

Anxiolytic and Anticonvulsant Activity of Aqueous Extract of Stem Bark of *Erythrina variegata* in Rodents

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Abstract: *Erythrina variegata* and some other species of genus *Erythrina* are known to act on central nervous system. In India, *Erythrina variegata* is the species generally used by the traditional practitioners of south India for the treatment of mental illness. The anxiolytic effect of aqueous extract (100, 200 and 400 mg/kg) was evaluated by using elevated plus-maze (EPM) model, light-dark transition (LDT) model and open field test (OFT), while Pentylene tetrazole (PTZ), Maximal electroshock (MES) induced convulsions models were used to assess anticonvulsant activity in male Swiss albino mice. Pretreatment with aqueous extract and estimation of GABA in rat brain tissues also performed to study the effect of aqueous extract (100, 200 mg/kg) on GABA levels of brain. Aqueous extract showed significant anxiolytic and anticonvulsant activity and also showed significant modulation of GABA levels in cerebellum and also in whole brain other than cerebellum.

Key words: *Erythrina variegata*; Anxiolytic; Anticonvulsant; GABA

1. Introduction

Anxiety affects one-eighth of total population of the world and become a very important area of research interest in psychopharmacology during this decade (Hwa et.al., 2005). Currently, the most widely prescribed medications for anxiety disorders are benzodiazepines. However, the clinical uses of benzodiazepines are limited by their side effects such as psychomotor impairment, potentiation of other central depressant drugs and dependence liability. Therefore, the development of new medications processing anxiolytic effect without the complications of benzodiazepines would be of great importance in

the treatment of anxiety related disorders (Emamghoreishi et.al., 2005).

Epilepsy is the most common neurological disorder of the brain and is characterized by recurrent unprovoked seizures. The established antiepileptic drugs produce adverse effects such as ataxia, hepatotoxicity and megaloblastic anaemia (Shindikar et.al., 2006). The use of herbal medicines by physicians in Europe and Asia, exploring their traditional remedies to find a suitable cure of these 'mind affecting diseases' and herbal medicines are often considered to be gentle and safe alternative to synthetic drugs (Rabbani et.al., 2004; Marjan et.al., 2007).

At least 110 species of the genus *Erythrina* have been identified and many of them are native to the American continent. *Erythrina* plants produce alkaloids, flavonoids and terpenes and are also commonly used in folk medicine due to their tranquillizing effects (Ribeiro *et al.*, 2006).

Erythrina variegata belongs to the plant family Fabaceae. A wide spectrum of biological activities has been reported for different parts of the plant. Further folkore medicine suggests that *Erythrina variegata* barks acts on the central nervous system so as diminish or abolish its function. However there was lack of scientific data regarding its effect on the central nervous system.

Hence the present study was designed to evaluate the anxiolytic and anticonvulsant activity of aqueous extract of stem bark of *Erythrina variegata* and also to study the effect on GABA levels of the rat brain as this neurotransmitter plays an important role in the pathogenesis of anxiety and epilepsy.

2. Materials and methods

2.1. Drugs and chemicals

Phenytoin (Sun Pharmaceuticals India.Ltd, Halol, India). Diazepam (Ranbaxy Laboratories Ltd, New Delhi, India), Pentylene tetrazole (Sigma-Aldrich, St.Louis, USA) were used for the study. All the solvents used for the extraction process are of Laboratory grade and they are purchased from local firms.

2.2. Plant material and preparation of extract

The stem bark of *Erythrina variegata* was supplied and authenticated by Dr. Siddamallayya N, Regional Research Institute (RRI), Bangalore. The shade dried plant material was powdered. The coarse powder was subjected to successive extraction with petroleum ether, alcohol (70%) in soxhlet apparatus and the marc obtained after alcoholic extraction was macerated with chloroform water to obtain an aqueous extract.

2.3. Phytochemical investigation

The aqueous extract of *Erythrina variegata* (AQEEV) was subjected to preliminary qualitative investigations (Khandelwal.,1996.).

2.4. Experimental animals

Swiss albino mice of either sex (18-25g) was procured from Bioneds, Bangalore and all were acclimatized for 7 days in the animal house of P.E.S. College of Pharmacy, Bangalore. Male Wistar albino rats (120-150g) were obtained from National Toxicology Centre, Pune and were acclimatized at the animal house of Poona College of Pharmacy, Pune. All the animals were maintained under standard conditions, i.e. room temperature $26 \pm 1^{\circ}$ C, Relative humidity 45-55% and 12:12 h light- dark cycle. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of PES College of Pharmacy, Bangalore and all the experiments were conducted

according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

2.5. Acute toxicity studies

Acute oral toxicity of aqueous extract of stem bark of *Erythrina variegata* was determined by using female, nulliparous and non pregnant mice weighing 18-22 g. The animals were fasted for 3 hrs prior to the experiment. Up and down procedure OECD guideline no. 425 was adopted for toxicity studies. Animals were administered with single dose of extract and observed for their mortality during 48 hours study period (short term) toxicity. LD₅₀ was calculated as per OECD guidelines 425 using AOT 425 software (OECD guidelines no.425, 2001).

2.6. Pharmacological experiments

2.6.1. Anxiolytic activity

(i) Elevated Plus Maze model

The Elevated plus-maze test is described elsewhere (Hogg, 1996; Rodger *et al.*, 1997). The apparatus comprises of two open arms (35 cm × 5 cm) and two closed arms (30 cm × 5 cm × 15cm) that extend from a common central platform (5 cm × 5 cm). The floor and walls of the closed arms is wooden and painted black.

The entire maze is elevated to height of 50 cm above the floor level (Lister, 1987). Mice of 18-22 were housed in pair for 10 days prior to testing in the apparatus. During this time the mice are handled by the investigator on alternate days to reduce stress. A group consists of 6 mice for each dose. One hour after the Vehicle/Standard/Extract treatment, each mouse was placed in the center of the maze facing one of the open arms. During a five minutes test period the following measures were taken: the number of entries into and the time spent in the open and the closed arms, time spent in central zone and rearing. The procedure was conducted preferably in a sound attenuated room.

(ii) Open Field

Spontaneous motor activity was evaluated in open field test (Bhattacharya *et al.*, 1997). The open field apparatus is made up of plywood and consisted of square 56 cm x 56 cm. The entire apparatus was painted black and 6mm thick white lines divided the floor into 16 square of identical dimension.

Open field was lighted by 40 W bulb focusing on to the field from the height of about 100cm. The entire room, except the open field was kept dark during the experiment. One hour after Vehicle/Standard/Extract treatment each animal was placed at one corner of the apparatus and the following behavioral aspects were noted in the next 5min.

- a) Latency: Time taken by animal to leave square in which it was placed.
- b) Time taken to enter central compartment.
- c) Total locomotion and central locomotion.

d) Ambulation: Number of squares passed by animal.

e) Rearing: Number of times animal stood on its hind leg.

(iii) Light-Dark model

Natural aversion of animal for brightly lit places was evaluated in light-dark transition model (Crawley and Goodwin, 1980). Light dark box is a rectangular box of 46 x 27x 30cm (l x b x h), which is divided into 2 compartments. 1/3rd is for the dark compartment and 2/3rd is served as light compartment. One hour after the Vehicle/Standard/Extract treatment, the mouse were placed individually in the light compartment and observe for a period of 5 min. Parameters like total locomotion in light zone, number of rearing, time spent in light and dark zones and number of crossings/entries in the each zone were observed during this period.

2.6.2. Anticonvulsant activity

(i) PTZ (Pentylene tetrazole) induced convulsions

Effect of AQEEV was tested against chemically induced convulsions (Khosla and Pandhi, 2001). Male albino mice weighing between 20-25g were divided into five groups each comprising of six animals. One hour after Vehicle/Standard/Extract treatment, PTZ (Pentylentetrazole 80 mg/kg s.c.) was administered to all groups of mice. Each animal was placed in to individual plastic cage and were observed initially for 30min and later up to 24 hrs. The following parameters were recorded during test session of initial 30min and at the end of 24 hrs; Latency (onset of clonus), Onset of tonic convulsions and status of animal after 24 hrs.

(ii) Maximal Electro Shock (MES) induced convulsions

Effect of AQEEV was tested against electrically induced convulsions (Swinyard et.al., 1952). Briefly, a 60mA current was delivered transauricularly for 0.2 sec in mice via small alligator clips attached to each pine by electroconvulsometer, this current intensity will elicit complete tonic extension of the hind limbs in control mice.

For recording various parameters, 30 mins after the Vehicle/Standard/Extract treatment the mice were placed in a rectangular plastic cage with an open top, permitting full view of animal's motor responses to seizure in the pilot study of various phases of convulsions, viz. tonic flexion, extension, stupor and mortality due to convulsions are selected as the parameters (Vogel and Vogel., 2000.).

2.6.3. Estimation of GABA levels in rat brain

Effect of AQEEV on GABA levels in brain was also studied (Suher et.al., 2000). Briefly, after three days treatment with aqueous extract/standard, animals were killed by euthanasia and the body was exposed to a microwave irradiation for 4 sec.

The brain was rapidly removed and the cerebellum, the tissue other than cerebellum were dissected on an ice-cold petri dish. The tissues were placed after weighing

in pre-cooled 100 ml plastic tubes. Ice-cooled 0.1M perchloric acid (10 ml) which contained valine (internal standard) at a concentration of 15 µg/ml was added to the tissue. The tissues were homogenized for one minute during which the tube was embedded in an ice bath and then centrifuged at 5,000 rpm for 10 min at 4°C. The supernatants were stored at -20°C until assayed. Dansylation reaction was induced. Dansylation was carried out by adding 100 µl of each supernatant of the samples or the standards to a micro-tube containing 100 µl of 0.1M potassium carbonate solution. These solutions were mixed using vortex and then centrifuged using microcentrifuge at 10,000 rpm for 10 min. 100 µl of each supernatant was transferred into a pyrex tube containing 100 µl of 0.1M sodium hydrogen carbonate solution, to which 400 µl of working dansyl chloride solution (1.25 mg/ml anhydrous acetone) was added. The tubes were shaken for 30 sec using vortex and then incubated at 90°C in benchtop oven for 30 min.

The tubes were not capped during the incubation to allow most of the solvent to be evaporated. This did not appear to adversely affect the progress of the dansylation reaction and served to concentrate the samples.

After getting the tubes out of the oven, they were left to cool down to room temperature and the dansylated derivatives were transferred to 1.5 ml microtubes and stored at -20°C until assayed. C8 reversed-phase HPLC columns (5 µm, 250 x 3.2 mm) were used to resolve and quantify the samples. The HPLC mobile phase consisted of deionized helium degassed water-acetonitrile (HPLC grade) mixture (65:35 v/v) containing 0.15% v/v phosphoric acid. The flow rate was kept at 0.5 ml/min.

The detector excitation was at 333 nm and emission at 532 nm. 25 µl of the dansyl derivative of the GABA samples were transferred to HPLC micro-sample vials and injected into the column. Retention time of GABA and internal standard were determined.

The peak ratios of the samples were calculated with reference to the internal standard. GABA levels were expressed as ng/g of tissue.

2.6.4 Statistical Analysis:

Values were expressed as mean ± SEM from 6 animals. Statistical difference in mean was analyzed using one way ANOVA (analysis of variance) followed by followed by Tukey-kramer test and p<0.05 was considered significant. All the analysis was made using the INSTAT statistical software package.

3. Results

3.1 Phytochemical investigation

This study revealed that aqueous extract of *E.variegata* contains alkaloids, flavonoids, terpenes and glycosides as major constituents.

3.2 Acute toxicity

No mortality was observed upto 2000 mg/kg dose. Hence aqueous extract of *E.variegata* was found to be safe upto 2000 mg/kg.

3.3 Pharmacological behavioral screening

3.3.1 Anxiolytic activity

(i) Elevated plus-maze (EPM) behavior

The aqueous extract at medium (200 mg/kg) and high (400 mg/kg) dose levels showed significant increase in the number of entries into open arm and time spent in open arm compared vehicle control group and these results were comparable with the reference drug diazepam.

On the other hand the effect of low dose (100 mg/kg) on time spent in open arm was significant but not on number of entries into open arm and these results were shown in **Table 1**.

(ii) Open-Field Test

Significant increase in the total locomotion, time spent in central compartment and ambulation was observed with 200 mg/kg and 400 mg/kg doses of extract but the effect of low dose (100 mg/kg) of AQEEV on these parameters was insignificant. The other parameters including rearing were not significantly modified by all three doses used. These effects were shown in **Table 2**.

(iii) Light-Dark Transition model

Aqueous extract (100, 200 and 400 mg/kg) had increased the latency to the first crossing to the dark zone. AQEEV at medium (200 mg/kg) and high (400 mg/kg) doses showed significant increase in the time spent in light zone, total locomotion time in light zone along with number of crossings between light and dark zones compared to control group which indicates the reduced fear of animal. These effects were shown in **Table 3**.

3.3.2 Anticonvulsant activity

(i) PTZ (Pentylene tetrazole) induced convulsions

Pretreatment with aqueous extract (100, 200, 400 mg/kg) showed significant alteration in the onset of tonic - clonic seizures compared to control group animals and also showed protection against PTZ induced mortality in a dose dependent manner but the extract was failed to abolish the seizures. These effects were shown in **Table 4**.

(ii) MES (Maximal Electro Shock) induced convulsions

Aqueous extract (200 and 400 mg/kg) of *E. variegata* significantly increased the onset of clonus and decreased the duration of extensor phase along with abolition of flexion phase of grandmal seizures. Extract also showed protection against mortality in a dose dependent manner.

Low dose (100 mg/kg) of AQEEV had not shown significant effect on these parameters. These effects were shown in **Table 5**.

3.3.3 Estimation of GABA levels in rat brain

Pretreatment with AQEEV (100 and 200 mg/kg) for three days had increased the GABA levels in cerebellum and also in whole brain other than cerebellum. Similar effect was observed with diazepam (2 mg/kg). However, the effect of AQEEV was less than diazepam (2 mg/kg). These effects were shown in **Table 6**.

4. Discussion and Conclusion

In our study, the anxiolytic effect of aqueous extract of stem bark of *Erythrina variegata* was studied in elevated plus-maze, open field test and light-dark transition models. These are the classical animal models to evaluate anxiolytic effect of a compound. Diazepam, a standard anxiolytic used clinically and is also employed in behavioral pharmacology as a reference compound for inducing anxiolytic-like effects, even when the compound being screened does not act via benzodiazepine receptors (Soderphalam et.al., 1989). Measuring anxiety like behavior in mice has been mostly undertaken using a few classical animal models of anxiety such as the Elevated Plus Maze (EPM) Light Dark Model (LDM) and Open Field Test (OFT). All these procedures are based upon the exposure of subject to unfamiliar aversive place (Belzung and Griebel., 2001.).

Pretreatment with aqueous extract stem bark of *Erythrina variegata* showed significant anxiolytic activity in EPM by increasing the number of entries and time spent in open arm, and in LDM by increasing the time spent in light zone and number of crossings between light and dark zones, and in OFT model by increasing the time spent in central compartment and ambulation.

Epilepsy is one of the most common serious neurological conditions. Seizure refers to a transient alteration of behaviour due to disordered, synchronous and rhythmic firing of populations of brain neurons (Noel et al.,2008.)

For measuring the anticonvulsant activity in mice has been mostly undertaken using a few classical animal models such as the PTZ induced convulsions are assumed to identify anticonvulsant drugs effective against generalized tonic-clonic partial seizures and MES induced convulsions are assumed to identify generalized clonic seizures respectively (Madhavan et al., 2008; Cristiana et.al., 2006; Atif et al.,2005.). Studies have proved that the agents which increase the brain GABA content and administration of centrally active GABA mimetic agents have been used as an effective therapeutic approach for treatment of epilepsy Hence to see the effect of the extract on

GABA levels different parts of the brain, the animals are treated with the extracts and GABA levels are estimated by HPLC method (Suher et.al., 2000.).

Aqueous extract of stem bark of *Erythrina variegata* showed significant anticonvulsant activity by increasing the onset of clonus and tonic phases in PTZ induced convulsion model, and in MES induced convulsion model by increasing the duration of tonic extensor phase.

GABA appears to play an important role in the pathogenesis of several neuropsychiatric disorders. Many of the traditional agents used to treat psychiatric disorders are known to act, at least in part, by enhancing GABA activity, while some of the newer agents may exert their therapeutic effects exclusively via GABAergic actions. In our present study, three days treatment with AQEEV and further GABA

estimation in brain showed significant enhancement of GABA levels in cerebellum and whole brain other than cerebellum compared to control group.

These findings suggests that the aqueous extract of stem bark of *Erythrina variegata* possess significant anxiolytic and anticonvulsant activity and modulation of GABA levels in brain might be the reason for anxiolytic and anticonvulsant activities.

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Table 1: Effect of AQEEV on elevated plus maze model in mice

TREATMENTS	Elevated plus maze model					
	NUMBER OF ENTRIES (COUNTS/5MIN)		TIME SPENT IN (SEC/5MIN)		TIME SPENT IN NEUTRAL ZONE (COUNTS/5MIN)	REARING (Counts/5min)
	OPEN ARM	CLOSED ARM	OPEN ARM	CLOSED ARM		
Control (3% tween 80)	3.66±0.55	13.16±1.22	29.16±7.94	218.16±14.28	52.66±7.67	4.83±0.47
Diazepam (2 mg/kg)	12.16±1.04 ^{**}	15.16±1.04	137.16±9.33 ^{***}	142.83±7.71 ^{***}	20.0±3.49 ^{**}	7.33±0.55
AQEEV (100 g/kg)	5.66±1.02	13.16±1.01	76.5±7.89 [*]	183.83±12.39	39.33±7.11	5.16±0.94
AQEEV (200mg/kg)	10.33±1.14 [*]	4.16±1.16	129.5±11.00 ^{***}	148.66±8.60 ^{***}	21.83±5.16 [*]	5.83±1.01
AQEEV (400mg/kg)	10.66±1.35 ^{**}	13.5±1.05	133.66±9.97 ^{***}	145.16±8.07 ^{***}	21.16±5.12 ^{**}	5.83±0.79

Values are expressed as mean ±SEM from 6 mice. $P < 0.05^*$, $< 0.01^{**}$ and $< 0.001^{***}$ as compared to control group

Table 2: Effect of AQEEV on Open Field Test in mice

Treatments	Time spent in square where it is placed (Sec/5min)	Time taken to enter central compartment (Sec/5min)	Total locomotion (sec/5min)	Central locomotion (sec/5 min)	Ambulation (Counts/5min)	Rearing (Counts/min)
Control (3% tween 80)	9.33±1.78	44.50±8.08	164.16±19.52	11.16±2.52	61.83±6.95	7.33±1.60
Diazepam (2 mg/kg)	3.83±0.79*	24.16±4.23	271.66±8.77***	44.16±6.43**	173.33±16.20***	14.66±1.54*
AQEEV (100 mg/kg)	6.66±1.58	32.50±5.01	206.33±22.76	26.16±5.78	111.66±11.39	8.83±0.90
AQEEV (200 mg/kg)	4.50±0.99	26.16±4.61	262.50±12.66**	40.16±4.59*	168.50±14.32***	11.50±0.80
AQEEV (400 mg/kg)	4.16±0.70	23.33±6.19	263.83±11.28**	39.66±7.88*	161.16±21.85**	11.16±1.49

Values are expressed as mean ±SEM from 6 mice. $P < 0.05^*$, $< 0.01^{**}$ and $< 0.001^{***}$ as compared to control group

Table 3: Effect of AQEEV on Light-dark transition model

Treatments	Latency (sec)	Time spent in dark zone (sec/5 min)	Time spent in light zone (sec/5 min)	No. of crossings (Counts/5min)	Total locomotion time in light zone (sec/5min)	Rearing (Counts/5min)
Control (3% tween 80)	10.16±1.86	206.83±9.30	93.16±9.30	4.83±0.70	60.33±6.31	7.66±1.40
Diazepam (2 mg/kg)	19.16±3.13	107.83±8.91***	192.16±8.91***	11.33±0.98*	174.33±10.51***	9.66±0.95
AQEEV (100 mg/kg)	13.33±1.94	170.16±16.22	129.83±16.22	7.16±1.37	106.33±16.59	8.83±1.30
AQEEV (200 mg/kg)	18.33±2.98	141.16±18.60*	158.83±18.60*	9.16±1.62	141.83±19.22**	8.83±0.87
AQEEV (400 mg/kg)	21.66±3.20	103.66±14.07***	196.33±14.07***	12.83±1.62**	177.33±15.35***	9.16±1.30

Values are expressed as mean ±SEM from 6 mice. $P < 0.05^*$, $< 0.01^{**}$ and $< 0.001^{***}$ as compared to control group

Table 4: Effect of AQEEV on PTZ-induced convulsions model

Treatments	Latency (onset of Clonus) (Sec/30min)	Onset of Tonic (Sec/30min)	% Protection against seizures	% Protection against mortality
Control (3% tween 80)	51.83±5.48	369.16±23.64	0	0
Diazepam (5 mg/kg)	No clonus	No tonus	100	100
AQEEV (100 mg/kg)	110.66±11.23*	440.16±40.97	0	16.66
AQEEV (200 mg/kg)	155.83±17.53***	503.16±27.30*	0	50
AQEEV (400 mg/kg)	211.16±16.50***	570.16±39.64***	0	66.66

Values are expressed as mean±SEM from 6 mice. $P<0.05^*$ and $<0.001^{***}$ as compared to control group

Table 5: Effect of ALEEV and AQEEV on MES induced convulsions

Treatments	Duration of tonic flexion (Sec/30min)	Duration of tonic extensor (Sec/30min)	Latency (onset of clonus) (Sec/30min)	% Protection against mortality (24h)
Control (3% tween 80)	Not observed	15.33±0.55	2.16±0.47	0
(Phenytoin (25 mg/kg)	6.83±0.79***	Not observed***	13.16±1.35***	100
AQEEV (100 mg/kg)	Not observed	13.16±1.19	4.16±0.79	33.33
AQEEV (200 mg/kg)	Not observed	9.66±1.02**	8.16±1.49**	50
AQEEV (400 mg/kg)	Not observed	7.66±1.08***	9.16±1.01***	83.33

Values are expressed as mean±SEM from 6 mice. $P<0.01^{**}$ and $<0.001^{***}$ as compared to control group

Table 6: Effect of AQEEV on GABA levels in brain.

Treatments	In cerebellum (ng/g of tissue)	In whole brain other than cerebellum (ng/g of tissue)
Control (3% tween 80)	423.83±16.61	2228.83±48.01
(Diazepam 2 mg/kg)	1929.13±51.86***	5946.74±56.46***
AQEEV (100 mg/kg)	772.90±25.05***	3248.74±60.03***
AQEEV (200 mg/kg)	1299.74±73.61***	4835.36±96.72***

Values are expressed as mean ±SEM from 6 mice. $P < 0.001$ *** as compared to control group

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