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Anxiolytic effect of leaf galls extracts of *Pipernigrum* Linn. in Swiss Albino mice

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SUMMARY

Anxiety disorders are one of the serious problems which need proper therapy devoid of side effects of presently available medicines. The present study evaluates the anxiolytic and sedative activity of leaf galls of *Piper nigrum* Linn. in Swiss Albino mice. The pet. ether, chloroform, ethyl acetate and ethanol extracts of leaf galls of *Piper nigrum* Linn were obtained by continuous soxhlet extraction. The prepared extracts were found to be safe up to 2000 mg/kg body weight of mice in the acute toxicity study. Each extract was assessed for anxiolytic activity in Swiss Albino mice by elevated plus Maze, open field test, rota rod test and phenobarbitone induced sleeping time test. In the Elevated Plus Maze test, the pet.ether extract and chloroform extract at a dose of 50 mg/kg b.w. orally, significantly ($P < 0.01$) increased the number of entries and time spent in open arm comparable with standard diazepam at the dose of 10 mg/kg. b.w. p.o. In the open field test, pet. ether extract (50 mg/kg b.w. p.o.) showed significant increase ($P < 0.01$) in ambulation and activity in the center. Chloroform extract (50 mg/kg b.w p.o.) was significant ($P < 0.05$) for both ambulation and center activity. Pet. ether extract (50 mg/kg b.w. p.o) also showed significant activity ($P < 0.01$) in rota rod test. All the results are comparable with standard diazepam at the dose of 1 mg /kg b.w, p.o. Moreover all the extracts showed significant ($P < 0.01$) increase in the phenobarbitone induced sleeping time among which pet.ether showed more prominent activity (36%) comparable with control. The results revealed that, the active pet.ether extract and chloroform extract of leaf galls of *Piper nigrum* Linn is worthwhile to develop the bioactive principle for anxiolytic activity.

Key words: Anxiolytic; *Piper nigrum*; Leaf galls; Elevated plus maze

INTRODUCTION

Anxiety disorders in a modern society have a relative high prevalence and command considerable financial resources. Benzodiazepines are the most widely prescribed class of psychoactive drugs in current therapeutic use, despite the important unwanted side-effects that they produce such as sedation, myorelaxation, ataxia, amnesia, ethanol and barbiturate potentiation and tolerance (File, 1987). Therefore the development of new medications possessing anxiolytic effect without the complication

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of benzodiazepines would be of great importance in the treatment of anxiety related disorders. Medicinal plants are a good source to find new remedies for these disorders. Worldwide researchers are emphasizing herbal-based therapy for the treatment of such disorders. It is well known that some plant extracts and isolated constituents from plants possess anxiolytic and sedative activities (Peng *et al*., 2000; Cha *et al*., 2004).

Piper nigrum Linn belongs to the family Piperaceae (Trease and Evans, 1985), a stout glabrous climber is found in South Western parts of India. This plant finds extensive use in the Ayurvedic system of medicine (Chopra *et al*., 1958). This plant has been used as a stimulant, carminative, antitubercular (Chopra *et al*., 1956, 1958), oxytocic (Banerjee and Dandia, 1967), and insecticide (Chandhoke *et al*., 1968). A number of piperidine and pyrolidine alkamide present in piper species are known to posses a variety of biological properties like CNS stimulant, analgesic, antipyretic, and antifeedant activities (Miyakado *et al*., 1979). Galls are pathologically developed cells, tissues, and organs of plants, which have risen mostly under the influence of parasitic organism. Recently we have demonstrated the anti-inflammatory activity of the leafgalls of *Piper nigrum* Linn in Albino wistar rats (Rajesh *et al*., 2007). Literature survey revealed that tannins and flavonoids possess activity against many CNS disorders (Takahash *et al*., 1986, Aguirre-Hernández *et al*., 2007). Phytochemical studies revealed the presence of tannins and flavonoids in the leafcgalls of *Piper nigrum* Linn. (Rajesh *et al*., 2007). Although many medicinal properties have been attributed to the extracts of this plant, there is a scarcity of scientific data in leaf galls of *pipernigrum* Linn and there is no substantiation about the anxiolytic and sedative activity of the leaf galls extracts of this plant. On this basis, the present study is deliberated to investigate the anxiolytic and sedative effect of various extracts of leaf galls of *Piper nigrum* Linn in mice.

MATERIALS AND METHODS

Fresh leaf galls of *Piper nigrum* Linn. were collected from the local areas of Kanyakumari district, Tamilnadu state in the month of November and were identified by Prof. Pamela Mohandoss, Dept. of Botany, Lady Doak College, Madurai. A voucher specimen (LPN-01) has been deposited at the departmental herbarium, K.M.College of Pharmacy, Madurai. Diazepam injection (calmpose, Ranbaxy, India) and phenobarbitone sodium were purchased commercially. All the reagents used were of analytical grade (Merck) purchased from local stockist, Tamilnadu, India.

Healthy, female Albino mice weighing between 20 - 25 g, acclimatized to our laboratory condition under standard animal house conditions, were used. CPCSEA guidelines were adhered to during the maintenance and experiment. Experiment protocol was submitted to Institutional Animal Ethics Committee and approval was taken.

Preparation of extract

The leaf galls (500 g) were shade dried and reduced to coarse powder, subjected to continuous hot extraction with petroleum ether, chloroform, ethyl acetate and 90% ethanol in a Soxhlet extractor for 72 h. The obtained extracts were filtered and evaporated to dryness at 40 °C under reduced pressure in a rota evaporator. The yields of petroleum ether, chloroform, ethyl acetate and ethanol extracts were found to be 10 g, 14 g, 15 g and 17 g, respectively. All the extracts were kept in a dessicator till experimentation. The extracts were suspended in 0.5 % w/v of sodium lauryl sulphate (SLS) for the pharmacological studies.

Preliminary phytochemical studies

The preliminary qualitative phytochemical screening of the extracts was performed to identify the presence of possible phytoconstituents (Clarke, 1975).

Acute toxicity studies

Acute toxicity study was carried out for all the extracts following OECD guidelines (OECD, 2001). Overnight fasted, healthy mice (n = 3) were administered orally the respective extracts in the dose of 2000 mg/kg body weight and observed continuously for 4 h and 24 h for any abnormality and mortality.

Drug treatment

The animals were divided into six groups each of six animals. Group III, IV, V and VI were administered with pet. Ether, chloroform, ethyl acetate and ethanol extracts respectively at the dose of 50 mg/kg b.w orally. Group II received standard drug diazepam (1 mg/kg. b.w) and vehicle 10 ml/kg b.w. Group I act as control received vehicle 10 ml/kg b.w. alone. The above drug treatment was followed for elevated plus maze test, open field behaviour and rota rod test.

Assessment of CNS activity: anxiolytic activity on elevated plus maze (Gorbett *et al.*, 1991)

The elevated plus maze consists of two open arms (15 × 5 cm) and two closed arms (15 × 5 × 12 cm) with the open air perpendicular to the closed one. The maze was made of clear acrylic and was located 29 cm above a black floor. After 30 min of the drug treatment, the animals were individually placed at the center of the plus maze and observed for 5 min. The time in seconds, spent by the animals in the open arm, closed arm and number of entries in both the arms were noted. Anxiolytic compounds reduce the animal's natural aversion to the open arms and promoted exploration. Therefore, increased number of entries and time spent in the open arm was considered to reflect an anxiolytic effect, in comparison with the control group. The results are tabulated in Table 1.

Open field test

The open field test was carried out by the method suggested by Ambavade *et al* (Ambavade *et al.*, 2006). The dark grey floor subdivided into 16 equal parts in a wooden box was used. After 45 min of the treatment with the extracts of *Piper nigrum* Linn at the dose of 50 mg/kg b.w, the animals were individually placed in the corner square of the open field. Spontaneous ambulation (number of segments crossed at periphery), activity in the centre (number of central squares crossed) and total locomotion (total number of squares crossed), were observed for 5 min. The results are tabulated in Table 2. Rota-Rod Test: (Muscle relaxant activity) (Dunham and Miya *et al.*, 1957).

Untreated mice were placed on a horizontal wooden rod (32 mm diameter), rotating at a speed of 5 rpm. The animals remaining on the rod for 3 min or more in successive trial were selected for the study. After drug treatment, each group was placed on the rod at intervals of 30, 60, 90 and 150 min after treatment. The time taken for the mice to fall from the rotating rod was noted. The results are tabulated in Table 2. Effect of Phenobarbitone

Table 1. Table showing results of Elevated Plus Maze test

Treatment	Time spent in Open Arm	No. of entries in open arm	No. of entries in Closed arm
Control	98.67 ± 5.287	2.833 ± 0.3073	3.5 ± 0.2236
Petroleum Ether (50 mg/Kg)	176.84 ± 3.547**	7 ± 0.2582**	2.83 ± 0.1667
Chloroform (50 mg/Kg)	148 ± 1.255**	6.33 ± 0.21**	2.83 ±0.3073
Ethyl Acetate (50 mg/Kg)	110.67 ± 1.235	3.85 ± 0.2108*	3.166 ± 0.307
Ethanol (50 mg/Kg)	101.67 ± 2.365	3.96 ± 0.212*	3.83 ± 0.307
Diazepam (1mg/kg, i.p.)	165.72 ± 3.548**	8 ± 0.2582**	4.56 ± 0.2236*

Values are mean ± S.E.M, (n = 6). *$P < 0.05$, **$P < 0.01$ compared to control group (One-way ANOVA followed by Dunnet's Multiple Comparison test).

Table 2. Table showing results of Open Field Test

Treatment	Open Field Behaviour		
	Ambulations (Number of squares crossed at periphery)	Activity at centre (number of central squares crossed)	Total locomotion (total number of squares traveled)
Control	77.66 ± 3.59	3 ± 0.2582	62.33 ± 0.2682
Petroleum Ether (50 mg/Kg)	98 ± 1.528**	11.67 ± 0.5**	92.16 ± 0.2236**
Chloroform (50 mg/Kg)	89 ± 1.438*	8.66 ± 0.426*	72.26 ± 0.2108**
Ethyl Acetate (50 mg/Kg)	79 ± 2.95	8 ± 0.4472*	70.28 ± 0.4492**
Ethanol (50 mg/Kg)	72.66 ± 2.108	7 ± 0.4472	73.5 ± 0.3416**
Diazepam^{+} (1 mg/kg, i.p.)	100.16 ± 2.286**	13.166 ± 0.4014**	112.5 ± 0.5**

V alues are mean ± SEM, (n = 6).
*P < 0.05, **P < 0.01 compared to control group (One-way ANOVA followed by Dunnet's Multiple Comparison test).

sodium induced sleeping time (Turner, 1965).

This test was conducted as suggested by Turner. Healthy albino mice weighing between 20 - 25 g were fasted for 24 h before the experiment and were divided into 5 groups of 6 animals each. The pet. ether, chloroform, ethyl acetate and ethanol extracts were administered to group II, III, IV, and V at a doe of 50 mg/kg b.w, and control group I received vehicle (10 ml/kg) alone. 30 min after administration, each animal was administered with phenobarbitone sodium (40 mg/kg b.w. p.o). The sleeping time was noted by recording the time interval between loss and return of righting reflex. The results are tabulated and shown in Table 3.

Statistical analysis

All the results were analyzed for statistical significance by performing one-way ANOVA followed by Dunnet's multiple comparison test. P < 0.05 implies significance.

Table 3. Table showing results of Rota rod test

Treatment	Time Spent on Rota rod (s)
Control	19.5 ± 1.38
Petroleum Ether (50 mg/Kg)	14.4 ± 0.98**
Chloroform (50 mg/Kg)	15.7 ± 0.49
Ethyl Acetate (50 mg/Kg)	15.2 ± 1.3*
Ethanol (50 mg/Kg)	17.2 ± 1.2
Diazepam^{+} (1 mg/kg, i.p.)	10.25 ± 0.56**

Values are mean ± S.E.M. (n = 6). *P < 0.05, **P < 0.01 compared to control group (One-way ANOVA followed by Dunnet's Multiple Comparison test).

RESULTS

Neither mortality nor any visible changes were observed during the acute toxicity studies. Hence the extracts in the dose up to 2000 mg/kg body weight were found to be safe in laboratory animals.

The preliminary qualitative phytochemical study showed the presence of triterpenoids, flavonoids and tannins in all the extracts (Rajesh *et al.,* 2007).

In the elevated plus maze, the pet.ether extract and chloroform extract at a dose of 50 mg/kg significantly (P < 0.01) increased the number of entries and time spent in the open arm. The standard Diazepam at a dose of 1mg/kg significantly (P < 0.01) increased the number of entries and time spent in the open arm (Table 1).

In the open field test, pet. ether extract showed a significant increase (P < 0.01) in ambulation and activity in the center. Chloroform extract was significant (P < 0.05) for both ambulation and center activity. Other extracts are not significant except ethyl acetate extract, which was significant (P < 0.05) only in the central activity. All extracts showed significant (P < 0.01) total locomotion.

In rota rod test, Pet. ether extract and ethyl

Table 4. Table showing phenobarbitone induced sleeping time of Leaf galls of *Piper nigrum* Linn

Treatment	Phenobarbitone sodium	Sleeping time (min)	Increase in sleeping time
Control	40 mg/kg	44.8 ± 1.934	-
Petroleum Ether (50 mg/kg)	40 mg/kg	70.8 ± 1.772**	36.72 %
Chloroform (50 mg/kg)	40 mg/kg	62.8 ± 2.288**	28.66 %
Ethyl Acetate (50 mg/kg)	40 mg/kg	63.2 ± 3.277**	29.72 %
Ethanol (50 mg/kg)	40 mg/kg	64.6 ± 2.654**	30.64 %

ANOVA followed by Dunnet's *t* test . **$P < 0.01$ compared to control group. Values are expressed as mean ± S.E.M of six values.

acetate extract showed significant motor dis coordination of ($P < 0.01$) and ($P < 0.05$) respectively, where as other extracts were not significant. The reference drug Diazepam significantly ($P < 0.01$) showed motor dis co-ordination (Table 2).

The phenobarbitone induced sleeping activity showed that all the extracts produced a significant increase in the hypnotic effect. The duration of sleeping time expressed as percentage effect is maximum obtained by pet.ether extract (36%) followed by ethanol, ethyl acetate and chloroform extracts respectively (Table 3).

DISCUSSION

Anxiolytic compounds reduce the animal's natural aversion to the open arms and promoted exploration in the elevated plus maze test. Therefore, increased time spent in the open arms was considered to reflect an anxiolytic effect, in comparison with the control group. The decrease in the aversion to the open arm in the Elevated plus Maze test is the result of an anxiolytic effect, expressed by the increased time spent in the open arm. In the present study, Pet. ether and chloroform extracts significantly increased both the number of entries and time spent in open arm suggested that behaviour alterations induced by these extracts were consistent with anxiolytic activity. The number of entries in closed arm was reduced. Eventhough the reduction was not statistically significant, it was not increased indicating that the locomotor activity did not increased by the extracts treated group compared to control group. The standard drug diazepam increased the number of entries so that increased the locomotor activity as expected.

In the open field test, rodents show thigmotaxic behaviour identified by spontaneous reference to the periphery of the apparatus and reduced ambulation. (Bhattacharya and Satyan, 1997) The inhibition or marked decrease in the exploratory behaviour usually, is decreased by anxiolytic agents. In the present study the pet.ether extract showed prominent effect on ambulation than chloroform extract. All extracts other than ethanolic extract were significant in the central activity in which pet.ether extract was more prominent. The significant increase ($P < 0.01$) in locomotion was observed by all the extracts.

The increased ambulation, and higher central activity in the OFT further supports the results of Elevated plus maze for the anxiolytic activity. The rota rod test, which can explain the muscle relaxant activity of animals, showed similar type of activity like Diazepam. Diazepam significantly reduces the motor coordination. Diazepam is a very well known anxiolytic benzodiazepine (BDZ) which produces important sedative effects (Shader and Greenblatt, 1993). In phenobarbitone induced sleeping time, all the extracts significantly enhanced the percentage effect in which Pet.ether is more prominent. Such an effect might arise for a CNS depressant action (Fastier *et al.*, 1957) or tranquilizing action (Mukharjee *et al.*, 1996). It has been reported that an inhibition in hepatic enzymatic metabolism may prolong the sleeping time induced by barbiturate (Gyamfi *et al.*,

2000) and also the activation of the inhibitory GABAergic system is involoved in the effects of phenobarbital on the CNS (Steinbach and Akk, 2001). In the present study, the Pet. ether extract at the same dose exhibiting anxiolytic activity, motor in coordination and potentiating phenobarbitone sleeping time. Eventhough, from the rota rod test and phenobarbitone induced sleeping time, there may be a possibility of sedative effect for the extract, the increased ambulation and locomotion in the OFT is contradicted for the sedative effect. The total number of crossings in the field is an index of non specific locomotion activity (Schmitt and Hiemke 1998). But moderate dose of benzodiazepines are known to decrease activity in rats and to increase it in mice (Prut and Belzung, 2003). Similar activity was found in present study where diazepam 1mg/kg increased the total locomotion. Similarly the pet.ether and chloroform extracts showed activities which can be well correlated with diazepam. Also previous studies showed that plant extracts which possess anxiolytic effect at low doses exhibiting sedative effect at high doses (Ambavade *et al*., 2006). However at this position it is difficult to conclude for the sedative activity of the extract. Further dose dependant studies with higher doses and locomotor activity studies are required to confirm the sedative property, which are in process.

All the above observations are quite sufficient to conclude that the leafgalls of *pipernigrum* Linn possessing anxiolytic activity which we are reporting for the first time. The results are well agreed with the earlier report by Aguirre Hernandez *et al* (Aguirre-Hernández *et al*., 2007) in the case of evaluation of *Tilia americana* L. var. *mexicana.* Earlier studies revealed that tannins and flavonoids have been shown to possess activity against many CNS disorders. As the phytochemical studies shows the presence of tannins and flavonoids and the anxiolytic activity of any phytoconstituent of leaf galls of this plant is still remained unexplored, it is worthwhile to isolate the bioactive principle which is responsible for the activity and its possible mechanism to be studied thoroughly.

To summaries the pet.ether and chloroform extract of leaf galls of *pipernigrum* Linn at a dose of 50 mg/kg possesses more anxiolytic activity than the ethyl acetate and ethanol extracts. Diazepam at a dose of 1mg/kg showed more activity than all extracts. The above results suggest that the leafgalls of this plant can be used as an alternative for the currently available anxiolytic drugs.

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