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## Original Research Article

Evaluation of antianxiety properties of *Piper betle* L. leaf extracts on swiss albino miceUttara Krishna<sup>1</sup>, Roopa P Nayak<sup>1,\*</sup>, Chaitra S R<sup>1</sup><sup>1</sup>Dept. of Pharmacology, Yenepoya Medical College (Deemed to be University), Mangalore, Karnataka, India

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## ABSTRACT

**Background:** Many indigenous medicinal plants with natural neuroprotective and antioxidant properties have shown to be beneficial in a variety of behavioral disorders such as anxiety disorders.

**Objective:** To screen the Ethanolic and aqueous extracts of *Piper betle* L. leaf for its antianxiety activity in Swiss Albino mice.

**Materials and Methods:** Ethanolic and aqueous extracts of *Piper betle* L. leaf [PBEE and PBAE] at doses 100 mg/kg and 200 mg/kg were compared with standard (Diazepam 1mg/kg) and control (distilled water). Six Swiss albino mice (3 each of both sexes) weighing 25-30g were used for each group. Level of anxiety was studied using the behavioural models Elevated Plus Maze [EPM] and Light Dark Arena [LDA]. Phytochemical analysis of the extracts was done and compared.

**Statistical Analysis:** Data was analyzed by one-way ANOVA followed by Tukey Kramer's multiple comparison test at P = 0.05. The results were represented as Mean ± SEM.

**Results:** PBEE 100mg/kg has shown significant increase in time spent in open arm and number of entries to open arm when compared with control in EPM model. The same dose was found to be significant for time spent in bright area and number of bright area entries in LDA model. On the other hand the PBAE doses were not significantly different from the control. Preliminary phytochemical screening showed the presence of bioactive component flavonoids in both the extracts, but saponins were absent in aqueous extract.

**Conclusion:** The ethanolic extract of *Piper betle* L. at a dose of 100mg/kg has showed significant antianxiety activity.

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## 1. Introduction

Anxiety disorders are present in 15-20% of medical clinic patients, which makes it the most prevalent psychiatric illness in the general community. Anxiety is a feeling of fear, apprehension and uncertainty. It is a distressing state of tension with an anticipation of impending danger and can be defined as a subjective sense of unease, dread, or foreboding, can indicate a primary psychiatric condition or can be a component of, or reaction to, a primary medical disease.<sup>1</sup> The current allopathic framework utilizes benzodiazepines, antidepressants and azapirones for antianxiety medication, but are having limitations like

unfavourable energy, withdrawal side effects on sudden stoppage of the medications, intellectual, and psychomotor debilitation and gastrointestinal side effects.<sup>2</sup> Thus there is a need for alternatives in the treatment of anxiety disorders such as medicinal plants and plant-based formulations which are effective, cheaper as well as with lesser risks of side effects, dependence and abuse potential.

The medicinal plant *Piper betle* L., which is commonly found in humid and tropical regions of South India, has been reported to have a neuroprotective effect in rats.<sup>3</sup> Betel leaf has various medicinal properties like antioxidant, anti-bacterial, anti-fungal, anti-inflammatory, anti-diabetic, gastroprotective, radioprotective activities and contraceptive effects as revealed from past studies. The previous studies have shown a neuroprotective action in Hydroalcoholic

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extract of *Piper betle* leaf in mice but it was not comparable to standard drug.<sup>4</sup> This could be due to the inadequate quantity of the concerned bioactive constituents in the used extract. Hence to confirm the antianxiety properties of *Piper betle* leaf, two other types of extracts viz., ethanolic and aqueous were proposed and the effects were compared.

## 2. Materials and Methods

### 2.1. Experimental animals

Adult Swiss albino mice of either sex of 3-4 months of age, weighing 25-30g were used for the study. They were housed in clean polypropylene cages under standard housing conditions with free access to standard pellet diet and water ad libitum. The mice were marked with haematoxylin stain for the purpose of identification and were handled as per CPCSEA guidelines.<sup>5</sup> The study was conducted in the Ethnopharmacology lab, Department of Pharmacology, Yenepoya Medical College, Mangalore after obtaining Institutional animal ethics committee (IAEC) clearance (5/9.6.2016).

### 2.2. Inclusion criteria

1. Healthy Swiss albino mice of either sexes with normal behaviour and activity
2. Weight: 25-30g
3. Age: 3-4 months

### 2.3. Exclusion criteria

1. Unhealthy animal
2. Obese animal
3. Pregnant animal
4. Animals previously used in other experiments

### 2.4. Plant material

The Fresh leaves of *Piper betle* L. were procured from the local market of Kasaragod district, Kerala. It was authenticated from the Department of Applied Botany, Mangalore University. The betel leaves were cleaned under running water and leaves were cut into pieces and kept for shade drying. The dried leaves of *Piper betle* were then powdered in a mixer grinder.

#### 2.4.1. Ethanolic extract of *Piper betle* leaves (PBEE)

The dried powdered betel leaves weighed around 122g. It was wrapped in a muslin cloth and extracted using 95% ethanol v/v in Soxhlet apparatus maintained at around 60°C - 70°C for period of 3 days.<sup>6</sup> The extract was filtered, concentrated under reduced pressure and dried using Rota-vapour at 60°C and subsequently in the water bath for evaporation of solvent for a period of 3 days. Aromatic greenish extract was obtained which weighed 16.7g. The yield of betel leaves obtained was 13.65% w/w.

#### 2.4.2. Aqueous extract of *Piper betle* leaves (PBAE)

The dried powdered leaves weighed around 122g. It was wrapped in a muslin cloth and extracted using distilled water (1000ml) as solvent in Soxhlet apparatus. The solvent was heated at 70°C - 80°C to reflux for a period of 23 hours. The obtained extract was concentrated using Rota-vapor and water bath for evaporation of solvent for 3 days.<sup>7</sup> Brownish extract weighing 15.20g was obtained. The yield of leaves was 12.4% w/w.

### 2.5. Acute toxicity study of test drugs (PBEE and PBAE)

The acute toxicity study of ethanolic and aqueous extract of *Piper betle* leaves was done according to OECD guidelines 425 limit test at the dose of 2000mg/kg.<sup>8</sup> A single dose of 2000mg/kg of PBEE and PBAE was administered orally to five female Swiss albino mice (fasted overnight) each. Animals were observed individually once during the first 30 minutes after dosing, hourly in the first 4 hrs, at 24hrs and daily thereafter, for a total of 14 days. Changes appearance, general behaviour and movement and mortality were observed. The highest dose of PBEE and PBAE (2000 mg/kg) in mice did not cause any behavioural changes or mortality which implies that LD<sub>50</sub> of each extract is above 2000mg/kg. Accordingly, the dose was fixed at 100 and 200 mg/kg for each of the extract for comparison.

### 2.6. Experimental design

Number of experimental animals used was 72: Animals were divided into 12 groups of size 6; Antianxiety activities were observed in two experimental models: Elevated Plus Maze (EPM) and Light Dark Arena (LDA). All the experiments were carried out in the Etho-chamber (Figure 1) which is a fabricated dark and sound proof chamber.

From the 12 groups of animals formed, 6 at random were used for the EPM model and the remaining 6 groups were used in LDA model. The following six types of drugs and doses were compared in EMP and LDA models:

- Group I: Control – Distilled water (10ml/kg)
- Group II: Standard drug: Tab.Diazepam<sup>9</sup> (obtained from Yenepoya Hospital Pharmacy, Mangalore)
- Group III: Test drug (PBEE100) (100mg/kg)
- Group IV: Test drug (PBAE100) (100mg/kg)
- Group V: Test drug (PBEE200) (200 mg/kg)
- Group VI: Test drug (PBAE200) (200mg/kg)

The groups were treated with vehicle (Distilled water), test PBAE and PBEE (100mg/kg and 200mg/kg each) and standard drug Diazepam. The required amount of test drug was calculated according to the body weight of animals and was administered as once a day dose (O.D), per orally (p.o) for 14 days. On the 14<sup>th</sup> day, one hour after oral administration of drug, animals were screened

for antianxiety activity.

### 2.7. Elevated plus maze (EPM)

EPM consists of two open arms and two closed arms that are interconnected by central platform (Figure 2). It combines three factors that are considered to be anxiogenic – novelty, height and open space. The EPM was suspended 50cm above the ground level.<sup>10</sup> Each mouse was placed in the central platform facing one of the closed arms and observed for 5 minutes. The animal when placed in EPM, develop fear due to height which induces anxiety. Eventually it manifest as decrease in the motor activity and preference to remain at safer and dark places. Whereas, anxiolytic agents are expected to increase the motor activity, which is measured by time spent in open arms and closed arms and number of entries to open and closed arm during the 5 minute test.

### 2.8. Light dark arena (LDA)

The apparatus is divided into two compartments, 1/3 is dark chamber and 2/3 of the chamber is open and illuminated by a light source. The compartments are separated by a wall which has an opening that permits the movement of the mouse from one chamber to other (Figure 3).<sup>10</sup> This model was based on the tendency of rodents to explore a novel environment, but from an aversive sight of brightly illuminated open area they withdraw. Each mouse was placed in the light compartment and observed time spent in bright and dark chamber and number of bright area entries for 5 minutes. During the test period, the time spent in bright and dark chamber and number of bright area entries was recorded. Placing all four paws within the boundaries of the compartment was defined as an entry. Antianxiety activity was expressed by an increase in time spent in the illuminated compartment and an increase in bright area entries

### 2.9. Phytochemical analysis of PBEE and PBAE

The test sample was subjected to phytochemical analysis in order to find out the presence of phytochemical constituents. The phytochemical tests employed were: Wagner's test, Keller Killiani test, Fehling's test, Biuret test, Ninhydrin test, Lead acetate test, Foam test, Salkowski's test, Ferric chloride test and Spot test.<sup>11</sup>

### 2.10. Statistical analysis

One way analysis of variance (ANOVA) was used for simultaneous comparison test drugs and for significant variables Tukey Kramer test was used for pair-wise comparisons with level of significance  $P = 0.05$ . The statistical analysis was carried out using the software SPSS v24.0.

## 3. Results

### 3.1. Elevated plus maze

ANOVA of the four variables observed in EPM were found to be significant. The mean  $\pm$  standard error for the 6 drugs/doses is shown in Table 1. Highest value for the variable 'time spent in open arm' was observed in the group administered with standard drug and it was significantly higher than the other groups. The groups administrated with betel leaf extracts were not statistically significant. Further, these groups were significantly different from the control except for PBAE100 group. Time spent in closed arm was in the reverse order as that of open arm. In this case, PBAE200 was not significantly different from the control. With regard to number of entries to open arm, the lowest value was observed in control which was not significantly different from PBAE200 and PBAE100. The standard was significantly superior to the rest. Whereas number of entries to closed arm was not significantly different between the Standard and PBEE100; other three extracts were not significantly different from the control.

### 3.2. Light dark arena

Comparison of groups for variables observed in LDA is shown in Table 2. The groups were significantly different for all the three variables. Lowest time spent in bright area was in control which is significantly different from the rest. There was no significant difference between the Standard and Extract-administered groups for this variable. Comparison of groups for time spent in dark area was similar to that observed in bright area. With regard to number of entries to bright area there was no significant difference between the Standard, PBEE100 and PBEE200, but for the other two extracts, PBAE100 and PBAE200, it was not significantly different from the control.

### 3.3. Phytochemical analysis

Phytochemical screening of ethanolic and aqueous extracts of *Piper betle* leaves are shown in Table 3. The Lead acetate test indicated the presence of flavonoids, a bioactive compound in both type of extracts. However, glycosides (Keller Killiani test), saponins (Foam test) and phytosterols (Salkowski's test) were not found in PBAE.

## 4. Discussion

Current allopathic treatment for anxiety disorders are reported to have many limitations such drug dependency, sedation, muscle relaxation, memory disturbance and unwanted drug interactions. Thus, there is a need to investigate alternatives especially with known medicinal plants.<sup>12</sup> One such potential medicinal plant is *Piper betle* L. which is reported to have many therapeutic uses.

**Table 1:** Anxiolytic effect of *Piper betle* L. leaf extracts as observed in Elevated Plus Maze

Group	Time (seconds) spent in		Number of entries observed in 5 minutes	
	Open arm	Closed Arm	Open arm	Closed arm
Control	117.83 <sup>a</sup> ± 9.57	162.67 <sup>b</sup> ± 10.45	9.00 <sup>c</sup> ± 0.57	16.00 <sup>a</sup> ± 1.00
Standard	262.00 ± 6.66	32.50 ± 4.51	25.00 ± 1.84	9.83 <sup>b</sup> ± 0.98
PBEE100	200.00 <sup>b</sup> ± 11.35	92.33 <sup>a</sup> ± 9.84	16.17 <sup>a</sup> ± 0.60	11.17 <sup>b</sup> ± 0.87
PBEE200	197.33 <sup>b</sup> ± 8.38	89.50 <sup>a</sup> ± 4.77	14.33 <sup>ab</sup> ± 0.88	14.17 <sup>ab</sup> ± 0.60
PBAE100	161.67 <sup>ab</sup> ± 18.68	105.00 <sup>a</sup> ± 19.59	12.33 <sup>abc</sup> ± 0.67	13.33 <sup>ab</sup> ± 1.52
PBAE200	169.33 <sup>b</sup> ± 6.75	125.67 <sup>ab</sup> ± 7.78	11.83 <sup>bc</sup> ± 0.95	13.67 <sup>ab</sup> ± 1.33

Common alphabets in a column indicating that corresponding group means are homogeneous based on Tukey Kramer multiple range test (P=0.05). PBAE- *Piper betle* Aqueous extract PBEE- *Piper betle* Ethanolic extract

**Table 2:** Anxiolytic effect of *Piper betle* L. leaf extracts as observed in Light Dark Arena

Group	Time (seconds) spent in		Number of bright area entries
	Bright area	Dark area	
Control	51.00 ± 9.95	210.17 ± 11.88	9.17 <sup>a</sup> ± 0.60
Standard	196.83 <sup>a</sup> ± 3.84	95.00 <sup>a</sup> ± 2.54	16.83 <sup>b</sup> ± 2.72
PBEE100	192.00 <sup>a</sup> ± 15.29	99.50 <sup>a</sup> ± 12.69	19.33 <sup>b</sup> ± 2.73
PBEE200	189.83 <sup>a</sup> ± 8.94	103.28 <sup>a</sup> ± 6.35	16.33 <sup>b</sup> ± 1.28
PBAE100	184.50 <sup>a</sup> ± 6.02	101.33 <sup>a</sup> ± 13.99	13.67 <sup>a</sup> ± 1.05
PBAE200	158.50 <sup>a</sup> ± 18.32	128.33 <sup>a</sup> ± 13.93	13.83 <sup>a</sup> ± 0.94

Common alphabets in a column indicating that corresponding group means are homogeneous based on Tukey Kramer multiple range test (P=0.05). PBAE- *Piper betle* Aqueous extract PBEE- *Piper betle* Ethanolic extract

**Table 3:** Phytochemical constituents in ethanolic and aqueous extracts of *Piper betle* L.

S. No.	Phyto- chemicals	Test	Observation	Inference	
				PBEE	PBAE
1	Alkaloids	Wagner's test	Reddish brown precipitate	+	+
2	Glycosides	Keller Killiani test	Reddish brown ring at junction	+	-
3	Carbohydrates	Fehling's test	Orange red precipitate	+	+
4	Proteins	Biuret test	Violet color	+	+
		Ninhydrin test	Blue color	+	+
5	Flavonoids	Lead acetate test	Yellow precipitate	+	+
6	Saponins	Foam test	Bubbles persisted beyond 10 min	+	-
7	Phytosterols	Salkowski's test	Reddish brown ring	+	-
8	Tannins & Phenols	Ferric chloride test	Bluish black color	+	+
9	Fixed oil	Spot test	No staining	-	-

PBAE- *Piper betle* Aqueous extract

PBEE- *Piper betle* Ethanolic extract

The results of the study confirm the antianxiety properties of *Piper betle* L., but found to vary between the two types of extracts tested. In all the three variables observed in LDA (time spent in bright- and dark-area and number of entries to bright area) and one variable in EPM (number of entries to closed arm) the ethanolic extract was comparable with the standard (Diazepam). But with regard to other three variables observed in EPM (time spent in open- and closed- arm and number of entries to open-arm), the ethanolic extract was not as effective as the standard. The results of this study thus indicating the effectiveness of betel leaf ethanolic extract over aqueous extract.

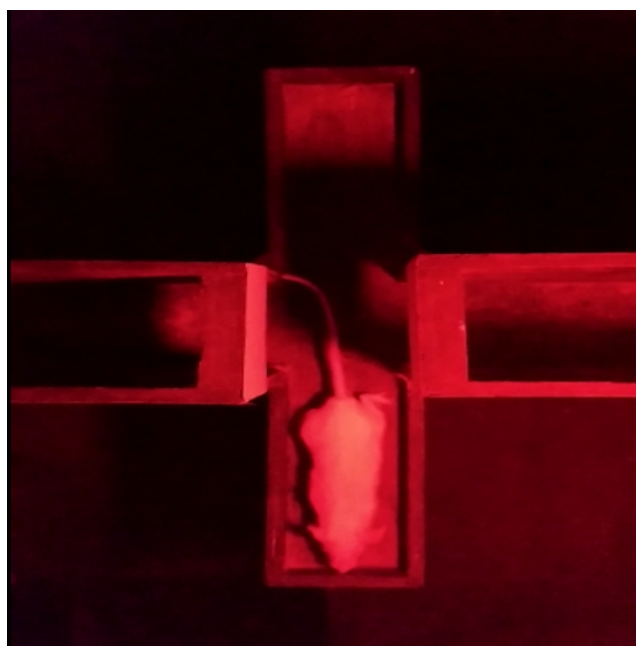
Flavonoids and saponins are known to have prominent anxiolytic effect.<sup>13</sup> Flavonoids were present in both ethanolic and aqueous extracts of betel leaf, but few other phytochemical constituents were not seen in aqueous extract, especially the saponins. Flavonoids have affinity to central benzodiazepine receptors, bind to gamma-amino butyric acid - GABA<sub>A</sub> receptor and pharmacologically act as a partial agonist.<sup>14,15</sup> Saponins effects through regulating neurotransmitter mechanism (5-Hydroxytryptamine, Dopamine, Norepinephrine), modulation of the GABA neurotransmission, glutamatergic system, hypo-thalamus-pituitary-adrenal (HPA) axis, brain-derived neurotrophic



**Fig. 3:** The light dark arena



**Fig. 1:** The etho-chamber used for the experiments



**Fig. 2:** The elevated plus maze

factor (BDNF), and its intracellular signalling pathways in the central nervous system.<sup>16</sup> Presence of saponins is probably the factor attributing to enhanced anxiolytic activity of ethanolic extract of betel leaves when compared to the aqueous extract. The usefulness of PBEE for treating anxiety disorders however needs isolation of bioactive compound(s) and molecular level information on pharmacological activities. Clinical studies are also required to confirm the safety, efficacy and adverse effects in humans.

## 5. Conclusion

The results of the study confirm the antianxiety properties of *Piper betle* L. The ethanolic extract has advantage over aqueous extract according to the observations on behaviour of Swiss albino mice in Elevated Plus Maze (EPM) and in Light Dark Arena (LDA) models. However, the difference between the two doses tried (100mg/kg and 200mg/kg) has not shown much difference in either of the extracts. The difference in response seen between the two types of extracts on mice could be due to quantitative difference in the phytochemicals available in the extracts or absence of some that are attributing the antianxiety properties.

## 6. Source of Funding

No funding sources.

## 7. Conflict of Interest

None declared

## 8. Ethical approval

The study was approved by the Institutional Animal Ethics Committee.

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