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Der Pharmacia Lettre, 2009, 1 (2) 172-181
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Investigation of anxiolytic effects of *Mitragyna parvifolia* stem-bark extracts on animal models

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Abstract

The purpose of this investigation was to characterize the putative anxiolytic-like activity of methanolic, ethyl acetate extract and alkaloid rich fraction prepared from the stem-bark of *Mitragyna parvifolia* (Roxb.) Korth (Rubiaceae) using the elevated plus maze (EPM) and marble burying test (MBT) in mice. The extracts increased the time spent on and the number of entries into the open arms of the EPM in doses of 200 and 400 mg/kg p.o., respectively. This effect was comparable to that of negative control group treated with 0.5 % CMC and positive control the benzodiazepine diazepam (1.0 mg/kg p.o.) was used as a standard. When evaluated by MBT the number of marbles buried by mice was decreased significantly as compared to control group CMC 0.5 %. Fluoxetine (10 mg/kg p.o.) was used as a standard for comparison. These results indicate that all the extract were effective in dose dependent manner and proved statistically significant at higher doses but alkaloid rich fraction was found to be more potent in producing anxiolytic effects by both test. It suggest that the anxiolytic-like activities of this plant are mainly mediated via the GABAergic system. Neither diazepam nor the test extracts produced any overt behavioral change or motor dysfunction in the performed tests.

Keywords: Anxiolytic effect, Benzodiazepine, Elevated plus maze, marble burying, GABA receptor, Fluoxetine.

Introduction

Anxiety disorders are the most common mental illness in the world and became a very important area of research interest in psychopharmacology. Benzodiazepines are among the first line of drugs that have been extensively used for the last 45 years to treat several forms of anxiety [1]. Benzodiazepines are the major class of compounds used in anxiety and they have remained the

most commonly prescribed treatment for anxiety [2]. Although benzodiazepines have well-known benefits, their side effects are prominent, including sedation, muscle relaxation, anterograde amnesia and physical dependence [3]. However, the realization that benzodiazepines present a narrow safety margin between the anxiolytic effect and those causing unwanted side effects has prompted many researchers to evaluate new compounds in the hope that other anxiolytic drugs will have less undesirable effects [4]. Thus, there is a need of robust anxiolytic compounds that have lesser side effects than benzodiazepines and a more immediate onset of action than currently available 5-HT_{1A} receptor acting drugs.

Mitragyna parvifolia (Roxb.) Korth (Rubiaceae) is commonly known as Kaim. It is a deciduous tree found in well drained deep soil. Common in deciduous hilly forest tracts [5]. It is credited with innumerable medicinal properties and is widely used by tribal peoples and other ayurvedic practitioners. The bark and roots are used to treat fever, colic, muscular pain, burning sensation, poisoning, gynecological disorders, cough, edema and as aphrodisiac. [6-9].

It is very rich in alkaloids and many known are isolated from the leaves. Various indolic and oxindolic alkaloids have been reported from this species are of significant biological importance [10, 11]. Several indigenous drugs are being evaluated because of their easy availability, lack of adverse effects and cost-effectiveness. Traditional medicines are used by about 60 percent of the world population in rural areas in the developing countries as well as in the developed countries where use of modern medicine predominates [12]. The historical use of such medicine provides the source to study the specific plant species with potential to be used in a particular disease. This study therefore carried out to evaluate the anxiolytic effects of *Mitragyna parvifolia* (Roxb.) Korth stem-bark extract on animal models.

Materials and Methods

Plant material

Mitragyna parvifolia (Rubiaceae) stem-bark was collected from Toranmal Hills of Satpuda region from Maharashtra, India, during the month of June. The plant got identified and authenticated by Botanical Survey of India, Pune, and a voucher specimen of the sample (MPVB11) has deposited in the Herbarium collection at Department. The stem-bark was cleaned and dried in the shade, and then it was cutted in small pieces and powdered to 40 mesh and stored in an airtight container.

Preparation of Extract

Methanolic extract (MPBM)

Dried stem-bark powder (900 gm) was extracted with 3 L of methanol by continuous hot extraction using soxhlet extractor. The methanol containing extract was filtered and distilled on a water bath. The resulting Methanol extract solution was concentrated in vacuum using a Rota vapor to obtain a brown powder 16% (144 g).

Ethyl acetate extract (MPBEA)

Total methanolic extract (60 g) was treated with ethyl acetate 2 L several times using a separating funnel. The resulting layer of ethyl acetate was separated, filtered and distilled using

water bath and finally extract was obtained concentrated in a Rota vapor to obtain light brown powder 10% (6 g)

Alkaloid rich Fraction (MPBalk)

45gm of methnolic extract was dissolved in 700 ml of 2% hydrochloric acid and filtered after 6 h. The filtrate was extracted with CHCl₃ thrice and ammonia was added to adjust pH 9. This was subsequently extracted with CHCl₃ several times until the CHCl₃ layer showed negative to the Dragendroff's reagent, to give alkaloid rich fraction [13]. The percentage yield of Alkaloid rich fraction (Chloroform part) was 13% (6.5 g) respectively. Whereas alkaloids estimated in plant part are 2.976%. [14]. The resulting Methanol (MPBM) extracts and Alkaloid rich fraction (MPBalk) was used for the pharmacological screening.

Animals

Swiss albino mice of either sex weighing 20–25 g, obtained from Institute of Animal Health and Veterinary Biological Products, Mhow, India were used for behavioral experiments. Animals were housed in acrylic cages (45×60×25 cm) with water and food available *ad libitum* under an artificial 12-h light/dark cycle (light on at 7:00) and at a constant temperature (22±2 °C). Mice were housed in the departmental room for 1 week before testing to ensure adaptation to the new environment. All of the behavioral experiments were performed between 10:00 and 17:00. All the experimental procedures and protocols used in the study were reviewed by the Institutional Animal Ethics Committee (Register Number: RCPIPER/IAEC/2008-09/13) and were in accordance with the guidelines of the CPCSEA, Ministry of Forests and Environment, Government of India.

Drugs and Chemicals

Diazepam and Fluoxetine were used as standard drugs for EPM and MBT methods respectively.

Acute toxicity test

Acute toxicity tests were performed according to OECD - 2001 guidelines. Animals were weighed and marked, A single high dose of MPBM, MPBEA and MPBalk as recommended by the OECD guidelines was administered to the first animal. After single administration, animals were observed for the sign of toxicity upto 24 hours. If mortality was observed, one step lesser dose of previous administration was given to the next animals. If the animal survived, the same dose was given to the next five animals. Further all the animals were observed for the presence of signs of toxicity and mortality for 14 days. The body weights of the animal were also recorded. The LD50 of the test drug was calculated using a computer assisted statistical programme-AOT425statPgm.

Elevated plus maze test (EPM)

The EPM for mice (Lister, 1987) was used with few modifications and designed as two perpendicular open arms (16cm×5 cm) and two closed arms (16cm×5cm× 12 cm) also in a perpendicular position. The open and closed arms were connected by a central platform (5cm×5 cm). The platform and the lateral walls of the closed arms were made of transparent acrylic. The floor was made of black acrylic. The maze was 25 cm above the floor. One hour after intraperitoneal and oral treatments, respectively, the animal was placed at the centre of the plus maze with its nose in the direction of one of the open arms, and observed for 5 min, according to

the following parameters: number of entries in the open and closed arms, and time of permanence in each of them. The plus maze was carefully cleaned with a wet towel after each animal test. The time of permanence measures the time spent by the animal in the open and closed arms. Anxiolytic compounds reduce the natural animal's aversion to the open arms and promote the exploration thereof. On the other hand, the forced or voluntary passages of the animal into the open arms of the elevated plus maze are associated with hormonal and behavioral changes indicative of increased anxiety. The mice were divided into eleven groups (6 animals/group). Standard drug Diazepam (1 mg/kg, p.o.) used as the positive control and CMC 0.5% was used as vehicle control. *Mitragyna parvifolia* stem-bark extracts at doses of 100, 200, and 400 mg/kg, p.o., were administered in remaining nine groups. All experiments were carried out between 10:00 am and 5:00 pm. After each trial, the EPM apparatus was wiped clean with alcohol (70%) solution [15].

Marble burying test (MBT)

The marble burying test was conducted as described by Yamada et al. (2002) with slight modifications. Individual mice were placed for 30 min in a plastic cage with the same dimensions as their home cage and that contained saw dust as a bedding material; they were then returned to their home cages (habituation trial). Twenty four clear glass marbles (diameter, 15 mm) were spaced 3 cm apart in four rows of six, on approx. 5-cm layer of sawdust bedding which was lightly pressed down to make a flat even surface, in a plastic habituation cages used for each individual test. Mice were reintroduced to these cages (each test mouse was returned to the same cage in which they had been habituated). After 30 min, the test was terminated, and the number of buried marbles (marbles more than 2/3 covered with bedding materials) was counted. After each trial, the sawdust was replaced, and the test apparatus and glass marbles were washed by water and cleaned with 70% alcohol [16,17,18,19]. All the doses of test extracts were same as used in EPM test except Fluoxetine (10 mg/kg, p.o.) which was used as standard drug for positive control and vehicle treated group CMC 0.5% was taken as negative control.

Results

Acute toxicity test

No mortality or any signs of toxicity was observed after the administration of *M. parvifolia* stem-bark extracts at the dose of 5000 mg/kg, p. o. From the results of acute toxicity study, three doses (100, 200, 400 mg/kg, p.o.) of were selected for further pharmacological studies. Thus the LD₅₀ of *M. parvifolia* may be assumed as > 5000 mg/kg body weight.

Effect of MP on the Elevated Plus-Maze (EPM)

In the elevated plus maze test, our results show (Table 1.) that similarly to diazepam which increased the time spent in the open arms, All the extracts of *M. parvifolia* in the doses of 100, 200 and 400 mg/kg also increased this parameter. The effect was dose-dependent and, at the doses of 200 and 400 mg/kg of MPBEA and MPBAlk it increased the time spent in the open arms significantly ($P < 0.01$). Furthermore, significant increases in the number of entrances in the open arms were also observed, after administration of MPBM, MPBEA and MPBAlk at both doses, as compared to control (Table 2.). The time spent in closed arm and numbers of entries in closed arm were also observed. The MPBEA and MPBAlk showed significant ($P < 0.01$) decrease in the time spent in closed arm (Table 3.), but number of closed arm entries were

increased significantly ($P < 0.01$) at dose of 400 mg/kg of MPBAIk and standard diazepam (1 mg/kg) (Table 4.).

Marble-Burying Test (MBT)

To examine this premise, we studied the effect of the representative of *M. parvifolia* extracts on burying behavior. MPBM at the dose of 400 mg/kg reduced number of marbles buried by mice significantly ($P < 0.01$), whereas MPBEA and MPBAIk at the dose of 200mg/kg and 400mg/kg reduced number of marbles buried very significantly ($P < 0.01$) as compare to vehicle control group. As expected, positive control Fluoxetine (10 mg/kg, p.o.) exhibited significant decrease in the marble burying behavior (Table 5.).

Table 1: Effect of oral administration of *Mitragyna parvifolia* stem-bark extracts of on Time spent (Seconds) in open arm in elevated plus maze

Sr. No.	Con trol	MPB M 100	MPB M 200	MPB M 400	MPBE A 100	MPBE A 200	MPBE A 400	MPBAI k 100	MPBAI k 200	MPB Alk 400	STD. (Diazepam) 1mg/kg
1	2	2	3	4	5	6	5	5	12	11	22
2	1	4	2	5	4	7	7	8	9	16	14
3	3	2	3	5	4	7	3	10	10	19	18
4	2	1	4	4	3	8	7	4	11	14	12
5	1	2	4	6	2	6	6	3	13	8	19
6	4	2	2	5	3	7	6	5	11	9	20
	2.2 ±0.48	2.2±0.40	3.0±0.37	4.8±0.31	3.5±0.43	6.8±0.31**	5.7±0.61*	5.8±1.1*	11.0±0.58**	13.0±1.7**	18.0±1.5**

Table 2:Effect of oral administration of *Mitragyna parvifolia* stem-bark extracts of on No. of entries in open arm in elevated plus maze

Sr. No.	Con trol	MPB M 100	MPB M 200	MPB M 400	MPBE A 100	MPBE A 200	MPBE A 400	MPBA lk 100	MPBAI k 200	MPBA lk 400	STD. (Diazepam) 1mg/kg
1	0	1	1	1	2	2	2	2	2	4	4
2	0	0	0	0	0	1	2	2	1	3	1
3	1	0	1	2	1	2	2	1	3	2	3
4	0	0	2	0	1	1	1	2	2	3	5
5	0	1	1	1	2	2	3	2	2	2	4
6	0	0	0	2	1	1	1	1	1	3	5
	0.17 ±0.17	0.33±0.21	0.83±0.31	1.0±0.37	1.2±0.31	1.5±0.22*	1.8±0.31**	1.7±0.21*	1.8±0.31**	2.8±0.31**	3.7±0.61**

Table 3: Effect of oral administration of *Mitragyna parvifolia* stem-bark extracts of on

Time spent (Seconds) in closed arm in elevated plus maze.

Sr. No.	Control	MP BM 100	MPB M 200	MPBM 400	MPBE A 100	MPBE A 200	MPBE A 400	MPBAI k 100	MPB Alk 200	MPBAI k 400	STD. (Diazepam) 1mg/kg
1	298	284	280	270	271	250	237	254	240	205	175
2	295	287	273	263	263	246	249	250	235	215	187
3	275	280	265	253	256	261	247	247	231	201	200
4	284	278	277	261	275	245	255	245	224	217	208
5	295	271	269	268	269	258	235	258	242	210	197
6	270	281	274	251	261	260	239	249	230	219	192
	290±4.8	280±2.2	270±2.2*	260±3.2**	270±2.9**	250±2.9**	240±3.2**	250±1.9**	230±2.7**	210±2.9**	190±4.7**

Table 4: Effect of oral administration of *Mitragyna parvifolia* stem-bark extracts of on No. of entries in Closed arm in elevated plus maze.

Sr. No.	Control	MP BM 100	MP BM 200	MP BM 400	MPBE A 100	MPBE A 200	MPBE A 400	MPBAI k 100	MPBAI k 200	MPBAI k 400	STD. (Diazepam) 1mg/kg
1	1	1	1	4	2	5	5	5	6	11	20
2	1	4	5	2	4	3	4	6	5	9	3
3	4	2	7	7	5	2	9	2	8	13	9
4	6	3	3	5	7	7	8	5	6	6	3
5	2	4	6	2	2	4	7	7	9	12	12
6	3	7	4	7	5	8	10	7	4	15	30
	2.8±0.79	3.5±0.85	4.3±0.88	4.5±0.92	4.2±0.79	4.8±0.95	7.2±0.95	5.3±0.76	6.3±0.76	11.0±1.3**	13.0±4.3**

Values represent mean ± SEM, n=6, One way ANOVA followed by Dunnett's multiple comparison test

*p < 0.05, **p < 0.01 compare with control group

Statistical analysis

The data are presented as mean ± SEM. The statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. $P < 0.05$ was considered statistically significant.

Table 5: Effect of oral administration of *Mitragyna parvifolia* stem-bark extracts of on number of marble burying in mice.

Values represent mean \pm SEM, n=6, One way ANOVA followed by Dunnett's multiple comparison test
*p < 0.05, **p < 0.01 compare with control group

S r . N o .	Contr ol	MPBM 100	MPBM 200	MPBM 400	MPBE A 100	MPBE A 200	MPBEA 400	MPBA lk 100	MPBAIk 200	MPBAIk 400	STD. (Fluoxe tine) 10 mg/kg
1	21.	13.	23.	5.	9.	9.	8.	2.	3.	7.	1.
2	22.	16.	15.	8.	21.	11.	7.	5.	8.	0.	2.
3	20.	17.	21.	11.	18.	12.	10.	7.	5.	4.	2.
4	23.	21.	8.	10.	12.	14.	13.	6.	5.	3.	1.
5	22.	12.	12.	14.	22.	8.	11.	4.	4.	5.	0.
6	20.	24.	18.	9.	6.	12.	10.	9.	6.	2.	1.
	21 \pm 0. 49	17 \pm 1.9	16 \pm 2.3	9.5 \pm 1.2 **	15 \pm 2.7 *	11 \pm 0.8 9**	9.8 \pm 0.87 **	5.5 \pm 0.9 9**	5.2 \pm 0.7* *	3.5 \pm 0.99 **	1.2 \pm 0.3 1**

Discussion

There has been a considerable popular interest in the use of the so-called natural remedies, or herbal products, to treat anxiety and depression. Recently, several plants have been reported to possess anxiolytic effects in different animal models of anxiety. Various traditional herbal medicines have also been suggested to possess anxiolytic activity. Some herbs such as St. John's wort and ginseng clinically have been introduced for the treatment of anxiety [20-23]. The elevated plus-maze is one of the many tests for the identification of anxiolytic or anxiogenic effect of a drug in rodents [24]. Several plants increase the exploration of open arms in the elevated plus-maze test and are used to diminish anxiety in folk medicine.

Effects of diazepam on mice anxiety behavior in EPM

The elevated plus-maze is currently one of the most widely used model of animal anxiety, having been employed by many research laboratories in the last decade and has been extensively validated for use with both rats and mice [15,25]. Its validity in our study was supported by the observation that diazepam, a classic anxiolytic, significantly increased the time spent in the open arms. The behavior observed using the EPM in the present study confirmed the anxiolytic activity of diazepam as reported previously [26]. The classic anxiolytic benzodiazepine, diazepam 10 mg/kg was used as positive control. Statistical analysis of plus maze data revealed that diazepam significantly increased the time spent in open arms. These results confirm the suitability of the method used in the present study.

Effects of MPBM, MPBAIk and MPBEA extract on the EPM

In the present study, MPBM extract does not produce open arm exploration significantly at all doses administered, since we have demonstrated that both MPBAIk (100, 200 and 400 mg/kg) and MPBEA (100, 200 and 400 mg/kg), following acute oral administration, produced a dose-

dependent anxiolytic-like effect in mice as measured by an increased open arm exploration in the EPM.

MPBAIk doses upto 400 mg/kg were chosen to clarify anxiety effects in EPM model. Benzodiazepines (BDZs) 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, muscle relaxation, ataxia, amnesia, ethanol and barbiturate potentiation and tolerance. Alkaloids are the most important secondary metabolites in many plants that are held responsible for their sedative and anxiolytic actions [27]. Since anxiolytic effect of *Mitragyna parvifolia* might be due to the presence of various indole and oxindole alkaloids [10,11]. These alkaloids are responsible for regulation of fear and anxiety which is strongly associated with the central GABA and serotonergic (5-HT) systems. Recently however, other neurotransmitter systems (such as cholinergic, dopaminergic and glutamatergic) in modulating emotional behavior have received attention. It has been suggested that NMDA antagonists are potential nonclassical anxiolytics [27].

The MPBAIk extract, similarly to diazepam, increased the time spent and number of entries in the open arms. Whereas time spent in closed arm was decreased significantly and in dose dependent manner. These results are suggestive that MPBAIk has an anxiolytic-like effect in the plus-maze test. The behavioral profile induced by MPBAIk extract is similar to that induced by diazepam. Our data represents that this alkaloid fraction of plant has a significant and very potent anxiolytic effect similar to diazepam.

Searching for safer BDZ-receptor (BDZ-R) ligands it has been demonstrated the existence of a new family of ligands which have a flavonoids structure, first isolated from plants used as tranquilizers in folkloric medicine, some natural flavonoids have been shown to for benzodiazepine receptors. Some of those compounds, such as 6,3-dinitroflavone were found to have a very potent anxiolytic effect [28]. These compounds exhibit a high affinity for the benzodiazepine receptors. Due to their selective pharmacological profile and low intrinsic efficacy at the benzodiazepine receptors, flavonoids derivatives, such as those described, could represent an improved therapeutic tool in the treatment of anxiety.

Since MPBEA doses upto 400 mg/kg were taken to clarify anxiety effects of flavonoids in EPM model. Some members of the family of flavonoids have been demonstrated to have moderate binding affinities for the benzodiazepine-site. In vivo studies revealed that these compounds were mostly partial agonists of GABA_A receptors, and only a few flavonoids were shown to possess antagonistic activities [29]. Considering some plant species, Wolfman et al. [30] have postulated that chrysin, a natural monoflavonoid, is a ligand for central benzodiazepine receptors. A major problem of anxiolytic compounds is that their anxiolytic activity cannot be easily separated from sedation [31, 32]. At high doses, for example, diazepam starts to reduce the activity of the rats, as hinted at by the significantly reduced unpunished licks in the Vogel conflict test, a parameter related to locomotor activity [33, 34]. The anxiolytic-like effect of MPBEA observed in the present study seems not to be associated with any motor effects, since no significant behavior change of mice was observed in the open field. This leads to the assumption that the anxiolytic-like effect of *Mitragyna parvifolia* extracts is selective without producing benzodiazepine-like side effects such as sedation, muscle relaxation or ataxia.

Using this test the MPBEA increased the time spent and number of entries in the open arms. The MPBEA extract, similarly to diazepam, increased the time spent and number of entries in the open arms. These results are suggestive that MPBEA has an anxiolytic-like effect in the plus-maze test. The behavioral profile induced by MPBEA extract is similar to that induced by diazepam. Our data represents that ethyl acetate extract of plant has a significant but not very potent anxiolytic effect.

Effects of MPBM, MPBAlk and MPBEA extract on the Marble burying test (MBT)

MPBM does not decreased number of marbles buried by mice significantly, while MPBAlk and MPBEA decreased the number of marbles buried. This activity is similar to marble burying which was reduced by acute administration of different classes of antidepressants with slow-onset anxiolytic properties in the clinic such as selective serotonin reuptake inhibitors, SSRIs (citalopram, paroxetine, fluoxetine), an serotonin and noradrenaline reuptake inhibitors, SNRI (duloxetine), an tricyclic antidepressants, TCA (clomipramine) and an MAOI (phenelzine) [35]. Our data represents that MPBAlk and MPBEA extract of plant has a highly significant and potent anxiolytic effect.

Conclusion

The *Mitragyna parvifolia* stem bark extracts produced significant anxiolytic effects when subjected to EPM and MBT models of anxiety. The MPBM extracts showed satisfactory effects but MPBEA and MPBAlk extracts showed highly significant activities. MPBAlk extract was very potent in effect similar to standard such as diazepam and Fluoxetine in experimental animals. Since MPBEA and MPBAlk contains flavonoids and alkaloids respectively, further investigation of constituents responsible for above activity can be done by isolating and characterizing them for future research.

Acknowledgements

The authors would like to thank Principal, R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur, India for the facilities provided.

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