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Evaluation of Anxiolytic Activity of Hydroalcoholic Extract of *Pimenta dioica* (Linn.) Merrill Leaves on Swiss Albino Mice

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ABSTRACT

Anxiety is a feeling of apprehension and fear and a group of mental disorders characterised by physical symptoms as palpitations, sweating and feelings of stress. Globally, the total number of people with anxiety disorder is estimated to be 264 million in 2015, equivalent to 3.6% of the world's population. The aim of this study was to evaluate the anxiolytic activity of hydroalcoholic extract of *Pimenta dioica* leaves (HAEPDL) (Family: *Myrtaceae*) on swiss albino mice. *Pimenta dioica* has been used as an important spice and also known for its medicinal qualities. The acute toxicity studies revealed that HAEPDL did not produced any abnormal behaviour or mortality even at the highest permissible dose of 2000 mg/kg in mice. The corticosterone is used to induce anxiety in dose depended manner. The anxiolytic activity of HAEPDL is evaluated using Locomotor activity, Elevated Zero Maze, Elevated Plus Maze, Dark-light compartment and Hole Board. After treated with HAEPDL shows significant reduction in anxiety activity with the extracts at 200mg/kg and 400mg/kg doses. The study concludes HAEPDL are suggestive of anxiolytic activity in dosages of 200 and 400 mg/kg when compared with the standard dose of Diazepam.

INTRODUCTION

Anxiety disorder is divided into generalized anxiety disorder, phobic disorder and panic disorder, each has its own characteristics and symptoms and they require different treatment. Anxiety is the most common psychiatric illness which is considered as a diffuse, unpleasant, elusive sense of apprehension[1]. Anxiety is tension or apprehension which is a normal response to certain situation in life. When it becomes excessive and disproportionate to the situation, it becomes a disorder that needs treatment. Anxiety disorders are a group of mental disorders characterized by significant feelings of anxiety and fear[2]. Some common symptoms are headache, perspiration, palpitations, tightness in the chest and mild stomach distress[3]. Anxiety can be either a short term "state" or a long term "trait". Whereas trait anxiety represents worrying about future events[4]. Anxiety disorders are partly genetic but may also be due to drug use including alcohol, caffeine and benzodiazepines (which are often prescribed to treat anxiety), as well as withdrawal from abuse of drugs. They often occur with other mental disorders, particularly bipolar disorder, major depressive disorder or certain personality disorders. There are many plants that show anxiolytic activity using experimental laboratory animal with different animal models. A good proportion of the world depends mostly on herbal drugs for health needs compared to synthetic drug. Drug derived from plants are generally considered to be less toxic with few side effects. Hence the herbal drug namely *Pimenta dioica* (L) Merr. leaves are used to treat anxiety. It possesses an aromatic taste and flavour resembling a mixture of cinnamon, cloves and nutmeg, hence the name allspice[5]. In India, the leaves of *Pimenta dioica* are used to flavour rice, aromatic stimulant and as an adjuvant to tonics and purgatives[6, 7] and are used in edible food plants, which may be used as iron rich leafy condiment in our country[8].

The other therapeutic properties of *Pimenta dioica* are anaesthetic, analgesic, antimicrobial, antioxidant, antiseptic, carminative, muscle relaxant, rubefacient, stimulant, tonic and also reported to have good antioxidant activity. Also help in cases of depression, nervous exhaustion, tension, neuralgia and stress and is used as natural repellent. It is also used in perfumes, aftershaves and commercial food flavouring. The present study was conducted to find out anxiolytic activity of hydroalcoholic extract of *Pimenta dioica* (Linn) Merr. leaves in mice model.

MATERIALS AND METHODS

Preparation of plant extract

The leaf of *Pimenta Dioica* was collected from Thalayolaparambu, Kottayam, Kerala. The specimen was identified and authenticated by Prof. P. Jayaraman, Ph. D. Director, Institute of Herbal Botany, Plant anatomy and research centre, Chennai, Tamil Nadu, India. All procedure described were reviewed and approved by the Institutional Animal Ethical Committee. Leaves are washed and cleaned in running water and shade dried at room temperature. The dried leaves are subject to size reduction to a coarse powder by using dry grinder and passed through sieve. This powder was packed into soxhlet apparatus and extracted with ethanol at a temperature range of 55^{o}C. The extract was dried at 45^{o}C in hot air oven till semisolid to solid mass was obtained and was stored in airtight containers in refrigerator below 8^{o}C till use. The percentage yield of the hydroalcoholic extract of *Pimenta dioica* leaves was found to be 40%w/w.

Phytochemical screening

The fresh crude hydroalcoholic extract was subjected to phytochemical screening for the presence of alkaloids, tannins, flavonoids, saponins, glycosides, terpenes.

Experimental animals

The Swiss albino mice weighing 18-25gm were used for this study. The inbreed animals were procured from the animal house of C. L. Baid Metha College of Pharmacy, Thoraipakkam, Chennai-97. They were housed six per mice cage under standard laboratory conditions at a temperature 22±2°C with 12:12 hrs light and dark circle in polypropylene cages. The animals were provided with standard pellet feed (Hindustan Lever Limited, Bangalore) and drinking water was provided *ad libitum* throughout experimentation period. All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).Reg.no: 321/PO/Re/S/01/CPCSEA. The study was approved by Institutional Animal Ethical Committee (IAEC).

Experimental design

On the day of experiment the animals were divided randomly into five groups of six animals each. Anxiety is induced by corticosterone for the II, III, IV, V groups and continued for 10

days. Control animals were given distilled water orally. The last dose was given 60 min prior to behavioural testing and on 21st day sacrification of animal was done for *in vitro* studies.

Drugs and chemicals

The standard drug diazepam is obtained from Ranbaxy laboratories Ltd., chemical division, New Delhi. Corticosterone is obtained from Sigma-Aldrich.

Acute Oral Toxicity Studies

The acute toxicity was done by using OECD guidelines (organization of economic corporation and development) 423 (acute toxic class method). Adult female Swiss albino mice weighing 18-25gm were used for the study. The starting dose level of 2000mg/kg body weight p.o of HAEPDL was given. Since most of the crude extracts possess LD50 value more than 2000 mg/kg, p.o.so starting dose 2000mg/kg body weight was used. The mice were fasted overnight with water *ad libitum*. Food was withheld for further 1-2 hrs after oral administration of drugs and observed for the signs of toxicity[9].

Treatment Schedule

The swiss albino mice were grouped into five groups (n=6) out of which four groups (Groups II, III, IV, V) were induced with corticosterone 30mg/kg treatment for 10 days, the behavioural parameters were observed. After the treatment of corticosterone, the plant extract was treated to group IV, V for 10 days. Simultaneously the standard group III was treated with Diazepam (2mg/kg i.p) and group I was treated with CMC 0.1% w/v, p.o. On the last day of drug treatment, the mice were again tested for behavioural parameters. The variances in the pre and post treatments were tabulated.

Assessment of Anxiolytic activity

Locomotor activity

Actophotometer which operates on photoelectric cells is connected with a counter. When a beam of light falling on the photocell is cut off by the animal a count is recorded and displayed digitally. Each mice was placed individually in the activity cage floor for 5 min and the locomotion count was observed from the digital reading displayed in the actimeter.

Elevated zero maze

Elevated zero maze is a modification of the elevated plus maze model for testing anxiety in mice. The elevated zero maze consists of annular platform with two opposite enclosed quadrants and two open quadrants. The maze is an elevated (40cm) white or black, annular having outer diameter of 45 cm and inner diameter of 30cm.After proper treatment each mice was placed individually in the open arm facing towards the closed arm. During the 5 min experiment, the behaviour of the mice was observed and the number of entries into the open or closed arm and time spent by the mice in each of the arms were recorded.

Elevated plus maze

The plus maze apparatus consists of a two open arm (30cm x 5cm) and two closed arms (30cm x 5cm x 5cm) extending from a central platform (95cm x 5cm) and raised 50cm above floor level. After proper treatment each mouse was placed at the centre of the maze with its head facing the open arm. During the 5 min experiment, the behaviour of the mouse was observed and the number of entries into the open or closed arm and time spent by the mouse in each of the arms were recorded.

Dark-light compartment test

The apparatus used was a box with an overall dimensions of 40cm x 60cm x 20cm (length, width, height) and a grid floor composed by bars spaced 5 cm apart. The box was further divided by a barrier possessing a hole (7cm round),which mice could cross in two chambers of measured (40cm x 20cm) painted black not illuminated and (40cm x 40cm) painted while illuminated with light source. The animals were places in the middle of the light compartment, facing the doorway separating the two compartments. The behaviour of animals was noted for 5 min.

Hole board model

The hole-board apparatus consists of Perspex box (60 x 60 x 35cm) with four equidistant holes of 2cm diameter on the floor. The floor of the box was positioned 12 cm above the ground and divided into nine (20 x 20cm) squares. The animal is placed at the edge of the board. The number of line crossing, head dipping and rearing was observed for 5min.

Statistical analysis

Results are expressed as mean± SEM. The data where analysed statistically using One–way ANOVA, followed by Dennett's T-test. P<6.0 indicated statistical significance.

RESULTS

The Acute Oral Toxicity Study was done according to the OECD guidelines 423 (Acute toxic class method). A single administration of starting dose of 2000 mg/kg body weight p.o hydroalcoholic extract of *Pimenta dioica* leaves was administered to three swiss albino mice and observed for 3 days. There was no considerable change in body weight before and after treatment and no sign of toxicity was observed.

The HAEPD showed the presence of various phytochemical constituents such as tannins, alkaloids, flavonoids, terpenoids, glycosides, anthraquinones, reducing sugar.

Locomotor Activity

Corticosterone induced animals showed significant increase in locomotor activity when compared with control group animals. Diazepam (2mg/kg) administered animals showed significant decrease in locomotor activity. The plant extract treated groups exhibited significant decrease in locomotor activity when compared with corticosterone induced animals as shown in table 1.

Elevated Zero Maze

Corticosterone induced animals showed decreased no of entries and time spent when compared with control group animals. Diazepam administered animals showed significantly increased number of entries and time spend. In the plant extract treated groups, significant increase in the number of entries and time spent in open arm were observed when compared to corticosterone induced animals as shown in table 2.

Elevated Plus Maze

Corticosterone induced animal showed significant decrease in the number of open arms entries and significant increase time spent in open arms, number of closed arm entries and time spent in closed arms when compared with control group animal. Administration of diazepam significantly increased the number of open arm entries and time spent in the open arms and

significant decreases in number of closed arm entries and time spent in closed arms were observed. The plant extract treated groups exhibited significant increase in number of open arm entries and time spent in the open arms and significant decrease in number of closed arm entries and time spent in closed arms were observed as compared to corticosterone induced animals as shown in table 3.

Dark-Light compartment Test

Corticosterone induced animal showed significant decreases of latency, time spent in light box and rearing as compared to control group animals. Administration of diazepam significantly increased the latency and rearing and time spent in light box. The plant extract treated group showed significant increase in latency and time spent in the light box and rearing were observed as compared to corticosterone induced animals as shown in table 4.

Hole - board model

Corticosterone induced animals showed significant increase in head dipping and non-significant increase in line crossings were observed as compared to control group animal. Administration of diazepam significantly decreased the head dipping and line crossing. The plant extract treated group showed significant decrease in head dipping and line crossing were observed as compared to corticosterone induced animals as shown in table 5.

Table No. 1: Locomotor activity test

S No.	Groups	No of movements
1	Control	340.5 ± 1.57
2	Negative Control	$401.83 \pm 1.35^{a***}$
3	Diazepam 2mg/kg	$103.5 \pm 1.88^{b***}$
4	HAEPDL200mg/kg	231.17 ± 0.99^{ns}
5	HAEPDL 400mg/kg	$198.67 \pm 1.34^{b**}$

All values were expressed as mean ± SEM (n=6). Comparison: (a) Group I vs. Group II and (b) Group II vs. Group III, IV and V. ns - non-significant, *p<0.05, **p<0.01, ***p<0.001.

Table No. 2: Elevated Zero Maze test

S No.	Groups	No. of entries in Open Arm	Time spend in Open Arm (in seconds)
1	Control	9 ± 0.149	40.17 ± 1.59
2	Negative Control	2.33 ± 0.172^{a**}	26.5 ± 1.48^{a*}
3	Diazepam 2mg/kg	11.67 ± 0.375^{b***}	91 ± 0.99^{b***}
4	HAEPDL 200mg/kg	8.5 ± 0.175^{b*}	59.83 ± 1.58^{b***}
5	HAEPDL 400mg/kg	10.17 ± 0.267^{b**}	70.33 ± 1.39^{b***}

All values were expressed as mean ± SEM (n=6). Comparison: (a) Group I vs .Group II and (b) Group II vs. Group III, IV and V. ns - non-significant, *p<0.05, **p<0.01, ***p<0.001.

Table No. 3: Elevated Plus Maze test

S No.	Groups	No of Arm entries		Time sent in Arms (in seconds)	
		Open Arm	Closed Arm	Open Arm	Closed Arm
1	Control	10 ± 0.279	8 ± 0.149	39.83 ± 1.99	122.67 ± 1.54
2	Negative Control	5.17 ± 0.306^{a*}	15.67 ± 0.251^{a*}	12.17 ± 0.98^{a**}	221.67 ± 1.00^{a***}
3	Diazepam 2mg/kg	11.5 ± 0.312^{b**}	9.33 ± 0.272^{b**}	195.5 ± 1.43^{b***}	47 ± 1.58^{b***}
4	HAEPDL 200mg/kg	10.33 ± 0.228^{ns}	10 ± 0.236^{b*}	85.5 ± 6.09^{b***}	119.33 ± 5.01^{b**}
5	HAEPDL 400mg/kg	10.67 ± 0.251^{b*}	8.5 ± 0.204^{b**}	166 ± 1.55^{b***}	83 ± 1.32^{b**}

All values were expressed as mean ± SEM (n=6). Comparison: (a) Group I vs. Group II and (b) Group II vs. Group III, IV and V. ns - non-significant, *p<0.05, **p<0.01, ***p<0.001.

Table No. 4: Light-Dark Compartment test

S No.	Groups	Latency	Time spend in Light box (in 5 mins)	Rearing
1	Control	7.33 ± 0.33	205 ± 19.854	6 ± 0.577
2	Negative Control	4 ± 1^{a*}	144 ± 6.364^{a*}	2.33 ± 0.33^{a*}
3	Diazepam 2mg/kg	12.66 ± 0.667^{b**}	230.75 ± 19.78^{b**}	8.66 ± 1.453^{b***}
4	HAEPDL 200mg/kg	7.33 ± 0.66^{b*}	206.75 ± 3.94^{b*}	5 ± 0.577
5	HAEPDL 400mg/kg	8.33 ± 0.882^{b**}	226.25 ± 9.43^{b**}	8 ± 0.57^{b**}

All values were expressed as mean ± SEM (n=6). Comparison: (a) Group I vs. Group II and (b) Group II vs. Group III, IV and V. ns - Non-significant, *p<0.05, **p<0.01, ***p<0.001.

Table No. 5: Hole Board test

S No.	Groups	Head Dipping	Line Crossing
1	Control	14.5 ± 1.87	61.67 ± 1.00
2	Negative Control	29.5 ± 0.86^{a***}	101.17 ± 1.54
3	Diazepam 2mg/kg	18.5 ± 0.90^{b***}	75 ± 1.40^{b***}
4	HAEPDL 200mg/kg	21.83 ± 1.00	82.17 ± 1.00^{b*}
5	HAEPDL 400mg/kg	19.83 ± 1.90^{b**}	80.17 ± 0.99^{b**}

All values were expressed as mean ± SEM (n=6). Comparison: (a) Group I vs. Group II and (b) Group II vs. Group III, IV and V. ns - non-significant, *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Anxiety is closely related to fear and is a normal evolved response in both humans and animals and the physical responses are linked to the "fight-or-flight" system. The autonomic nervous system controls the fight-or-flight response in the body[10]. The etiologies of anxiety disorders are not yet fully understood. The benzodiazepines (BZDs) are the most commonly used drugs for anxiety. They are CNS depressants[11]. Medicinal plants are a good source to find new remedies for these disorders. Despite the wide spread traditional use of *Pimenta Dioica* leaves for treating various disorders, there are no reports of scientific evaluation of its anxiolytic activity. The present work demonstrates that the HAEPDL has anxiolytic activity in mice through Locomotors activity, Elevated zero maze, Elevated Plus Maze, Light Dark Compartment model and Hole Board models.

Several plants have been reported to have CNS depressant and anxiolytic activity due to the presence of triterpenoids, saponins and flavonoids. Phytochemical analysis of HAEPDL also revealed presence of triterpenoid, saponin and flavonoids. They are reported to the hypothesis that they act as benzodiazepine-like molecules. This is supported by their behavioural effects in animal models of CNS depression and anxiety.

Locomotor activity test is used to evaluate the impulsive behaviour of mice[12]. Results showed that plant extract treated mice exhibited significant decrease in locomotor activity. This indicates the anxiolytic activity of the plant extract.

Elevated Zero Maze is a behavioural test of anxiety based on the naturalistic tendency of rodents to avoid open and elevated areas[13]. Results showed that plant extracts treated mice exhibited significant increase in number of entries and time spent in open arm observed. This points to the anxiolytic activity of plant extract.

Elevated Plus Maze is used to evaluate psychomotor performance and emotional aspects of rodents[14]. Results showed that plant extract treated mice exhibited significant increase in the number of open arm entries and time spent in the open arms and significant decreases in number of closed arm entries and time spent in closed arms were observed which reflects plant's anxiolytic property.

Light Dark Test is one of the most widely used tests to measure anxiety-like behaviour in mice[15]. The test is based on the natural aversion of mice to brightly illuminated areas and on their spontaneous exploratory behaviour in response to mild stressors, such as novel environment and light. This test is also sensitive to anxiolytic drugs treatment[16]. Results showed that plant extracts treated mice exhibited significant increase in latency and time spent in the light box and rearing were observed.

Hole-Board is used to evaluate Impulsive behaviour of mice[17]. The plant extract treated group showed significant decreases in head dipping and line crossing were observed, indicates the anxiolytic activity of plant.

CONCLUSION

The results of the present study conducted on hydroalcoholic extract of *Pimenta dioica* leaves shows that the HAEPDL has significant anxiolytic activity in animal models like corticosterone induced anxiety. The HAEPDL treated group showed anxiolytic activity results as comparable with the standards. The higher dosage of 400 mg/kg exhibited a higher anxiolytic activity compared to the lower dose of 200 mg/kg. However further studies are required to know the exact molecular level mechanism of action of *Pimenta Dioica* leaves for anxiolytic use in human.

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