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ANXIOLYTIC ACTIVITY OF *TABERNAEMONTANA DIVARICATA* (LINN) R. Br. FLOWERS EXTRACT IN MICE

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ABSTRACT

The present study was undertaken to evaluate **anxiolytic** activity of alcoholic flowers extract of *Tabernaemontana divaricata* (Linn) R.Br. (ALETD) by using mice. This plant contains carbohydrates, steroids, tannins, triterpenes, flavonoids, proteins, amino acids and glycosides. Anxiolytic effect of ALETD (100, 200 and 400 mg/kg) was studied and diazepam used as a standard drug by using following animal models, Elevated Plus Maze (EPM), Open-Field Test (OFT) and Light-Dark Transition Test (LDT**).** On EPM, the diazepam and all the doses of extract had showed significant anxiolytic activity by increasing open arm entries and time spent in open arm. In OFT increased total locomotion, central locomotion and decreased number of rearings, immobility time was observed with diazepam and all the doses of extract. In LDT model the anxiolytic activity was observed with diazepam as well as all the doses of extract by increasing latency, number of crossings, time spent in light box and decreasing rearings in light box was observed. The alkaloids, flavonides and other chemical constituents present in ALETD are speculated to account for the observed pharmacological effects of the plant's extract in the experimental animal paradigms used. The findings of this experimental animal study indicate that ALETD possess anxiolytic property and thus lend pharmacological credence to the folkloric and ethanomedical uses of the plant in the treatment of anxiety condition.

KEYWORDS

Tabernaemontana divaricata (Linn) R.Br. diazepam, anxiolytic activity, Elevated Plus maze.

INTRODUCTION

Anxiety is a cardinal symptom of many psychiatric disorders closely allied with appropriate fear and often serving psycho biologically adoptive purposes and is rather infrequently "disease" itself. It is typically associated with the former "psychoneurotic" disorders, hypothesis implicates over activity of adrenergic systems or dysregulation of serotonergic systems in the central nervous system (CNS) and symptoms are of anxiety commonly associated with depression[1].

Anxiety affects one-eighth of the total population worldwide and has become an important area of research in psychopharmacology during this decade. Benzodiazepines (BZDs) are the major class of compounds used in anxiety and they remain the most commonly prescribed treatment for anxiety. However, the realization that BDZs have a narrow safety margin has promoted many researchers to evaluate new compounds in the hope of identifying other anxiolytic drugs with fever unwanted effects[2].

In the present study we selected a plant namely *Tabernaemontana divaricata* (Linn.) R.Br. (*T.divaricata*) belonging to the family of Apocynaceae. It is distributed in the throughout India, also cultivated as an ornamental plant. The flowers white, sweetly fragrant in 1-8 flowered cymes at the bifurcations of the branches. In traditionally the plant is used as an emmenagogue, aphrodisiac, tonic, purgative, tonic to the brain, the liver and spleen. It is useful in paralysis, weakness of the limbs, cures scorpion-sting, epilepsy. Its charcoal is good in ophthalmia. The oil is good for epilepsy (Yunani) [3].

Earlier the plant has been studied for its, anti-inflammatory[4], acetyl cholinesterase inhibitory[5], blocking of cell proliferation, inhibition of amyloida β-peptide 35-25 induced cognitive deficits[6], anti-acne[7], antioxidant and anti-inflammatory[8] and antifertility[9] activities.

Considering the varied important activities reported in traditional system of medicine with this plant. It was planned to study the effects of flowers extract of *T.divaricata* on CNS mainly for its anxiolytic activity.

MATERIALS AND METHODS

1. ***Drugs and Chemicals:*** Diazepam (Ranbaxy Laboratories Ltd.), Tween 80 (Lobo Cheme PVT. Ltd., Mumbai).

2. ***Animals:*** Albino mice weighing between 18-22g of either sex were used for in this study. All the animals were procured from Shri Venkateswara Enterprises, Bangalore for experimental purpose. After procuring, all the animals were acclimatized for 07 days and housed in groups of 06 under standard husbandry condition[10] like room temperature 26 ± 2^0C, relative humidity 45-55% and light/ dark cycle of 12 h.

   All the animals were fed with synthetic standard diet (Pranava Agro Industries Ltd., Bangalore.) and water was provided *ad libitum* under strict hygienic conditions. After obtaining permission from Institutional Animal Ethical Committee (IAEC) of T.V.M. College of Pharmacy, Bellary (Karnataka), animal studies were performed as per rules and regulations in accordance to guideline of CPCSEA with R. No. 462/01/CPCSEA, 2001.

3. ***Plant material:*** The flowers of *T.divaricata* was collected from Bellary (Karnataka State) and authenticated and identified by a Botanist Dr. Govind Raju of A.S.M. College Bellary. The flowers were dried in shadow and slices of flowers were subjected to size reduction by using mixy, to coarse powder.

4. ***Preparation of alcoholic extract[11, 12]:*** The air dried flowers powder was extracted successively with the following solvents of their increasing polarity in a soxhlet extractor.
1) Pet. Ether (60-80%), 2) Chloroform, 3) Alcohol. After alcoholic extraction macerated the mark with chloroform water for 24 h to obtained the aqueous extract. Concentrated the each extract solvent by using flash evaporator to dryness on the water bath in low heat. Weighed the residue obtained with each solvent and determine its % in terms of air dried weight to the flower material (% w/w) to obtained successive solvent extractive values. On the basis of % yield highest percentage of the extract was selected for the study.

5. ***Preliminary phytochemical screening[11, 12]:***
The preliminary phytochemical investigation was carried out with ALETD for qualitative identification of phytochemical constituents. Phytochemical tests were carried out by standard methods**.** All the chemicals and reagents used were of analytical grade.

6. ***Determination of acute toxicity (LD_{50})[13]:*** The acute toxicity of ALETD was determined by using albino mice of either sex (18-22g). The animals were fasted 3 h prior to the experiment, Acute Toxic Class method (OECD guideline No. 423) of CPCSEA was adopted for toxicity studies. Animals were administered with single dose of extract and observed for its mortality during 48 h study period (short term toxicity). Based on short-term toxicity profile of extract the dose for the next animal was determined as per as OECD guideline No.423.

7. ***Anxiolytic activity***

7.1 Elevated plus-maze (Exteroceptive behavior model) [14-16]: The plus-maze apparatus comprises of two open arms (16×5cm) and two closed arms (16×5×12cm) that extend from a common central platform (5×5cm). The entire maze is elevated to a height of 25cm above the floor level. Albino mice (18-22g) of either sex were divided into 05 groups of 06 mice in each was fasted overnight prior to the test but water was supplied *ad libitum*. Group I was maintained as normal control which was given with 3% Tween 80 (10 ml/kg p.o.) only once daily for 7 days, group II received diazepam (2 mg/kg, i.p.) 30 min before the test. Groups III, IV and V were treated with different doses of ALETD i.e., 100, 200 and 400 mg/kg, p.o. respectively once daily for 7 days. On 7th day 60min after administration of the vehicle or extract and 30 min of standard each mouse was placed in the center of the maze facing one of the open arms. During a 5min test session, the following parameters were noted.

- Number of entries into open arm
- Number of entries into closed arm
- Time spent in the open arm
- Time spent in the closed arm and
- Total number of entries in open and closed arms

7.2 Open- Field Test[17]: This method is used to evaluate exploratory activity and emotionality of animal. The open field consisted of a white painted arena measuring 55 cm in diameter with 100 W lamp. The floor of the arena will be divided into several units by black painted lines. The apparatus will be placed in a sound attenuated room, 48 cm above the floor. Albino mice (18-22 g) of either sex were divided into 05 groups of 06 mice in each was fasted overnight prior to the test but water was supplied *ad libitum*. Group I was maintained as normal control which was

given with 3% Tween 80 (10 ml/kg p.o.) only once daily for 7 days, group II received diazepam (2 mg/kg, i.p.) 30 min before the test. Groups III, IV and V were treated with different doses of ALETD i.e., 100, 200 and 400 mg/kg, p.o. respectively once daily for 7 days. On 7th day 60min after administration of the vehicle or extract and 30min of standard each mouse was placed in the center of open field arena and the following parameters were recorded during a test session of 5 min.

- Total locomotion (number of units entered on the floor)
- Central locomotion
- Rearing frequency (number of times the animal stood on its hind legs)
- Immobility time

7.3 Light-Dark Transition test model[18-20]: The light-dark apparatus consists of two-compartment chamber (40×60×20cm/h) comprising of a brightly illuminated area (40×40 cm) and a dark area (40×20 cm) separated by a wall with a round hole (7 cm diameter) will be used. Albino mice (18-22 g) of either sex were divided into 05 groups of 06 mice in each was fasted overnight prior to the test but water was supplied *ad libitum*. Group I was maintained as normal control which was given with 3% Tween 80 (10 ml/kg p.o.) only once daily for 7 days, group II received diazepam (2 mg/kg, i.p.) 30 min before the test. Groups III, IV and V were treated with different doses of ALETD (100, 200 and 400 mg/kg, p.o.) respectively once daily for 7 days. On 7th day 60 min after administration of the vehicle or extract and 30 min of standard each mouse was placed in the center of open field arena and the following parameters were recorded during a test session of 5 min. At the starting of the experiment, the mouse was placed in the illuminated part of the cage. The following parameters were recorded during the test session of 5 min.

- Latency to the first crossing into the dark compartment
- Number of crossings between the light and dark areas
- Total time spent in the illuminated part of the cage

8. Statistical analysis: The results obtained with various experiments were subjected to statistical analysis by using One-way ANOVA followed by ***Dunnett*** test to assess the significance difference if any among the groups and $P<0.05$ was considered as significant.

RESULTS

1. Acute toxicity studies: An acute toxicity study of ALETD was determined in mice, as per OECD guidelines No. 423. The extract was administered orally to different groups of mice at different dose levels and extract produced no mortality up to 2000 mg/kg. So 1/5th, 1/10th, and 1/20th of LD_{50} doses were selected for the present study.

2. Elevated Plus Maze Model (Exteroceptive behavioral model): Diazepam has long been reported for its anxiolytic activity in mice with the EPM model. In our study also, a significant anxiolytic effect was recorded with diazepam as increased number of entries in to open and decreased number of entries in to closed arms and with increased time spent in open and central platform but not in closed arms. When different doses of ALETD i.e., 100, 200 and 400 mg/kg i.p. were administered daily once for 7 days, it was found that the extract had increased the number of entries and time spent in the open arm, central platform and decreased the number of entries and time spent in closed arm as compared to control group and exhibited statistically significant activity. No significant effect was observed with total number of entries with all doses.

Table - 1
Effect of ALETD on Elevated Plus Maze in mice.

Groups No.	Treatment	Dose (Per kg)	No. of entries in 5 min session		Time spent in 5 min session		Total No of entries
			Open arm Mean ± SEM	Closed arm Mean ± SEM	Open arm Mean ± SEM	Closed arm Mean ± SEM	Mean ± SEM
Group I	Control (3% Tween 80)	10 ml p.o.	3.33±0.42	16.66± 0.49	26 ± 0.36	16.66±0.49	19.66±0.33
Group II	Diazepam	2 mg i.p.	8.66±0.49**	2.66±0.21**	173±0.85**	2.66±0.21**	11.33±0.66**
Group III	ALETD	100 mg p.o.	5.83±0.75**	14.16±0.30**	45.33±2.56**	13.66±0.66**	20±0.25ns
Group IV	ALETD	200 mg p.o.	7.5±0.42**	10.66±0.33**	75.33±3.19**	12±0.57**	18.16±0.54ns
Group IV	ALETD	400 mg p.o.	7.83±0.30**	4.66±0.42**	135.5±3.31**	5.16±0.60**	12.5±0.42 **
One-Way ANOVA		F DF	28.456 29	268.14 29	664.51 29	121.75 29	76.831 29

n=6.Significance at P<0.05, <0.01** and ns-not significant.*

Open-Field Test: Diazepam has long been reported for its anxiolytic activity in mice with the OFT model. In our study also, a significant anxiolytic effect was recorded with diazepam as increased number of total locomotion, central locomotion and decreased number of rearings immobility time. Different doses i.e., 100, 200 and 400 mg/kg of ALETD were subjected for anxiolytic activity using open-field test. These doses when administered orally daily once for 7 days, a significant effect on total locomotion, central locomotion, rearings and immobility time were observed.

Table - 2
Effect of ALETD on Open Field Test in mice.

Groups No.	Treatment	Dose (Per kg)	Total locomotion Mean ± SEM	Central locomotion Mean ± SEM	Rearings Mean ± SEM	Immobility time Mean ± SEM
Group I	Control (3% Tween 80)	10 ml p.o.	101.16±1.27	13.16±0.30	28.5±0.99	19.5±0.42
Group II	Diazepam	2 mg i.p.	210.5±2.20**	46.66±0.42**	7.33±0.49**	7.33±0.42**
Group III	ALETD	100 mg p.o.	161.16±7.52**	27.83±1.62**	22±0.81**	13.16±0.98**
Group IV	ALETD	200 mg p.o.	190.66±4.80**	34.83±0.83**	14.66±0.80**	9.5±0.42**
Group IV	ALETD	400 mg p.o.	206.33±7.18**	39.66±0.49**	13.83±1.30**	8.5±0.42**
One-Way ANOVA		F DF	73.77 29	212.64 29	78.488 29	71.85 29

n=6.Significance at P<0.05, <0.01** and ns-not significant.*

4. Light-Dark Transition Test (LDT): These three different doses of ALETD (100, 200 and 400 mg/kg) when administered orally daily once for 7 days, produced an increase in number of crossings and time spent in light box and decrease in the number of rearings in light compartment. Standard drug diazepam (2 mg/kg) had exhibited significant anxiolytic activity.

Table - 3
Effect of ALETD on Light-Dark Transition Test in mice.

Groups No.	Treatment	Dose (Per kg)	Latency Mean ± SEM	Number of crossings in 5min session Mean ± SEM	Time spent in L. Box 5min session Mean ± SEM	No of Rearings Mean ± SEM
Group I	Control (3% Tween 80)	10 ml p.o.	10.5±0.56	7.5±0.50	97±0.44	12.83±0.54
Group II	Diazepam	2 mg i.p.	27.5±0.71**	11.66±0.55**	162.33±2.49**	1.83±0.30**
Group III	ALETD	100 mg p.o.	17.33±0.33**	12±0.57**	124.66±5.67**	7.5±1.05**
Group IV	ALETD	200 mg p.o.	19.66±1.30**	10.33±0.21**	151.33±2.75**	3.16±0.40**
Group IV	ALETD	400 mg p.o.	22.66±0.55**	10.16±0.47**	153.3±3.51**	2.16±0.30**
One-Way ANOVA		F	67.308	13.512	60.929	62.303
		DF	29	29	29	29

DISCUSSION

The etiology of most anxiety disorders are not fully understood, but various studies has shown the involvement of GABAergic serotonergic neurotransmission in etiology, expression and treatment of anxiety. The adrenergic and dopaminergic systems have also been shown to a role of in anxiety[21].

Serotonergic hypothesis of fear or anxiety behavior proposes that in stressonergic or threating situations the serotonergic system activity increases where as the reduction of serotonergic systems exerts anxiolytic like effects[22].

The EPM test is a well-established animal model for testing anxiolytic as well as nootropic drugs irrespective of parameters observed[23,24], using diazepam (2mg/kg), a standard anxiolytic drug. The EPM test is based on a premise where the exposure to an EPM evoked approach-avoidance conflict that was considerably stronger than that evoked by the exposure to an enclosed arm[25]. The decreased aversion to the open arm is the result of anxiolytic effect, expressed by the increase in time spent and total number of entries in to the open arms. The animals treated with ALETD had increased the time spent and number of entries in to the open arms with a decreased in total number of entries into the closed arms.

In the OFT, the confrontation with the situation induces anxiety behavior in rodents. The anxiety behavior is triggered by two factors, i.e., individual testing as the animal was separated from its social group and agoraphobia, as the arena is very large, relative to the animals breeding or the natural environment. In such situations rodents show thigmotaxic behavior identified by spontaneous preference to the periphery of the apparatus and reduced ambulation[17]. Anxiolytic treatment decreases this anxiety-induced inhibition of exploratory behavior. In all the doses of ALETD shown more profound effects on total locomotion, central locomotion and rearings than control group.

The **LDT** may be useful to predict the anxiolytic like activity of drugs in mice. It has the advantages of being quick and easy to use without food and water deprivation prior training of animals and natural stimuli are used. Transitions have been reported to be an index of activity exploration because of habituation over time and the time spent in each compartment to be a reflection of aversion[25].

In LDT test, the apparatus contains two compartments i.e., light and dark. Animals always try to spend more time in dark compartment because of fear about new environment. In this model, four behavioral events were observed i.e. latency, number of crossings to light compartment, time spent in light box and

number of rearings in light box. In this study all the doses of ALETD had significantly increased the time spent in light compartment along with reduced time spent in dark compartment, number of crossings and decreased the number of rearings in light compartments, indicating that extract had produced significant anxiolytic effect as compared with control.

It is well known that BZDs have sedative, anticonvulsant, anxiolytic and hypothermic effect. Anxiolytics are known to exert pharmacological action by causing an increase in GABA content in the cerebral hemisphere[26, 27].

Several lines of evidence show that natural and synthetic flavonoids are potent anxiolytic agents without sedative, myorelaxant or amnestic effects. It is known the participation of GABA in these effects[28].

Earlier reports on the chemical constituents of the plants and their pharmacology suggest that plants containing flavonoids and tannins possess activity against many CNS disorders[29]. Phytochemical tests of ALETD revealed the presence of flavanoids and tannins. It might be possible that the mechanism of anxiolytic action of ALETD could be due to the binding of any of these phytochemicals to the $GABA_A$-BZD complex. In support of this, it has been found that flavones bind with high affinity BZD site of the $GABA_A$ receptors[30]. The plant *Tabernaemontana divaricata* (Linn.) R. Br. also contains flavones which is seems to be responsible for its anxiolytic activity. So the anxiolytic activity of ALETD might be involved in the GABAergic transmission or due to its mixed aminergic potentiating effect.

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