Anti-Seizure Activity of *Tabernaemontana Divaricata* (L.) R.Br. Flower Methanolic Extract Against Maximal Electroshock and Pentylene Tetrazole Induced Convulsions in Experimental Animals

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Summary

Tabernaemontana divaricata (L).R.Br belonging to Apocynaceae family is traditionally used to treat various disorders including neurological problems. In the view of its application in the management of epilepsy, present investigation was carried out to assess the anti-seizure potential of *Tabernaemontana divaricata* flower methanolic extract (TDFME) against maximal electroshock (MES) and pentylene tetrazole (PTZ) induced convulsions in rats and mice respectively. Two doses of TDFME, 250 & 500 mg/kg were used in the experiments. Phenytoin (PHT) 25 mg/kg, i.p and Diazepam (DZP) 4 mg/kg, i.p were used as standard anti-convulsant drugs. TDFME at dose of 250 mg/kg, failed to reduce hind limb extensor phase in MES model. However, it significantly delayed onset of both myoclonic spasm and convulsions with $p<0.01$ and $p<0.05$ respectively. High dose of TDFME (i.e. 500 mg/kg) significantly ($p<0.05$) reduced hind limb extensor phase in MES model, delayed onset of myoclonic spasm & convulsions with significance ($p<0.01$) & ($p<0.001$) respectively in PTZ model. The incidence of convulsions & percentage mortality observed was 50% less when compared to the control group. Thus, the effect of TDFME (500 mg/kg) was similar to that of DZP in PTZ model. These findings suggest that TDFME possesses anti-seizure activity.

Keywords: *Tabernaemontana divaricata* flower methanolic extract (TDFME), anti-seizure activity, maximal electroshock (MES) and pentylene tetrazole (PTZ) induced convulsions.

Introduction

Seizures are associated with disordered and rhythmic high frequency discharge of impulses by a group of neurons in the brain and status epilepticus is characterized by repeated episodes of epilepsy without the patient having recovered from the previous attack (1). Epilepsy is the second most common neurological disorder which affects an estimated 7 million people in India and 50 million people worldwide (approximately 1-2% of the world population) (2,3). Although several anti-epileptic drugs are available to treat epilepsy, the treatment is still far from adequate (4).

Unfortunately most of the synthetic drugs not only fail to control seizures in some patients, but they frequently cause side effects (5). Due to these problems research focus has shifted towards natural products for new and better sources of drugs. In this process, medicinal plants serve as an alternative source for the development of new anti-convulsant drugs. Various plants are being studied based on the traditional knowledge of their pharmacological properties and confirmed to be useful in treating and managing various diseases (6).

Tabernaemontana is one of the genera that are used in Ayurveda, Chinese and Thai traditional systems of medicine (7,8). *Tabernaemontana divaricata* (L.) R.Br. is traditionally used by people in many parts of the world to treat various disorders like abdominal tumours, arthralgia, asthma, diarrhoea, epilepsy, eye infections, fever, fractures, headache, inflammation, leprosy, mania, oedema, paralysis, piles, rabies, rheumatic pain, skin diseases, ulceration and vomiting. It is also used as antihelmintic, antihypertensive, aphrodisiac, diuretic, emmenagogue, hair growth promoter, purgative, remedy against poisons and tonic for brain, liver & spleen (9,10).

Growing evidences suggests that this plant has medicinal benefits and its extracts could possibly be used as pharmacological intervention in various diseases. Phytochemical studies on various parts reveal that this plant contains at least 66 indole alkaloids, non-alkaloidal constituents like enzymes, flavonoids, hydrocarbons, phenolic acids, phenyl propanoids, steroids and terpenoids (11). Alkaloids, flavonoids and terpenoids are the main secondary metabolites that exhibit many physiological and pharmacological properties on living cells (12). *T. divaricata* flowers contain 3, 4, 14, 19 – tetrahydro-Olivacine, 11-methoxy-N-methyl dihydro-Pericyclivine, 19-epivoacangine, Apparicine, Isovoacangine, Isovoacristine, Tabernaemontanine, Tabersonine, Voaphylline, N-1-methyl-Voaphylline, Vobasine (13-20). There are still many *T.divaricata* alkaloids & their derivatives whose pharmacological activities are yet to be studied. They may contain beneficial pharmacological properties (11). In the view of these evidences, the present study was undertaken to evaluate the effect of TDFME on experimentally induced seizures.

Material and Methods

Plant material: The flowers of Crepe Jasmine (*Tabernaemontana divaricata*) were collected in the month of November 2009 from the local areas of Ameerpet and Mallepally (Hyderabad District) and authenticated by plant taxonomist Dr. Ramana, Department of Botany, Osmania University. A voucher specimen (0360-OUH) has been preserved in the herbarium for future reference.

Preparation of extract: After collection flowers were shade dried and coarsely powdered. Approximately 500 gm of powdered flowers were extracted using 99% pure methanol (Carbinol – SD Fine Chemicals) in a soxhlet apparatus. The extract was concentrated under reduced pressure and stored in an air tight container in a refrigerator at temperature below 10°C. Dried mass of crude *Tabernaemontana divaricata* flower methanolic extract (TDFME) was weighed and recorded. The solution of TDFME was prepared using distilled water for the evaluation of anti-seizure activity.

Experimental animals: Adult male Wistar rats (150-200gm) and Swiss albino mice (20-30gm) were used to evaluate anti-convulsant activity by maximal electroshock (MES) and pentylene tetrazole (PTZ) induced convulsions respectively. The animals were maintained under standard laboratory conditions in polypropylene cages under 12 hr light/dark cycle, controlled temperature (24±2°C), fed with commercial pellet diet and water *ad-libitum* in an animal house approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (Reg no. 1185/a/08/CPCSEA). All animals were acclimatized to the laboratory environment for at least 10 days before the commencement of experiments. The experimental protocol was approved by Institutional Animal Ethical Committee, MESCO College of Pharmacy, Mustaidpura, Hyderabad, Andhra Pradesh, India.

Acute toxicity study: Acute toxicity test was performed in mice according to staircase method. Mice were divided into four groups with five animals per dose. The dose was increased from 250-1000 mg/kg, i.p. Mice were observed individually after dosing with special supervision given during the first 4 hrs, periodically during the first 24 hrs (21).

Evaluation of anti-convulsant activity:

1. Maximal Electroshock (MES) induced convulsions: An electroshock of 150 mA, 0.2 second was delivered through ear-electrode to induce hind limb tonic extensor phase (HLTE) in rats using electroconvulsiometer (INCO, Ambala, India). The current was delivered 30 mins after intra-peritoneal administration of respective treatments. Different parameters observed were the time spent by rats in flexion, extensor, clonus and stupor phases of tonic-clonic seizures. The occurrence and duration of HLTE and incidence of mortality were noted(21).

Experimental Design: The rats were divided into 4 groups (n=6) and treated with the respective test solutions as given below:

Group I (Control): Tween 80 (1% w/v, i.p)

Group II (Standard): Phenytoin (25 mg/kg, b.w, i.p)

Group III (Test-I): TDFME (250 mg/kg b.w, i.p)

Group IV (Test-II): TDFME (500 mg/kg b.w, i.p)

2. Pentylene tetrazole (PTZ) induced convulsions: 30 mins after administration of standard drug and test extract doses, clonic seizures and tonic-clonic convulsions were induced in mice by intra-peritoneal injection of Pentylene tetrazole (80 mg/kg). The latency to the onset of myoclonic spasm, onset of convulsions and mortality in treated mice was recorded(21).

Experimental Design: The mice were divided into 4 groups (n=6) and treated with the respective test solutions as given below:

Group I (Control): Tween 80 (1% w/v, i.p)

Group II (Standard): Diazepam (4 mg/kg, b.w, i.p)

Group III (Test-I): TDFME (250 mg/kg b.w, i.p)

Group IV (Test-II): TDFME (500 mg/kg b.w, i.p)

Statistical Analysis: The values are expressed as Mean ± Standard Error of Mean. $P< 0.05$ was considered statistically significant. Data obtained was analyzed by One-way ANOVA with Dunnett's multiple comparison post test using GraphPad Instat version 3.10 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

The percentage yield of TDFME (dried crude extract) obtained was 14%. The acute toxicity study reveals that the maximum tolerable dose of TDFME is more than 1000 mg/kg as all the test doses were found to be safe and no mortality or toxicity was observed. Two test doses i.e. (250 and 500 mg/kg) of TDFME were chosen for the evaluation of anti-convulsant activity based on the acute toxicity testing. The methanolic extract at dose of 500 mg/kg b.w, i.p has shown a significant ($p<0.05$) decrease in duration of hind limb extensor phase. Complete abolition was also observed in some of the animals in MES. Whereas in PTZ induced convulsions it delayed onset of myoclonic spasm as well as onset of convulsions by $p<0.001$ & $p<0.01$. The recorded incidence of convulsions and mortality rate decreased by 50% when compared to control (PTZ) group. At dose of 250 mg/kg, TDFME did not exhibit significant effect of hind limb extensor

phase of MES induced convulsions. But significantly ($p<0.01$) & ($p<0.05$) delayed onset of both myoclonic spasm and convulsions. Thus, the results of present study indicate that TDFME exhibits significant anti-seizure effect against MES & PTZ induced convulsion models.

Table-1. Effect of TDFME on maximal electroshock induced seizures

Groups & Treatments	Flexion	Extensor	Clonus	Stupor	Percentage Protection
I. Control: Tween 80 (1%w/v)	3.83±0.4014	11.00±0.9309	1.05±1.118	151.66±11.081	83.34
II.Standard: Phenytoin 25 mg/kg	13±1.653**	1.33±1.33***	2.83±0.7032**	70.83±10.374***	100
III.Test-I: TDFME 250	5±0.3651	8.16±0.7032	8.33±0.7601	139.16±9.867	100
IV.Test-II: TDFME 500	10.83±1.014**	4.16±1.558*	3.5±0.500**	88.33±13.208**	100

Table-2. Effect of TDFME on pentylene tetrazole induced seizures

Groups	Onset of Myoclonic spasm (in seconds)	Onset of Convulsions (in seconds)	Incidence of Convulsions	Percentage Mortality
Control	75.0±4.351	87.16±4.199	6/6	100
Standard	353.33±6.280***	-	0/6	0
TDFME 250	98.33±4.014**	165±8.06*	6/6	66.66
TDFME 500	191.66±3.33***	183±36.87**	3/6	50

Note: Data is expressed as Mean ± SEM; n=6 per group *p indicates <0.05, **p indicates <0.01 and ***p indicates <0.001 when compared to the control group.

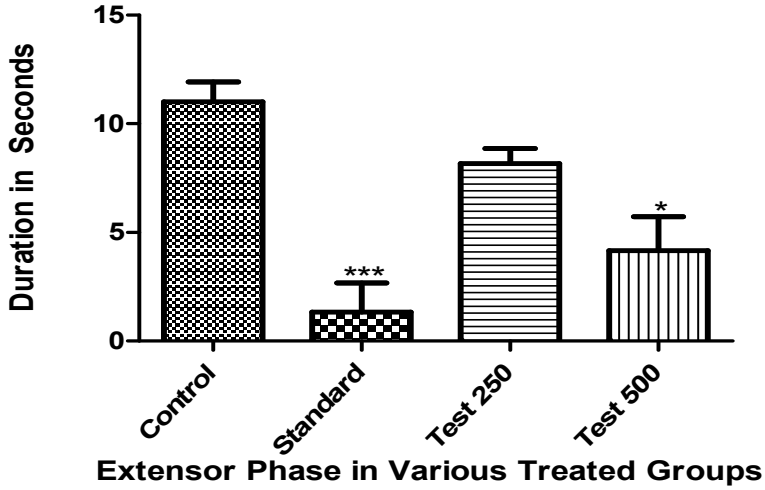

Figure 1: Effect of various treatments on hind limb extensor phase in MES induced convulsions in rats. **Note** - * & *** indicates P-values < 0.05 and < 0.001 respectively.

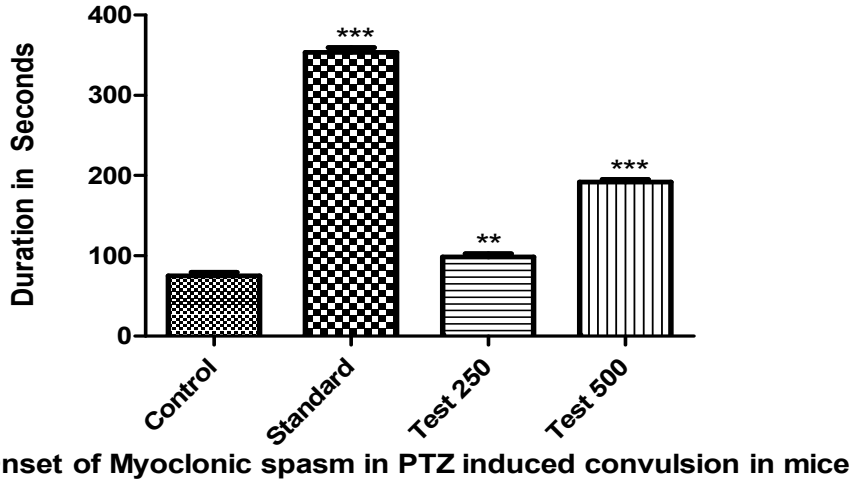

Figure 2: Effect of various treatments on onset of myoclonic spasm in PTZ induced convulsions in mice. ** indicates p< 0.01 & ***indicates p<0.001.

Discussion

Plants belonging to Tabernaemontana genus are used in folk medicine for the treatment of high blood pressure, pain, inflammation as well as topical application for healing wounds. *Tabernaemontana divaricata* exhibits different roles in CNS, cardiovascular, gonadotropic, anti-tumor, anti-infectious, anti-oxidant activities and enhances cholinergic activity in nervous system (11). Most common medicinal use of crude *T.divaricata* extract involves its anti-microbial action against infectious diseases such as gonorrhea, leprosy, syphilis, as well as anti-parasitic action against diarrhea, dysentery, worms and malaria (8).

The anti-convulsant potential of TDFME was assessed by MES & PTZ models. MES & PTZ – induced seizure models are the most commonly used preliminary screening tests to evaluate anti-convulsant potential of drugs. MES model characterizes for the assessment of generalized tonic-clonic seizures, whereas PTZ model is considered to be a predictor of generalized absence seizures. PHT reported to act by blocking voltage-dependent Na^{2+} channel, was employed as a standard drug to prevent MES- induced generalized tonic-clonic seizures (22,23). Reduction and abolition of extensor phase is documented to be an index of increase in seizure threshold (24).

PTZ test represents a valid model for human generalized myoclonic seizures and also generalized seizures of the petitmal (absence) type (25). PTZ enhances the basal activity and the sensitivity of dopaminergic neurons to PTZ in rat brain and the nigrostriatal dopaminergic neurons contribute to the central alterations associated with experimental epilepsy (5). PTZ induces convulsion by antagonizing γ- amino butyric acid $(GABA)_A$ receptor chloride (Cl^-) channel complex to attenuate GABA dependent inhibition(26). Drugs protecting against tonic-clonic seizures induced by PTZ are considered useful in controlling myoclonic and absence seizures in humans(27). Thus, demonstration of activity in this model suggests that the plant possesses anti-convulsant activity which validates the traditional use of this plant for the treatment of epilepsy. Since TDFME antagonized PTZ induced seizures, this suggests enhancement of GABAergic transmission with general depression of the central nervous system.

GABA is a major inhibitory neurotransmitter, the enhancement and inhibition of GABA neurotransmission has shown to attenuate and enhance the convulsions respectively (28,29). DZP, a benzodiazepine, is reported to prevent PTZ-induced absence seizures by enhancing $GABA_A$ receptor mediated inhibitory neurotransmission (22). Hence, in the present investigation, DZP has been employed as a standard drug in PTZ model. In addition, N-methyl-D- Aspartic acid (NMDA) receptor activation has also been implicated to mediate the occurrence of PTZ –induced seizures (22, 30, 31). Further, NMDA receptor antagonists such as agmatine, ketamine, diazocilpine & 2-amino-5-phosphovaleric acid (APV) have been reported to be effective against PTZ-induced convulsions (32-34). However, the acceptability of NMDA receptor antagonists as anti-epileptic drugs is limited as they are often associated with intolerable

side effects (34-37). Hence, the observed ameliorative effect of TDFME against PTZ-induced convulsions model may be due to potentiation of GABAergic system or by inhibition of glutamatergic excitation through blockade of NMDA receptor. Further, in PTZ model TDFME at 500 mg/kg was found to be more beneficial as compared to 250 mg/kg.

In PTZ model, TDFME was more effective at dose of 500 mg/kg indicating CNS depressant action as consequence of its GABAergic transmission (since PTZ is selective $GABA_A$ receptor antagonist). There are some evidences of anti-convulsant effect produced by the flavonoidal compounds (38,39). Anxiolytic effects of some natural & synthetic flavonoids were studied in rats. It was found that the compounds exerted effect through central benzodiazepine receptors (40). Moreover, previous studies have shown that flavonoids may cause facilitation of GABAergic system as they are structurally similar to benzodiazepines like molecules present in CNS (41). Kaempferol is reported to be present in *T.divaricata* flowers. It could be responsible for neuroprotective effect against experimentally induced convulsions (42).

It is often stated that anti-epileptic drug that either prevent or delay PTZ induced convulsions, act by elevating the seizure threshold, whereas drugs that block MES tonic extensor phase act by blocking spread of seizure (43,44). Moreover, the tonic extensor phase can be prevented either by drugs that inhibit voltage-dependent Na^{2+} channels, such as phenytoin, valproate, lamotrigine or by drugs that block glutamatergic excitation mediated by NMDA receptor such as felbamate(45,46). It is well established that facilitation of gluatamate release from nerve terminals and astrocytes results into glutamate induced neurotoxicity (47). On the other hand, drugs that reduce T-type Ca^{2+} currents like ethosuximide or drug that enhance $GABA_A$ receptor mediated inhibitory neurotransmission, such as benzodiazepines & Phenobarbital elevate the seizure threshold & thereby prevent seizures induced by PTZ (48).

Alkaloids are responsible for the medicinal properties of the plant. Alkaloidal component of *T.divaricata* could play important role in its pharmacological activities on cardiovascular and nervous systems (11). In the previous study *T.divaricata* has shown CNS depressant effect. Loss of screen grip and decreased muscle tone was observed in the rat model following *T.divaricata* administration suggesting *T.divaricata* has skeletal muscle relaxant property (49). Roots and stems of *T.divaricata* have been used in Thai traditional medicine as a component of rejuvenating & neurotonic remedies. It is believed to prevent forgetfulness & improve memory as well as being a CNS stimulant (50). *T.divaricata* root extract at concentration of 0.1 mg/ml inhibited more than 90% of acetylcholinesterase (AChE) activity (51). *T.divaricata* administration in various doses can significantly decrease neuronal AChE activity in the cerebral cortex (52). *T.divaricata* can also be a new therapeutic target for Alzheimer's disease (50,53).

The active anticonvulsant alkaloid might not have reached the effective dose in TDFME 250 mg/kg crude extract. Hence, failed to exhibit significant effect in MES model. The total yield of

alkaloid fraction obtained from dry stems of *T.divaricata* was about 0.98% and the total alkaloidal content of flowers is lesser than that of stems (54). In previous studies, compounds like terpenoids, phenolic acids and plant metabolites exhibited pharmacological properties such as in-vitro anti-inflammatory and anti-oxidant activities (55). *T.divaricata* may be useful therapeutic agent for several neurodegenerative diseases such as Alzheimer, vascular dementia & delirium as the possible cause of these disorders is cholinergic deficiency. According to previous studies several alkaloids in *T.divaricata* enhance cholinergic activity. The possible cholinergic candidates are coronaridine, isovoacristine, 19, 20 – dihydrotabernamine and 19, 20-dihydroervahanine A (11).

T.divaricata contains many neuropharmacologically important indole alkaloids. Catharanthine inhibited the calcium-calmodulin-stimulated activity of brain cyclic adenosine monophosphate (cAMP) phosphodiesterase in an in-vitro study, resulting in an increased intracellular level of cAMP. The increased intracellular level of cAMP in neurons may lead to improved neuronal activity (56, 57). Coronaridine, found in leaves, stem bark & roots has demonstrated an effect on autonomic and CNS activity (8). Dregamine found in leaves, stem bark, roots of *T.divaricata* has shown convulsive & respiratory stimulating effects. It has been used for the treatment of muscular & nervous asthenia and respiratory depression (58). Ibogamine, an indole alkaloid found in roots causes blockade of nicotinic receptors at the neuromuscular junction. It demonstrated weak anti-convulsant property in a mouse model. It is reported to reduce drug craving, with drawl symptoms, tremorigenic, hallucinogenic, neurotoxic & cardiovascular side effects in addicts. Ibogamine produces anti-addictive effect possibly by blocking kappa opioid receptors, N-methyl-D-aspartate (NMDA) receptors, the serotonin uptake sites and nicotinic receptors (59-62).

Tabernaemontanine obtained from whole plant exhibited vasodilatory effect in dogs. It is used to dilate blood vessels in human beings following cases of atherosclerosis, cerebral trauma & circulatory irregularities (8). Voacangine exists in whole plant, potentiated hypnotic effects of barbiturates and had an analgesic as well as local anesthetic activity in mice (63). Voacristine exhibited a weak stimulating effect on CNS (head shaking behavior in mice) (8). Vobasine at dose of 300 mg/kg caused respiratory and CNS depression in mice (64). However, Isovoacristine acts as anti-cholinergic agent. It also produced anti-histaminic activity on isolated guinea pig ileum and relaxation of skeletal muscles in rabbits (65). In-vivo investigations regarding their effects could provide insights into benefits of *T.divaricata* for future clinical management of many human diseases (11).

Conclusion

The results obtained from the present study indicate that TDFME inhibits electrically and chemically induced seizures in rats and mice respectively. The observed anticonvulsant activity

may be due to potentiation of GABAergic neurotransmission and or increase in neuronal seizure threshold by decreased Na^{2+} channel activity, which may be attributed to the alkaloids. There is also a possibility of synergism between alkaloids and flavonoids present in TDFME.

In conclusion, the present study provides preliminary data on anticonvulsant activity of *T.divaricata* flowers and highlights the presence of anticonvulsant alkaloids in *T.divaricata* flowers apart from Ibogamine isolated and characterized from the stem bark. However, detailed pharmacological studies on isolated pure indole alkaloids from flowers is essential to confirm the exact mechanism of action, which is underway.

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