml) acetic acid (12 ml) and water (60 ml). The chamber was saturated for half hour with mobile phase. The paper chromatogram was developed with ascending technique. The paper 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 ^{0}C. Blue color 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 kept for 1h Supernatant was decanted and absorbance was measured at 570 nm.

2.4.1.3 Standards and calculations:

Stock solution of standard GABA, 1 mg/ml was prepared in 0.01N HCl. Serial dilutions were prepared to get concentrations 1ng/10µl to 1000ng/10µl. To obtain a standard concentration curve for GABA same procedure was followed replacing brain homogenate with standard GABA solutions. [11]

2.4.2 Estimation of total protein:

Protein concentration was estimated according to previously described method [12], using BSA (bovine serum albumin) as a standard.

2.4.3 Estimation of nitrite/nitrate level:

The NO level was estimated as nitrite by the acidic Griess reaction after reduction of nitrate to nitrite by vanadium trichloride according to previously described method. [13] The Griess reaction relies on a simple colorimetric reaction between nitrite, sulfonamide and N-(1- naphthyl) ethylenediamine to produce a pink azo-product with maximum absorbance at 543 nm. The concentrations were determined using a standard curve of sodium nitrate and the results were expressed as µg/ml. [14]

2.4.4 Estimation of Xanthine oxidase (XO) level:

Xanthine oxidase activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbency at 293 nm, according to previously described method. [15] The concentrations were determined using a standard curve of XO solutions and results were expressed as units per gram protein in brain homogenate.

2.5 Statistical analysis:

Data were expressed as mean ± standard error mean (S.E.M.). The data of 'Brain GABA', 'Nitric oxide' and 'Xanthine oxidase' was analyzed using one-way analysis of variance (ANOVA),

Dunnett's multiple range test was applied for post hoc analysis. Data of 'incidence of convulsion' was analyzed by nonparametric Kruskal–Wallis ANOVA. Data of 'mortality' was analyzed by Fisher's exact test. Data of 'percentage seizure free rats' was analyzed by using Kaplan–Meier analysis, Log-rank (Mantel-Cox) test was applied for post hoc analysis. Analysis of all the statistical data was performed using GraphPad Prism 5.0 (GraphPad, San Diego, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

3.1 Effects of Phylanthus amarus and diazepam on pentylenetetrazole induced convulsions in mice:

Mice pretreated with PAA (60 and 120 mg/kg, i.p.) significantly (P<0.05) increased latency to tonic and clonic convulsions and reduced mortality to 83.33 and 66.67% respectively. However PAA 30 mg/kg, i.p. did not show significant effect on latency to tonic and clonic convulsions and protection from mortality Diazepam 5 mg/kg, i.p. significantly (P<0.001) increase in latency to tonic and clonic and 16.67% mortality induced by pentylenetetrazole in mice. (Fig 1-4).

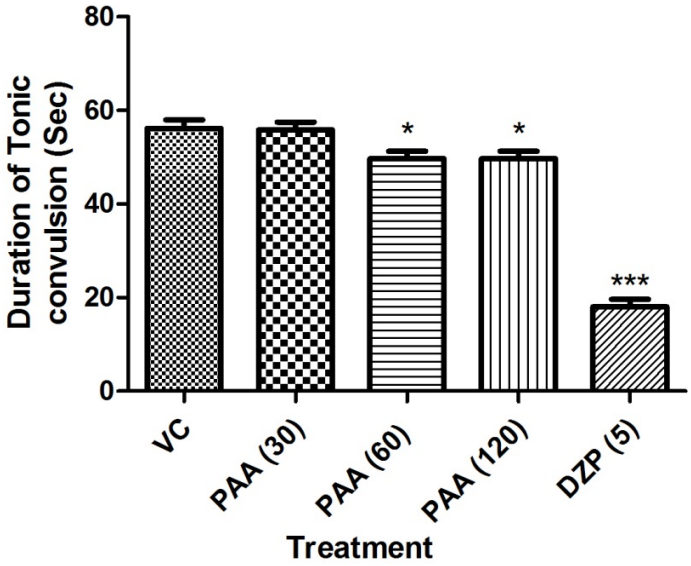
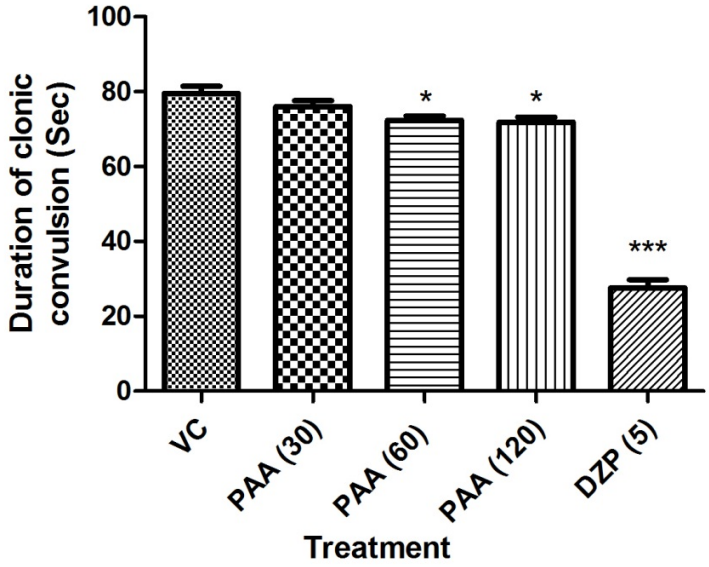


Fig-1


Duration of clonic convulsion (Sec)
100
80
60
40
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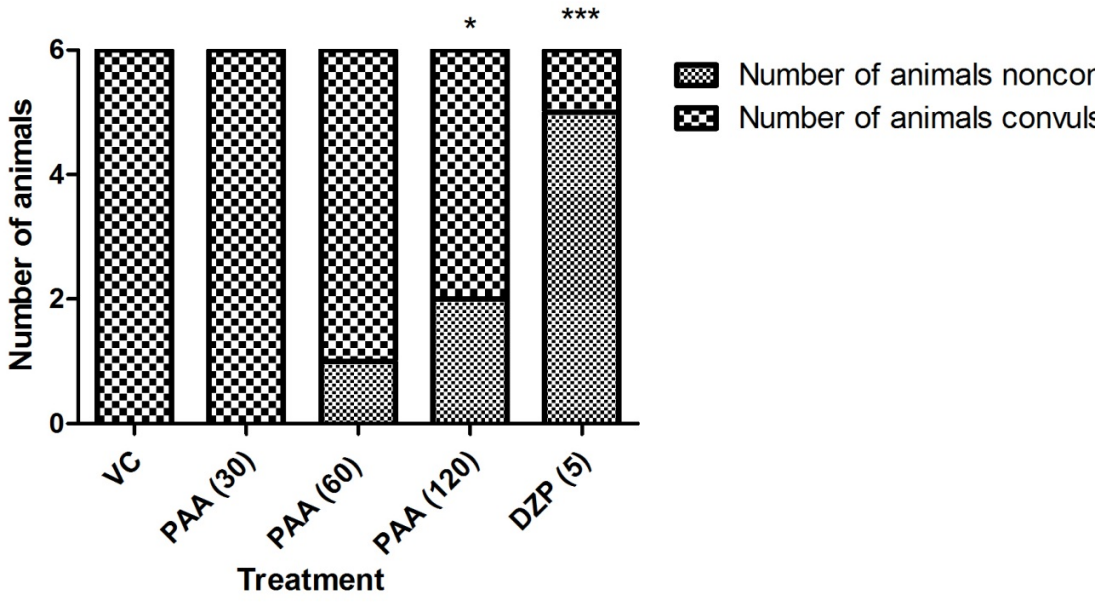
VC
PAA (30)
PAA (60)
PAA (120)
DZP (5)
Treatment

Fig-2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PAA (120)
DZP (5)
Treatment

Fig-3

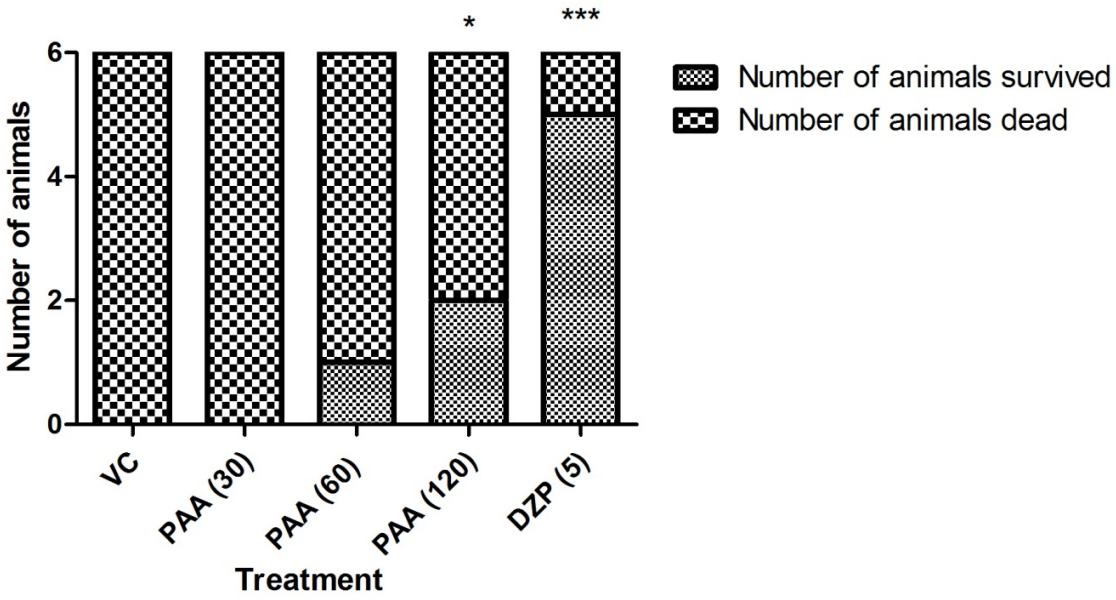


Fig-4

Data of 'latency to tonic & clonic convulsions' was analyzed by one way ANOVA followed by Dunnett's test. Data of 'incidence' and 'mortality' was analyzed by Fisher's exact test. *p<0.05, **p<0.01, ***p<0.001 when all the experimental groups were compared with vehicle control.

3.2 Effects of P. amarus and phenytoin on M.E.S. induced convulsions in mice:

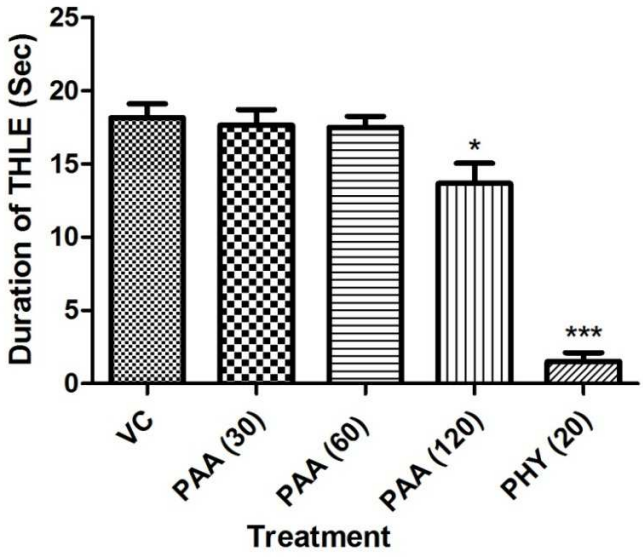


Fig-5

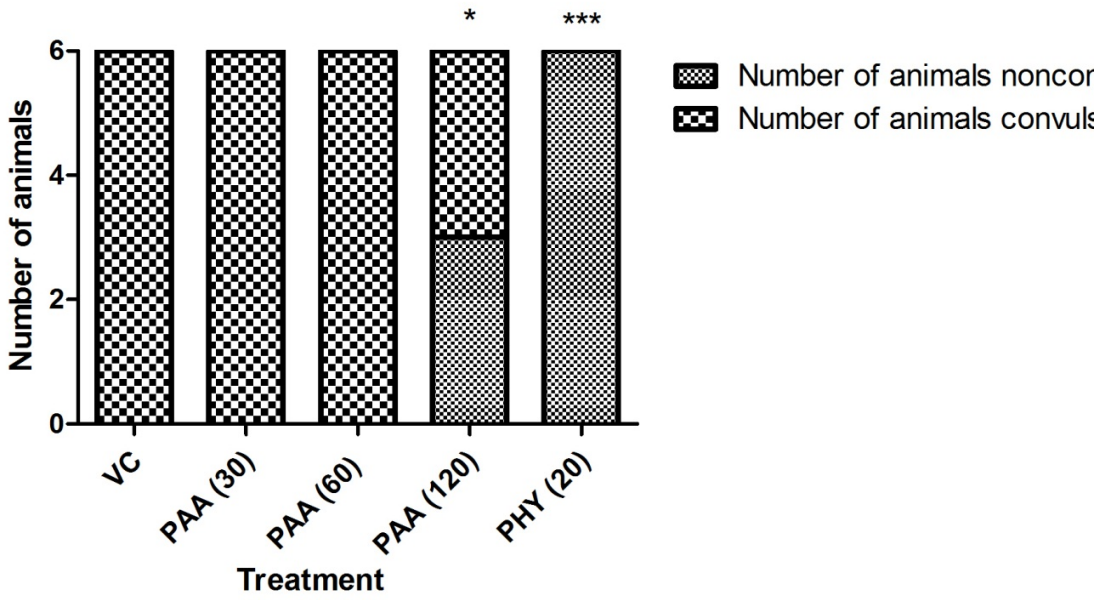


Fig-6

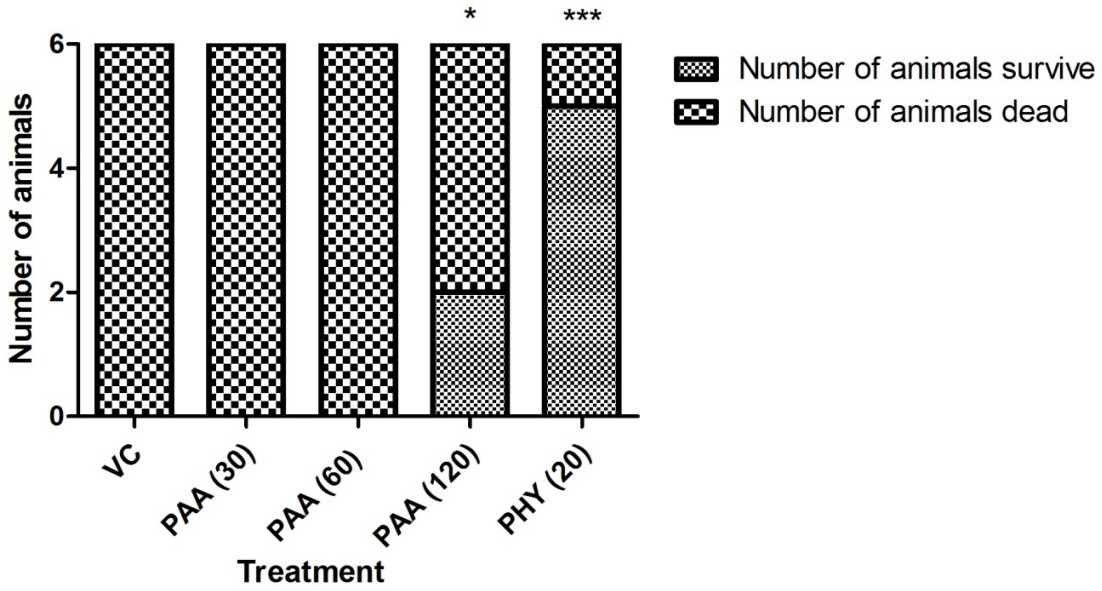


Fig-7

Data of 'latency to tonic & clonic convulsions' was analyzed by one way ANOVA followed by Dunnett's test. Data of 'incidence' and 'mortality' was analyzed by Fisher's exact test. *p<0.05, **p<0.01, ***p<0.001 when all the experimental groups were compared with vehicle control.

Animals treated with PAA 120 mg/kg, i.p. show significant (P<0.05) effect on latency to THLE and mortality was reduced to 66.67%. PAA (30 and 60 mg/kg, i.p.) did not show significant effect on latency to THLE and mortality. Phenytoin 20 mg/kg, i.p. significantly (P<0.001) blocked the MES induced THLE and mortality 16.67 %. (Fig 5-7)

3.3 Effect of extract of P. amarus on picrotoxin induced convulsions in mice:

Animals treated with PAA (60 and 120 mg/kg, i.p.) show significant (P<0.05) increase in latency to PTX induced tonic and clonic convulsions and mortality was reduced upto 66.67 and 50%. PAA 30 mg/kg, i.p. did not show significant effect on episodes of tonic and clonic convulsions and mortality. Diazepam 5 mg/kg, i.p. significantly (P<0.001) increase in latency to tonic and clonic and 0% mortality induced by picrotoxin in mice. (Fig 9-11)

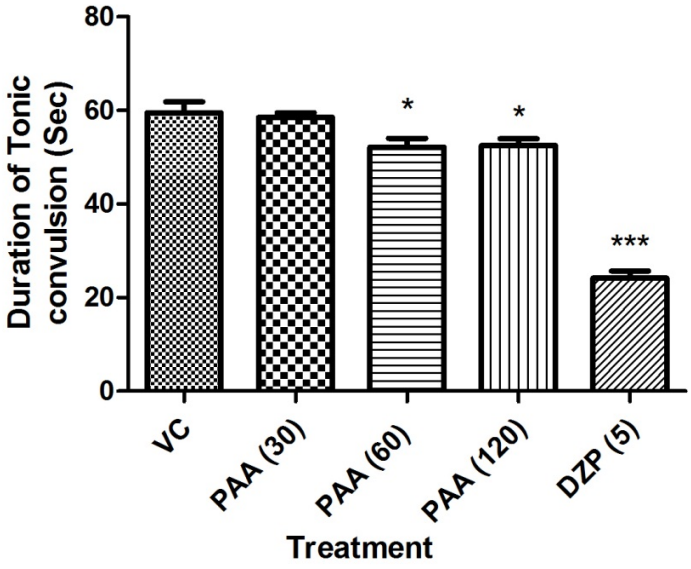
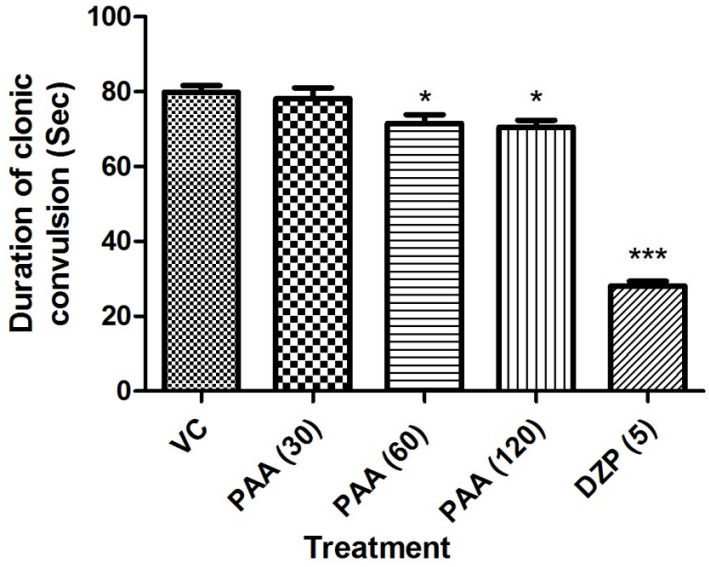


Fig-8


Duration of clonic convulsion (Sec)
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VC
PAA (30)
PAA (60)
PAA (120)
DZP (5)
Treatment

Fig-9

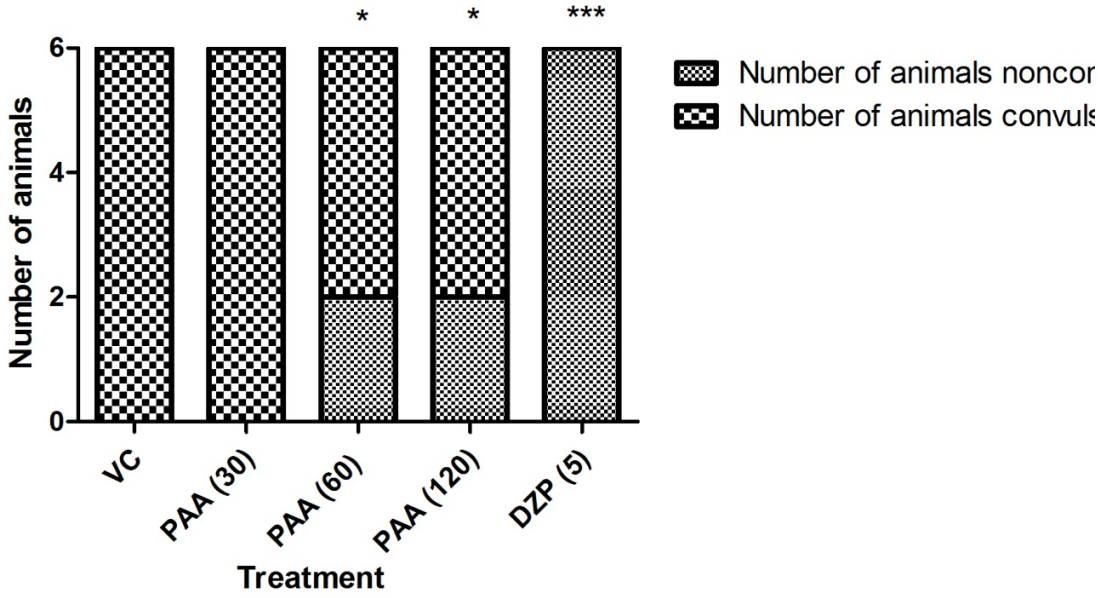
Number of animals
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Fig-10

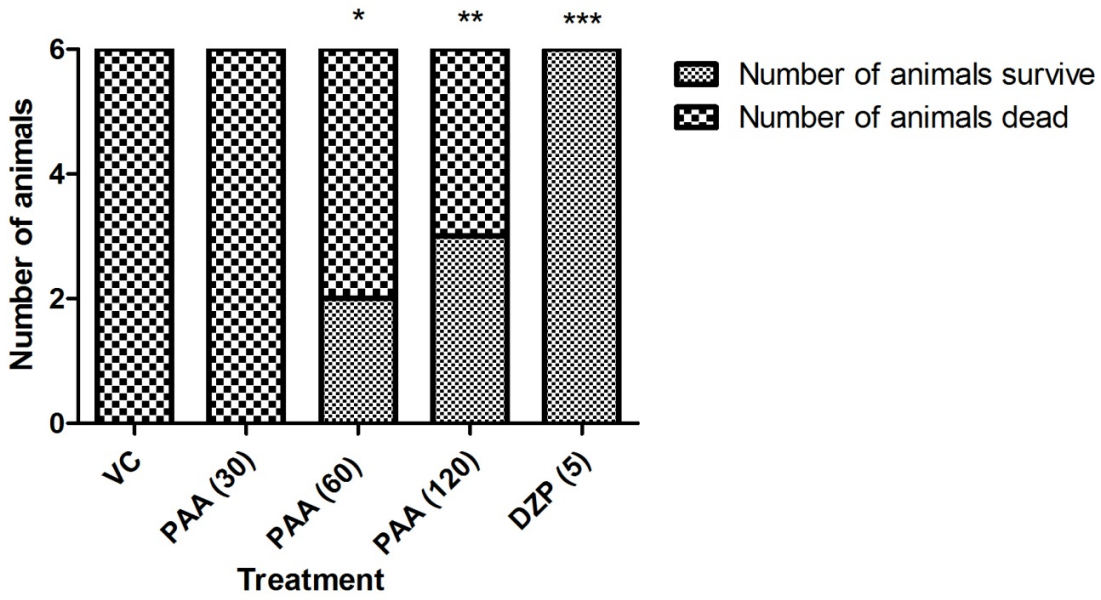

Fig-11

Data of 'latency to tonic & clonic convulsions' was analyzed by one way ANOVA followed by Dunnett's test. Data of 'incidence' and 'mortality' was analyzed by Fisher's exact test. *p<0.05, **p<0.01, ***p<0.001 when all the experimental groups were compared with vehicle control.

3.4 Effects of P. amarus and diazepam on brain GABA level:

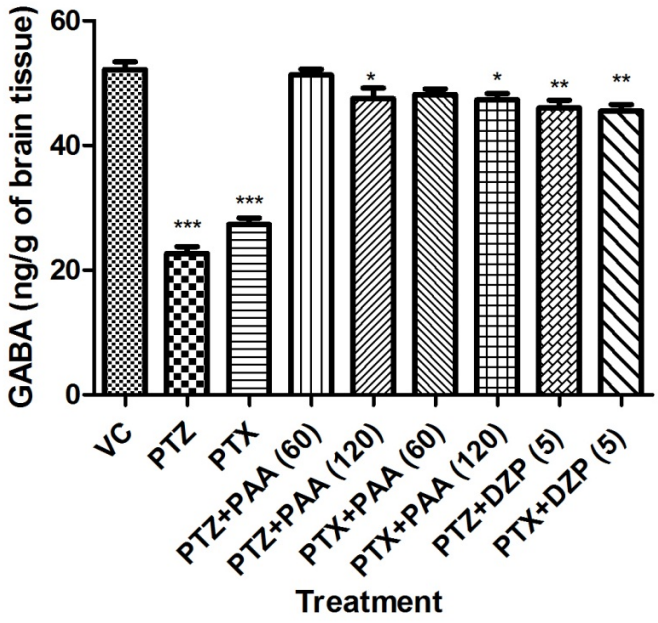

Fig-12

All data analysed using one way ANOVA followed by Dunnet's's post hoc test.
*p<0.05, **p<0.01, ***p<0.001 when all the experimental groups were compared with vehicle control.

Intraperitoneal administration of PTZ andPTX resulted in significant decreased (P < 0.001) in brain GABA level as compared to vehicle control mice. Mice treated with P. amarus (120 mg/kg) significantly increase brain GABA level in PTZ treated mice (P < 0.05) as compared to vehicle control mice. Mice treated with P. amarus (60 mg/kg) failed to produce any significant change in brain GABA level in PTZ and PTX treated mice as compared to vehicle control mice. When compared with vehicle control mice, diazepam (5 mg/kg) treated mice produced significant (P < 0.01) increase in brain GABA level in PTZ and PTX treated mice. (Fig. 12)

3.4 Effects of P. amarus and diazepam on brain nitric oxide level:

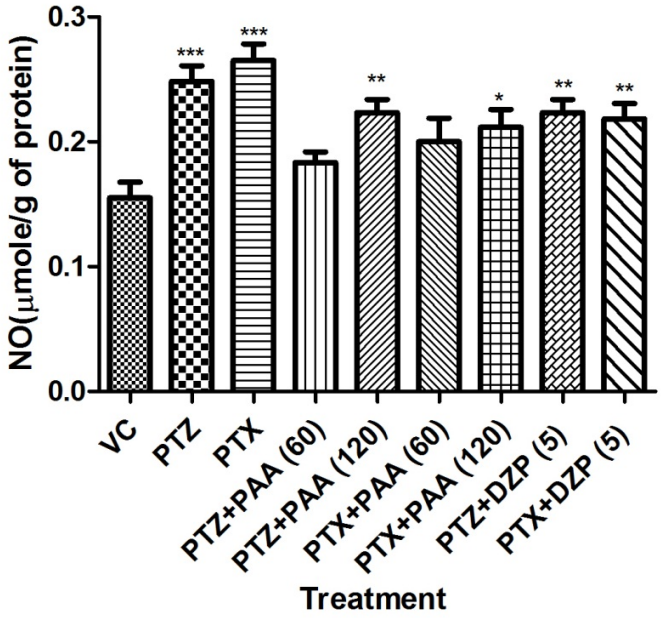

Fig-13
All data analysed using one way ANOVA followed by Dunnet's's post hoc test.
*p<0.05, **p<0.01, ***p<0.001 when all the experimental groups were compared with vehicle control.

Brain NO level in PTZ and PTX treated mice was significantly increased (P < 0.001) as compared to normal mice. Treatment with P. amarus (120 mg/kg) significantly attenuated this elevated level of brain NO in PTZ and PTX treated mice (P < 0.05 and P < 0.01 respectively) as compared to vehicle control mice. Mice treated with P. amarus (60 mg/kg) failed to do so as compared to vehicle control mice. Mice treated with diazepam (5 mg/kg) significantly antagonized elevated level of NO in brain in PTZ and PTX treated mice (P < 0.01). (Fig. 13)

3.5 Effects of P. amaru , diazepam and phenytoin on brain xanthine oxidase level:

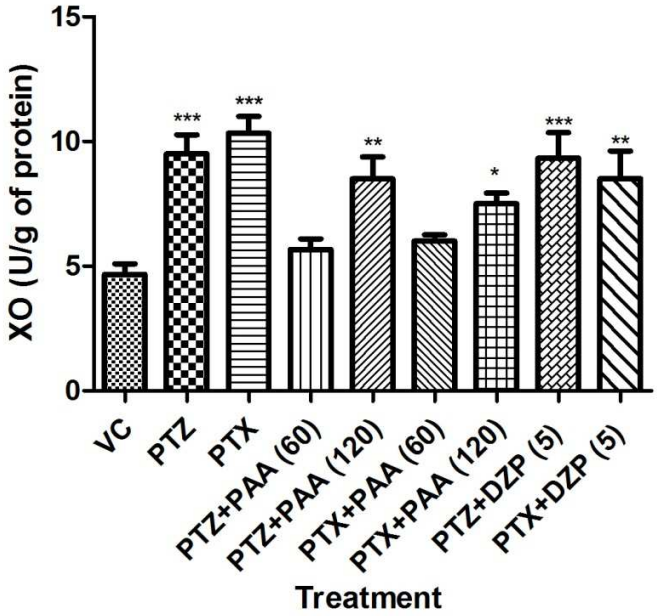

Fig-14

All data analysed using one way ANOVA followed by Dunnet's's post hoc test.
*p<0.05, **p<0.01, ***p<0.001 when all the experimental groups were compared with vehicle control.

Mice treated with PTZ and PTX results significant increase (P < 0.001) in brain XO level as compared to normal mice. Treatment with P. amarus (120 mg/kg) resulted significant decrease in brain XO level (P < 0.01 and P < 0.05 resp.) as compared to vehicle control mice. When compared with vehicle control mice, treatment with diazepam (5 mg/kg) significantly attenuated this elevated level of brain XO in PTZ and PTX treated mice (P < 0.01, P < 0.001 respectively). (Fig. 14)

DISCUSSION

Epilepsy is a common chronic neurological disorder caused due to tumours, degenerative conditions or cerebrovascular diseases. The imbalance between excitatory and inhibitory neurotransmission in the brain is an important characteristic of experimental and clinical seizure. [16] Nerve cell depolarization occurres due to the predominancy of excitatory postsynaptic potentials (EPSP) over the inhibitory (IPSP) resulting in the generation of the seizures in the brain. Epilepsy is precipitated due to an array of factors including elevated electrolytes (Na^+, K^+, Ca^{2+}) levels, excitatory amino acids (glutamic acid), and inhibitory amino acids (GABA), irregular interneuron connections and abnormal afferent connections from subcortical structures which modulate various intertwining biochemical pathways giving rise to discharges of large numbers of neurons resulting in an epileptic seizure.

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Depending upon their mechanism of action classical anticonvulsant drugs has been broadly classified into various categories and they are effective against partial and tonic-clonic seizures. The potent antiepileptic drugs act by either inhibiting the voltage gated Na^+ channels activation resulting in inhibition of firing of neurons or facilitating pre or postsynaptic gamma aminobutyric acid (GABA) mediated synaptic transmission and inhibition. This cause enhanced membrane polarization by influx of Cl^- ions through $GABA_A$ receptor. Some of them also act by inhibiting the GABA metabolism. [18]

In the present investigation the pharmacological screening models were selected on the basis of mechanisms involved in the anticonvulsant activity of drugs. PTZ induced convulsion in mice has been used to screen various drugs as it mimics an array of clinicopathobiological feature of human syndrome. [19] It has been reported that PTZ-induced convulsion model is widely used to identify compounds that are effective against absence and myoclonic seizures. In PTZ induced convulsions model, PTZ imparts convulsions by its inhibitory effect on GABA -mediated Cl^- influx through an allosteric interaction in the Cl^- channel. [20] Previously it has been shown that diazepam enhances the GABAergic neurotransmission and gives protection against the PTZ induced convulsion. [21] *P. amarus* provides protection against the PTZ induced convulsions suggesting anticonvulsant property by modulation of GABAergic pathways in brain. In the present investigation it was found that *P. amarus* increases the level of the brain GABA which provides credence to its anticonvulsant activity by GABA level regulation in brain.

The drugs which produce effect on Na^+ channels are widely used to screen against M.E.S. induced convulsions. These drugs are proven to be effective against partial and tonic-clonic seizures in humans. [22] Phenytoin and *P. amarus* exhibited anticonvulsant profile effect via inhibition of the Na^{2+} channels in partial and tonic-clonic seizures.
The mechanism of block by PTX and other related compounds is still equivocal. Based on the use-dependent characteristic of PTX. It may act within the channel lumen to block the channel. However, single-channel study has demonstrated that PTX does not affect channel burst duration. Moreover, PTX-induced inhibition of $GABA_A$ receptor is voltage dependent. These results are inconsistent with the conclusion that picrotoxin inhibits the receptor via a traditional open channel blocking mechanism. In addition, although it has been demonstrated that mutations of amino acids in the second trans membrane domain of the receptor inhibit the actions of PTX, it is not known if these amino acids are involved in binding, or even accessibility of picrotoxin to its site of action. In addition to blocking $GABA_A$ receptors, picrotoxin blocks number of other ion channels, including $GABA_C$ receptors and glutamate gated Cl^- channels. [23]

It has been experimentally studies that oxidative stress is an important player in central nervous system in various models epilepsy in laboratory animals. [24,25]

Xanthine oxidase exerts an important role in formation of ROS [26] which cause an array of reactions detrimental to neuronal tissue.

Hence antioxidants quench the superoxide anion, hydroxyl and peroxyl radicals and protect the neuronal tissues against damage. *P. amarus* is a potent antioxidant. The mice pretreated with *P. amarus* demonstrated downregulated activity of this xanthine oxidase enzyme, indicating that *P. amarus* may have ability to protect the cellular components against seizures exerted by oxidative stress. [27]

Nitric oxide is intercellular signaling molecule which is produced endogeneously which may have both inhibitory and elevator activity of epilepsy. The present investigation shows that the epilepsy is inversely related to levels of NO and the epileptic seizures are modulated by phyllanthus amarus via NO dependent pathway.
Therefore our results suggest that *P. amarus* may be useful in the above stated seizure types in human beings. [28] The drugs that are effective against tonic hind limb extension induced by electroshock generally have proven to be effective against partial and tonic-clonic seizures and those in PTZ model are effective against absence seizure in human beings.

Phytochemical studies have shown the presence of many valuable compounds such as lignans, flavonoids, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and alkaloids. The extract and the compounds isolated from *P. amarus* show a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective nephroprotective and diurectic properties.
It could be concluded from the present investigation that *P. amarus* protects endogenous enzyme level, and modulates GABAergic transmission to exhibit anticonvulsant effect. Further studies are needed to unravel its mechanism of action.

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