Research Article

In vivo evaluation of anxiolytic activity of aqueous and ethanolic extracts of *Litsea floribunda* (Bl.) Gamble -Lauraceae

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Abstract

Objective: The objective of the present study was to evaluate the anxiolytic potentials of the leaf and stem bark aqueous and ethanol extracts of *Litsea floribunda* (LF) using EPM and LDT in mice. **Materials and methods**: Albino mice were treated with extracts of *LF* (100 and 200 mg/kg, p.o.) and standard drug Diazepam (20 mg/kg, i.p.) for 14 days and after last dose administration on 14th day, all behavioural studies were performed and evaluated. **Results and conclusion**: The standard drug Diazepam increased the time spent in light arm in EPM model and light chamber in LDT model. In EPM model the anxiolytic effect was exhibited by group III, IV (ethanolic leaf extract 100 and 200 mg/kg), VI (aqueous leaf extract 200 mg/kg), VII (ethanolic stem bark extract 100 mg/kg), IX and X (aqueous stem bark extracts 100 & 200 mg/kg) was comparable to that of group III i.e. standard drug diazepam where as in LDT model, the anxiolytic effect exhibited by III, IV (ethanolic leaf extract 100 and 200 mg/kg), V (aqueous leaf extract 100 mg/kg), VII (ethanolic stem bark extract 100 mg/kg), IX and X (aqueous stem bark extracts 100 & 200 mg/kg) groups was comparable to that of standard drug diazepam. In conclusion LF extracts possessed potential anxiolytic effects which could be of therapeutic interest for using in the treatment of anxiety disorders.

Keywords: Anxiolytic, *Litsea floribunda,* ethanol, aqueous extracts, Diazepam

Introduction

According to WHO, 450 million people suffer from mental disorders but only few of them receive even the most basic treatment (WHO 2001). Anxiety and depression are the most prevalent stress related psychiatric disorders of present human population in the world. About two third of the patients suffering from anxiety and depression respond to the currently available treatment but with disappointing results (Onasanwa et al., 2010).

Anxiety is defined as feeling of apprehension, uncertainty or tension stemming from the anticipation of imagined or unreal threat. Synthetic drugs like Benzodiazepam (BZDs), these drugs have many side effects like drowsiness, muscle relaxation, insomnia, withdrawal and tolerance. Natural drugs from plant source are presumed to have less side effects but with

same ability of curing the disorder. Therefore there is significant progress in search for novel drug of natural source for psychiatric disorders. Plant secondary metabolites are used in the treatment of psychiatric disorders like anxiety in traditional medicine (Kothari et al., 2010).

Litsea is a genus of family Lauraceae comprising about 200 species mainly growing in the tropical and subtropical Asia, some distributed in Australia and from North America to subtropical South America. Out of 200 species of *Litsea* in the world, 45 are found in India and 18 of them are endemic (Bhuniya et al., 2010).

Litsea species have been used globally in traditional medicine for the treatment of various diseases including influenza, stomach aches, diarrhoea, diabetes, vomiting, bone pain, inflammation, illness related to the central nervous system and other ailments. Crude extracts, fractions and phytochemical constituents isolated from *Litsea* show a wide spectrum of in vitro and in vivo pharmacological activities including anticancer, anti-inflammatory, antimicrobial, antioxidant, antidiabetic, anti-HIV,

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insecticidal, etc. (Wang et al., 2016) and also wound healing, antidepressant, analgesic, cardioprotective and cytotoxic activity (Agarwal et al., 2011).

Litsea floribunda (Bl.) Gamble is an endemic tree species, predominantly found in the Western Ghats, a 'hotspot' of biodiversity centers of India (Bhagawat et al., 2005). Western Ghats being one of the hotspots of India harbor thousands of medicinal plants and *Litsea* in one such traditionally used plant which has not been studied scientifically waiting for scientific validation. A survey of literature shows that no scientific work is reported regarding anxiolytic effects of *L. floribunda*. Till date, there is no scientific report in literature about anxiolytic activity of *Litsea* species. In our previous studies we have reported antioxidant, hepatoprotective and antidepressant activity of *L. floribunda*. Since there are reports that antidepressant drugs are found effective against anxiety disorder also, the present study is undertaken to investigate the anxiolytic activity of aqueous and ethanolic leaf and stem bark extracts of *L. floribunda* using animal models.

Materials and methods

Collection of the plant material and preparation of extract

Litsea floribunda (Bl.) Gamble was collected from the forests of the Western Ghats ($012^0 17$" to $012^0 27$"N and $075^0 26$" to $075^0 33$"E), Kodagu District, Karnataka, India and identified based on taxonomical parameters. A herbarium specimen of the species is deposited in the herbarium collection of the Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore, India. Plant parts like healthy leaves and stem bark were collected in zip lock polyethylene bags and brought to the laboratory.

Collected plant parts were washed, shade dried, powdered and subjected to exhaustive soxhlet extraction in the increasing order of polarity of solvents (Hexane <chloroform <ethyl acetate < methanol < ethanol). The liquid obtained after solvent extraction was subjected to drying using a rotary flash evaporator (Superfit Model PBU-6D, India). The residue obtained after flash evaporation of solvents was designated as the dry extracts (Akshatha et al., 2015). The extracts were stored in pre-weighed glass vials and labeled. The aqueous extracts of leaf and stem bark were prepared according to the procedure of Hebbar *et al*. (2015) by stirring 500 g of the materials in distilled water and boiling for an hour. The extract was filtered using a double layer cheese cloth and the filtrate was evaporated to dryness in a temperature controlled water bath for 72 h (Fisher Scientific, Mumbai, India). The dried powder was scraped, quantified and designated as the dry aqueous extract and used throughout the studies.

Experimental animals and housing conditions and ethical approval

Albino mice weighing from 20 – 28 g were selected, housed in the animal house of Sarada Vilas College of Pharmacy, Mysore. The animals were maintained at a temperature of $23\pm2^\circ$C, relative humidity 55±2% and light and dark cycles of 12L: 12D. They were provided with standardized pellet feed and drinking water *ad libitum.* The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) Reg. No. 706/CPCSEA dt. 1.10.2002. All the experimental procedures were carried out in accordance with the guidelines of CPCSEA.

Drugs and chemicals

The standard drug Diazepam (DZP) was obtained from Ranbaxy laboratories Ltd. India. All other chemicals used were of analytical grade.

Preliminary phytochemical screening

Qualitative phytochemical screening of solvent extracts have been reported previously (Devika and Nalini, 2016).

Acute toxicity study, determination of LD_{50} of leaf and stem bark extract of *L. floribunda* and standard drug preparation

Acute toxicity study was carried out according to the Organization of Economic Corporation Development (OECD) guidelines No. 425. Healthy albino mice were divided into four groups (n=5 in each group), fasted overnight and administered with ethanolic and aqueous extracts of *L. floribunda* orally in a single increasing dose of 1000 mg/kg b.w., 1500 mg/kg b.w., and 2000 mg/kg b.w. of the mice, respectively. The mice were observed continuously for 24 hours. Since no mortality was observed up to 2000 mg/kg, LD_{50} dose, 100 mg/kg and 200 mg/kg doses were selected and considered as low and high doses respectively. Diazepam was used as reference standard drug for evaluating anxiolytic activity, and the drug suspension was prepared using distilled water.

Experimental design

The anxiolytic activity of the test drug was evaluated using the following experimental models of anxiety such as Elevated Plus Maze (EPM) and Light Dark Test (LDT). Thirty animals with body weight 20-28 g were divided into ten groups each consisting of three mice. All the mice were subjected to daily treatment for the period of 14 days as follows.

The present study design is as follows:

Group I: Normal was given only vehicle (distilled water) for 14 days.

Group II: Drug control was administered with standard drug Diazepam i. p. (2.0 mg/kg b.w.) for 14 days because

2.0 mg/kg dose was proved to be an active dosage without toxic effects.

Group III & IV: were administered leaf absolute ethanol extract p.o.100 and 200 mg/kg b.w. respectively for 14 days.

Group V & VI: were administered leaf aqueous extract p.o., 100 & 200 mg/kg b.w., respectively, for 14 days

Group VII & VIII: were administered stem bark absolute ethanol extract p.o. 100 & 200 mg/kg b.w., respectively, for 14 days

Group IX & X: were administered stem bark aqueous extract p.o. 100 & 200 mg/kg b.w. respectively, for 14 days

Anxiolytic Activity

Elevated Plus Maze (EPM)

Elevated plus maze is a rapid and selective test for detecting anxiolytic drug effects under identical conditions (Pellow et al., 1985; Lister et al., 1987). The elevated plus maze model apparatus consists of two open arms (35 x 5 cm^2) crossed with two closed arms (35 x 5 x 20 cm^3) with open roof. The arms were connected together with a central square of 5x5cm^2. The apparatus was elevated to the height of 25 cm in a dimly illuminated room as fear due to height induces anxiety in animals when placed on EPM. During the entire experiment, mice were allowed to socialize. Every precaution was taken to ensure that no external stimuli, other than the height of the plus maze, could invoke anxiety in mice. Doses were administered orally tuberculin syringes fitted with an oral canula. The aqueous and ethanolic leaf and stem bark extracts (100 and 200 mg/kg b.w. p.o.) of LF and the standard drug Diazepam (2 mg/kg b.w. i.p.) were administered for 14 days once daily and the last dose was given on 14th day 30 min before the experiment. Later each animal was placed in the centre of the apparatus, facing the closed arm. Time spent and number of entries in both open and closed arms was recorded for 5 minutes and all the sessions were videotaped. The apparatus was cleaned with 10% ethanol after every test to remove any residue or odour. An entry was defined as the entry of all four paws within the arms. The percentage of open arm entries (100 x open/total entries) and the percentage of time spent in the open arms (100 x open/open + closed arm) were calculated for each animal.

Light Dark Test (LDT)

The apparatus consists of two boxes (25 x 25 x 25cm^3) joined together with a hole (4.5 cm) in the middle of the separating wall. One box was illuminated with white light while the other was made dark by covering its top with plywood. The light chamber was painted with white inside and dark chamber was painted with black inside and also on the inner surface of the plywood roof (Young et al., 1991; Crawley et al., 1980). Distilled water (normal group) and the aqueous and ethanolic leaf and stem bark

extracts (100 and 200 mg/kg b.w. p.o.) of LF and the standard drug Diazepam (2 mg/kg b.w., i.p.) were administered for 14 days once daily and the last dose was given on 14th day 30 min before the experiment. Each animal was placed at the junction of the light dark box compartment facing illuminated compartment. The time spent in illuminated chamber, dark chamber, as well as number of entries into each chamber was recorded and videotaped for 5 minutes. The apparatus was cleaned with 10% ethanol after every test to remove any residue or odour. An entry was defined as the entry of all four paws into the chamber.

Statistical analysis

Results were expressed as mean ± SEM. The statistical significance was determined by one – way analysis of variance (ANOVA) followed by Dunnet's test. P>0.05 was considered as statistically significant.

Results

Phytochemical screening

The phytochemical analysis of *L. floribunda* carried out for the various solvent extracts of leaf and stem bark indicated that both the extracts contained saponins, tannins, terpenoids, flavonoids, glycosides and reducing sugars in common.

Acute toxicity studies

All the extracts of *L. floribunda* neither exhibited signs of acute toxicity nor mortality the dose of 2000 mg/kg, p.o. was not found to be toxic.

Evaluation of anxiolytic like activity through animal models

Elevated plus maze and light dark test are the two well established and most widely used models in contemporary clinical research on anxiety. Anti-anxiety activities of all the test extracts were compared with the standard anti-anxiety drug Diazepam. The ultimate manifestation of anxiety fear in the animals is exhibited by decrease in motor activity and preference to remain at safe place i.e. closed arm in EPM and dark chamber in LDT (Kumar et al., 2015).

Elevated plus maze

Results showed that the number of open arm entries and the time spent in open arms were increased and the number of closed arm entries and time spent in closed arms were decreased significantly in group III and IV (ethanolic leaf extract 100 and 200 mg/kg), VI (aqueous leaf extract 100 mg/kg), VII (ethanolic stem bark extract 200 mg/kg), IX and X (ethanolic stem bark extract 100 and 200 mg/kg) compared with the standard drug diazepam (G II) (Table 1).

Table 1. Effect of extracts of *L. floribunda* (100 and 200 mg/kg) and Diazepam in elevated plus maze anxiolytic model

Groups	Treatment	Dose mg/kg	Time spent (Seconds)		Number of entries	
			open arms	closed arms	Open arms	Closed arms
G I	Water	-	103.00 ± 4.04	189.31±1.23	1.93±0.33	20.01±0.56
G II	Diazepam	002	172.33±3.33	119. 21±2.33	6.01±0.11	16.33±1.20
G III	LE	100	159.00 ±2.04	135.14±18.11	5.01±1,21	15.03±2.88
G IV	LE	200	165.21±18.16	131.11±11.21	5.66±2.33	15.66±4.37
G V	LA	100	135.02±15.27	162.41±5.27	2.66±2.08	17.33±1.85
G VI	LA	200	160.66±17.66	141.34±11.22	4.33±1.20	15.66±0.88
G VII	BE	100	166.33±3.88	133.18±4.56	6.33±1.76	17.03±3.51
G VIII	BE	200	138.66±3.33	163.11±4.31	3.02±0.57	17.33±1.45
G IX	BA	100	169.33±3.71	126.31±4.17	5.93±0.57	17.33±2.40
G X	BA	200	162.33±8.81	125.56±6.41	5.92±0.43	16.53±1.15

Values are expressed by mean ± SEM. P<0.05 on the ANOVA followed by post hoc Dunnet's test; LA= Leaf aqueous, LE= Leaf ethanol, BA= Stem bark aqueous, BE= Stem bark ethanol; G= Grouping of animals; mg=milligram; kg=kilogram

Table 2. Effect of *L. floribunda* extracts and Diazepam in light dark test of anxiolytic model in mice

Groups	Treatment	Dose mg/kg	Time spent (Seconds)		Number of crossings
			Light box	Dark box	
G I	Water	-	120.61±12.77	120.67±11.56	10.66±1.20
G II	Diazepam	002	83.33±32.84	154.67±35.61	11.33±5.20
G III	LE	100	76.33±10.63	164.33±20.12	9.33±1.66
G IV	LE	200	79.34±9.40	163.67±9.40	10.01±2.08
G V	LA	100	89.67±58.45	110.67±58.71	4.33±2.33
G VI	LA	200	49.66±18.33	193.33±8.81	4.01±2.51
G VII	BE	100	73.02±31.74	167.33±18.26	13.02±5.56
G VIII	BE	200	58.33±26.78	181.33±25.46	6.66±3.52
G IX	BA	100	85.03±10.21	155.33±9.95	10.66±3.17
G X	BA	200	84.66±5.04	158.67±8.02	9.13±1.01

Values are expressed by mean ± SEM. P<0.05 on the ANOVA followed by post hoc Dunnet's test; LA= Leaf aqueous, LE= Leaf ethanol, BA= Stem bark aqueous, BE= Stem bark ethanol; G= Grouping of animals; mg=milligram; kg=kilogram.

Light dark test

Results showed that the number of illuminated chamber entries and the time spent in illuminated chamber were increased and the number of dark chamber entries and time spent in dark chamber were decreased significantly in group III, IV, V, VII, IX and X compared with Group II (diazepam 2.0 mg/kg). III and IV (ethanolic leaf extract 100 and 200 mg/kg), VI (aqueous leaf extract 100 mg/kg), VII (ethanolic stem bark extract 200 mg/kg), IX and X (ethanolic stem bark extract 100 and 200 mg/kg) compared with the standard drug diazepam (G II) (Table 2).

Discussion

Anxiety, a particular form of behavioural inhibitor occurs in response of environmental events. It is established that there are a lot of plant secondary metabolites employed in the treatment of psychotic disorders specially for anxiety in traditional medicines as they directly or indirectly affect CNS, Noradrenalin, Serotonin, GABA and BDZ neurotransmitters activities (Ayoka et al., 2005; Dhawan et al., 2003). Aggression is a form of anxiety and muricidal effect is a sign of aggression mediated either by nor-epinephrine or serotonin in the brain. The anxiolytic activity of the extracts from natural products is based on the fact that constituents from plant extracts could modify muricidal actions exhibited in rats (Bhattacharya, 1994).

EPM test has been recognized as a valuable model able to predict anxiolytic effects of drugs in rodents (Lister et al.,

1987; Pellow et al., 1985). EPM is based on natural aversion of rodents for open spaces. Rodents tend to avoid the open arm especially when they are brightly lit favouring darker and more enclosed spaces. EPM test is based on the premise where the exposure to an open arm of EPM evokes an approach avoidance conflict that is considerably stronger than that evoked by the exposure to an enclosed arm. The decrease in aversion to the open arm is the sign of anxiolytic effect expressed by an increase in time spent and entries into open arms. Drugs that increase open arm exploration are considered as anxiolytic and the reverse holds true for anxiogenic (Chakraborty et al., 2010).

The anxiolytic effect was evidenced through LDT as it is also a model useful for testing anxiety level in mice. In LDT, animals always try to spend more time in dark chamber compared to light box out of fear of exposure to the new environment it has been assumed that the time mice spend in the illuminated side of the box is the most useful and consistent parameter of anxiety (Young et al., 1991;Barua et al., 2012). If there is any lack of dose dependent effect, it could be attributed to the biological variability as well as to the chemical complexity of crude extracts (Ruiz et al., 2006)

In LDT, animals always try to spend more time in dark compartment compared to light box out of fear of exposure to new environment. Transitions have been reported to be an index of activity exploration because of habituation over time and time spent in each compartment are reflection of aversion (Barua et al., 2012).

Anxiolytic compounds typically increase the percentage of open arm entries as well as time spent in open arms. In addition, the number of closed arm entries has been used as a parameter reflecting general motor activity (Bhattacharya, 1994). Confirming previous results, the i.p. administration of Diazepam (2 mg/kg) resulted in anxiolytic effect characterized by an increase in the percentage of exploration in the open arms of the plus maze. Administration of *L. floribunda* extracts showed significant increase in time spent in lighted box, number of crossings with decrease in time spent in the dark box. Both aqueous and ethanolic extracts of *L. floribunda* increased the entries and time spent in open arms in the present study.

The effect of most of the anxiolytic agents is to enhance the response to GABA, by facilitating the opening of GABA-activated chloride channels. GABA$_A$ receptors were involved in anxiety and their direct activation would have an anxiolytic effect. GABA receptors are involved in anxiety and their direct activation would have an anxiolytic effect. Anxiolytic property of extracts demonstrates that extracts may be facilitating GABA transmissions. GABA is the primary inhibitory neurotransmitter known to counterbalance the action of the excitatory neurotransmitters; glutamate and

noradrenaline (Ayoka et al., 2005)

The biological effects of aqueous and ethanol leaf and stem bark extracts of *L. floribunda* observed in their study may be attributed to phytochemicals found in the plant. Flavonoids present in the extracts may be responsible for the biological activities like antidepressant and anxiolytic activities as it is reported that some flavonoids bind with high affinity to benzodiazepine site of GABA receptor (Foyet et al., 2012). In support of this, it has been found that flavones bind with high affinity BZD site of GABA$_A$ receptor (Adeyemi, 2006). There is correlation between content of flavonoids and their psychotropic activities in the plant extracts. Possibly the anxiolytic activity observed in this study was not only dependent on flavonoid but also on other phytochemicals of different classes involved in the biological responses (Dhawan et al., 2003).

It may be possible that the mechanism of anxiolytic action of *L. floribunda* aqueous and ethanolic leaf and stem bark extracts could be due to the binding of any of these phytochemicals like saponins, flavonoids, terpenoids to the GABA$_A$ - BZD complex. So the anxiolytic activity of *L. floribunda* might involve an action as GABAnergic transmission or effects on serotogenic transmission or due to its mixed aminergic potentiality effect (Chakraborty et al., 2010). Present study demonstrates that the extracts of *L. floribunda* reduced aggressive behaviour in mice. This effect may be due to the interaction of extracts with neural substrates or chemical mediators like NE, SE, GABA, BZD, hormone (testosterone) which are said to be responsible for aggressive and anxiety like condition.

The monoamine hypothesis of anxiety and depression-like states remain largely accepted. Most of the antidepressant drugs are found to be effective against anxiety disorder in addition to depression (Zohar et al., 2000; Kothari et al., 2010). It is interesting to note that species of *Litsea* is traditionally used in mental disorders and our study results support those reports. The phytochemicals present in *Litsea* species may be responsible for anxiolytic property. However precise mechanism underlying *L. floribunda* anxiolytic activity and identification of bioactive compounds in particular extracts responsible for those biological activities requires further investigations.

Conclusion

In conclusion, the present study hypothesizes that LF shows significant psychotherapeutic effects as anxiolytic agent with intact motor co-ordination in all the animal models. LF extracts have possibly exerted its effects through diverse mechanism possibly due to the presence of phytochemicals like flavonoids, terpenoids present there in the extracts and

their influence on the level of monoamines. Detailed laboratory analysis is required for a definitive conclusion and isolation of major secondary metabolite responsible for these therapeutic actions.

Conflict of interest

The authors have no conflict of interest.

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