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Pharmacology, Biochemistry and Behavior 71 (2002) 239–244

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Anxiogenic activity of *Myristica fragrans* seeds

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Received 28 March 2001; received in revised form 7 August 2001; accepted 11 September 2001

Abstract

In the present study, the *n*-hexane extract of *Myristica fragrans* (MF) seeds, acetone-insoluble part of the *n*-hexane extract (AIMF) and trimyristin (TM) were assessed for their anxiogenic activity. The MF (10 and 30 mg/kg), AIMF (30, 100, and 300 mg/kg), and TM (10, 30, and 100 mg/kg) administered intraperitoneally exhibited anxiogenic activity in elevated plus-maze (EPM) paradigm. The open-field test and hole-board test were also used to assess anxiogenic activity of AIMF and TM. In the EPM test, MF, AIMF, and TM decreased the time spent by mice in the open arm and the entries in the open arm. Further, the effect of diazepam (1 mg/kg ip), serotonin 5-HT3 receptor antagonist, ondansetron (1 mg/kg ip), and 5-HT1A receptor agonist, buspirone (1 mg/kg ip), on the occupancy in open arm and entries in open arm was significantly reduced by TM. In the open-field test, AIMF as well as TM reduced the number of rearing and locomotion. Both TM and AIMF reduced the number of head pock in the hole-board test. Inhibition of anxiolytic activity of ondansetron (5-HT3 receptor antagonist), buspirone (5-HT1A receptor agonist), and diazepam [acting on γ-aminobutyric acid (GABAA) receptor] suggests a nonspecific anxiogenic activity of TM and also a link between 5-HT and GABA systems in the anxiogenic activity of TM.

Keywords: Anxiogenic; *Myristica fragrans*; Trimyristin; Diazepam; Ondansetron; Buspirone

1. Introduction

Anxiety, which may be understood as the pathological counterpart of normal fear, is manifested by disturbances of mood, as well as of thinking, behavior, and physiological activity. The etiology of most anxiety disorders, although not fully understood, has come into sharper focus in the recent past. The benzodiazepines (BZs) are a large class of relatively safe and widely prescribed medications that exhibit rapid and profound anti-anxiety and sedative–hypnotic effects. There is sufficient evidence for the role of γ-aminobutyric acid (GABAA) receptor function in anxiety and a significant proportion of anxiety patients has a reduced sensitivity to BZs. When BZ binds to the subunit of GABA receptor complex, it causes a conformational change in the receptor complex that leads to increased affinity between GABA and its corresponding receptor site on the β subunit. The increased affinity between GABA and the receptor leads to a greater possibility of GABA binding. Thus, BZs are the allosteric modulators of GABA and the end result is increased hyperpolarization of the cell (Fogelman and Greenblatt, 2000). Sibille et al. (2000) have recently reported that serotonin 5-HT1A receptor knockout mice display BZ-resistant anxiety and they have suggested a link between 5-HT and GABA. In the search for an alternative to the BZs, 5-HT1A receptor agonists and 5-HT3 receptor antagonists are currently being considered for their potential use in the treatment of fear and anxiety-related disorders. Multiple serotonin receptors such as 5-HT1A, 5-HT2A, 2C, and 5-HT3 receptors are implicated in modulation of anxiety (Blackburn et al., 1993; Fuller, 1991; Moser, 1989; Nutt and Glue, 1991; Rocha et al., 1994; Nutt, 1999). Recently, a functional polymorphism in the promoter region of the serotonin transporter gene has been linked to anxiety (Menza et al., 1999).

Plants have multiple pharmacological actions as they contain numerous constituents of diverse chemical nature and have been an important source of medicines. Some plants like *Hunteria zeylanica* exhibit stimulant as well as depressant actions. Such stimulatory and inhibitory effects

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0091-3057/01/$ – see front matter
PII: S0091-3057(01)00660-8

are induced simultaneously in the late state of anxiety (Leewanich et al., 1996; Nutt and Glue, 1991). Seeds of *Myristica fragrans Houtt* (MF; Family: *Myristicaceae*) are psychotomimetic and possess both stimulatory and depressant activities. In addition, MF, commonly known as nutmeg, also possesses carminative, astringent, hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, and anti-inflammatory activities (Evans, 1996; Nadkarni, 1998). The kernel contains volatile oil, fats, starch, and mucilage. Fixed oil contains myristicin, myristic acid while volatile oil contains pinene, sabinene, camphene, elemicin, isoelemicin, eugenol, methoxyeugenol, isoeugenol, safrole, etc. (Isogai et al., 1973; Evans, 1996; Janssen and Lackman, 1990). Eugenol, the major constituent of volatile oil inhibits lipid peroxidation and maintains activities of superoxide dismutase, catalase, glutathione peroxidase, glutamine transferase, and glucose 6-phosphate dehydrogenase (Kumarvelu et al., 1996). Chloroform extract of nutmeg showed analgesic and anti-inflammatory activity in rodents (Olajide et al., 1999; Ozaki et al., 1989). It has been suggested that nutmeg, when consumed in sufficient quantities (about 20 g or two to three nutmeg seeds), acts as a hallucinogen and nutmeg devoid of volatile oil retains the gastrointestinal properties but not the psychotropic property (Truitt et al., 1961). Although the anxiogenic principle may not find any use in therapeutics, it can be a useful research tool in experimental pharmacology.

The preliminary studies on the neuropharmacological actions of the *n*-hexane extract produced twitches, palpebral closure, hypersensitivity to touch, vocalization when being approached, and decreased rearing. Several reports suggest that both stimulatory and inhibitory effects are induced simultaneously in the late state of anxiety (Leewanich et al., 1996; Nutt and Glue, 1991). Therefore, we have studied the anxiogenic activity of crude *n*-hexane extract of MF, the acetone-insoluble part of MF (AIMF), and a pure compound trimyristin (TM). The anxiogenic activity was studied using different animal models of anxiety based on exploratory behavior.

2. Materials and methods

2.1. Preparation of extract and isolation of TM

Nutmeg seeds were purchased from a commercial source. Seeds (1.0 kg) were crushed to a coarse powder and extracted with *n*-hexane using Soxhlet's extractor. The *n*-hexane extract (470 g) was concentrated under reduced pressure and then dried in air. The extract was then partitioned into acetone-soluble (70.5 g) and acetone-insoluble parts (399 g). AIMF was concentrated under reduced pressure and it was charged into the chromatography column of neutral alumina. The AIMF was eluted with *n*-hexane (yield 0.8% w/w of the seeds). The eluent was concentrated under reduced pressure and spotted on the thin-layer chromatography plate of silica gel-G using *n*-hexane as a mobile phase showed a single spot indicating its purity. On the basis of melting point, infrared (IR) spectroscopy, mass spectrum and chromatographic comparison with standard TM, the *n*-hexane fraction was identified as TM.

2.2. Animals

Male Albino mice weighing 22–25 g were obtained from the National Institute of Toxicology, Pune. Animals were housed into groups of six to eight per cage at an ambient temperature of 25 ± 1 °C and relative humidity 45–55%. A 12:12-h dark/light cycle was followed during the experiments. Animals had free access to food (Hindustan Liver, India) and water. Animals were deprived of food but not water 12 h before the experiments. The person unaware of treatments observed the animals. All experiments were carried out between 1000 and 1500 h. The Institutional Animal Ethical Committee approved the protocol of this study.

2.3. Drugs and chemicals

meta-Chloro phenyl piperazine (*m*-CPP, Sigma, USA); ondansetron (Cheminor Laboratories, India); diazepam (Anxol inj, Sigma, India); buspirone (Sun Pharma, India) were used in this study. The drugs were dissolved in water for injection, diluted to the required strength, and administered intraperitoneally in a volume of 5 ml/kg. The *n*-hexane extract of MF seeds (10 and 30 mg/kg), AIMF (30, 100, and 300 mg/kg) as well as TM (10, 30, and 100 mg/kg) were suspended in polyethylene glycol 400 (PEG) and administered intraperitoneally. The pH of MF, AIMF, and TM suspensions was 7.2, 6.8, and 7.1, respectively. The solvents such as acetone and *n*-hexane were purchased from Modern Scientific, Nashik, India.

2.4. Behavioral studies

2.4.1. Elevated plus-maze (EPM)

The EPM consisted of two open arms (25×5 cm) crossed with two closed arms ($25 \times 5 \times 20$ cm). The arms were connected together with a central square of 5×5 cm. The apparatus was elevated to the height of 25 cm in a dimly illuminated room. Mice in groups of six were treated with vehicle or MF (10 and 30 mg/kg), AIMF (30–300 mg/kg), or TM (10–100 mg/kg) 30 min before placing individually in the centre of the EPM facing a closed arm and the time spent in both the open and closed arms was recorded for 5 min (Pellow and File, 1986). The entries into open and closed arms were also counted during this test interval (Pellow et al., 1985). An entry was defined as all four paws in the arm. The EPM was cleaned with hydrogen peroxide after each trial. The anxiogenic potential of TM was also studied in the presence of anxiolytics like ondansetron

Table 1
Effect of AIMF and TM on the time spent in open arm and entries in open and closed arms in mice ($n=6$)

Treatment (dose: mg/kg)	Time spent in open arm (s) (mean ± S.E.M.)	Entries in open arm (mean ± S.E.M.)	Entries in closed arm (mean ± S.E.M.)
Vehicle	26.0 ± 1.3	4.6 ± 0.2	9.0 ± 0.6
MF (10)	22.1 ± 1.9	3.7 ± 0.5	7.5 ± 0.4
MF (30)	9.0 ± 1.0	2.2 ± 0.3*	6.0 ± 1.1*
AIMF (30)	4.0 ± 0.8*	2.0 ± 0.2*	7.7 ± 1.4
AIMF (100)	2.0 ± 0.2*	1.0 ± 0.1*	6.2 ± 1.1*
AIMF (300)	0.8 ± 0.2*	0.8 ± 0.2*	3.7 ± 0.8*
TM (10)	8.2 ± 1.9*	2.6 ± 0.2*	3.2 ± 0.6*
TM (30)	3.0 ± 0.2*	0.8 ± 0.3*	2.4 ± 0.2*
TM (100)	4.2 ± 0.5*	0.7 ± 0.3*	3.0 ± 0.7*
m-CPP (1)	–	294.2 ± 2.5*	1.25 ± 0.38*

* $P<.05$, ANOVA followed by Dunnett's test.

(0.5 mg/kg), a serotonin 5-HT3 receptor antagonist, diazepam (1 mg/kg), and buspirone (1 mg/kg), a 5-HT1A receptor agonist which were administered 30 min before TM.

2.4.2. Open-field test (Turner, 1972)

2.4.2.1. Apparatus. The apparatus consisted of a wooden box (96 × 96 × 5 cm). The floor of the box was divided into 16 squares (6 × 6 cm). The apparatus was illuminated with a 40-W lamp suspended 100 cm above. Mice divided into groups of six each, received the AIMF (30–300 mg/kg)/TM (10–100 mg/kg) or vehicle. After 30 min, they were placed individually in one corner-square and the time required to leave the corner-square was recorded as transfer latency. The number of rearing, assisted rearing (forepaws touching the walls of the apparatus), and the number of squares traversed were counted for 5 min. Diazepam (1 mg/kg) and ondansetron (0.5 mg/kg) were used as standard anxiolytics.

2.4.3. Hole-board apparatus

The hole-board apparatus was used as described earlier by Clark et al. (1971). The apparatus consisted of wooden box (40 × 40 × 25 cm) with 16 holes (diameter 3 cm) evenly distributed on the floor. The apparatus was elevated to the height of 25 cm. Mice ($n=6$) were treated with the AIMF (30–300 mg/kg) or TM (10–100 mg/kg) or vehicle 30 min before placing in the apparatus and the number of head pokes during the 5-min period were recorded. *m*-CPP (1 mg/kg ip), an anxiogenic agent with 5-HT2 receptor agonist activity, was used as a reference anxiogenic.

2.5. Acute toxicity

The AIMF was administered in doses of 30, 100, 300, 1000, and 3000 mg/kg ip and TM was administered in doses of 10, 30, 100, 300, and 1000 mg/kg ip to groups of mice ($n=10$) and the number of animals dying in 24 h was noted in each group.

2.6. Statistical analysis

The results are given as mean ± S.E.M. The data obtained were analyzed by one-way analysis of variance (ANOVA) followed by Student's *t* test. Mann–Whitney *U* test was used for nonparametric data. Differences were considered significant at the 5% level.

3. Results

3.1. Elevated plus-maze

The vehicle-treated mice spent 26.0 ± 1.3 s in the open arm, whereas animals treated with MF, AIMF, and TM spent significantly less time in the open arm and the effect was dose dependent. The MF in a dose of 30 mg/kg, and the AIMF as well as TM in all doses tested, also reduced the entries in both the open and the closed arms significantly. The observations are given in Table 1.

In another experiment, TM significantly and dose-dependently reduced the effect of the reference anxiolytic agents, namely, ondansetron, diazepam, and buspirone on the duration of occupancy in the open arm. Ondansetron and diazepam significantly increased the number of entries in the open arm. The TM significantly decreased the entries in both the arms dose dependently. The observations are given in Table 2.

Table 2
Reversal of the effect of ondansetron, diazepam, and buspirone by TM on the time spent in open arm and entries in open and closed arms in mice ($n=5$)

Treatment (dose: mg/kg)	Time spent in open arm (s) (mean ± S.E.M.)	Entries in open arm (mean ± S.E.M.)	Entries in closed arm (mean ± S.E.M.)
TM (0)	22.3 ± 3.2	4.8 ± 0.8	10.0 ± 1.0
Ondan (0.5)	72.2 ± 5.9*	10.4 ± 1.2*	10.2 ± 1.3
Ondan (0.5) +			
TM (10)	16.2 ± 2.2@	6.5 ± 0.8@	8.0 ± 0.7
TM (30)	12.0 ± 0.9@	5.5 ± 0.8@	7.6 ± 1.0
TM (100)	5.0 ± 0.7@	3.2 ± 0.8@	6.4 ± 0.1@
Diazepam (1)	48.7 ± 3.9*	8.5 ± 1.0*	12.1 ± 0.4*
Diazepam(1) +			
TM (10)	8.0 ± 1.4#	4.0 ± 0.8#	8.5 ± 1.4
TM (30)	4.6 ± 0.6#	3.6 ± 0.3#	5.0 ± 1.0#
TM (100)	1.2 ± 0.3#	3.8 ± 0.3#	9.0 ± 1.2
Buspirone (1)	42.0 ± 5.6*	5.5 ± 0.7	12.0 ± 1.5
Buspirone (1) +			
TM (10)	8.6 ± 1.2†	2.6 ± 0.2†	6.3 ± 1.2†
TM (30)	2.3 ± 0.5†	3.0 ± 0.6†	9.6 ± 2.4
TM (100)	2.0 ± 0.2†	2.0 ± 0.8†	5.6 ± 1.0†

Ondan = ondansetron.
* $P<.05$, compared to vehicle-treated group.
@ $P<.05$, compared to ondansetron-treated group.
# $P<.05$, compared to diazepam-treated group.
† $P<.05$, compared to buspirone-treated group. ANOVA followed by Dunnett's test.

Table 3
Effect of AIMF on rearing and locomotion in the open-field test in mice ($n=6$)

Treatment (dose: mg/kg)	Rearing (mean ± S.E.M.)	Assisted rearing (mean ± S.E.M.)	Number of squares traversed (mean ± S.E.M.)
Vehicle	13.0 ± 2.5	31.5 ± 6.5	135.0 ± 8.5
AIMF (30)	5.2 ± 1.2*	9.5 ± 3.2*	63.7 ± 12.2*
AIMF (100)	3.5 ± 0.5*	10.0 ± 3.7*	59.7 ± 8.8*
AIMF (300)	1.2 ± 0.2*	2.4 ± 0.6*	30.2 ± 5.7*
m-CPP (1)	5.0 ± 1.2*	9.3 ± 2.9*	85.2 ± 4.6*

* $P<.05$ (Student's t test).

3.2. Open-field test

The mice treated with vehicle reared for 13.0 ± 2.5 times during the test interval of 5 min. The animals showed increased tendency to take support of the walls of the apparatus as indicated by the greater number of rearing touching the walls (assisted rearing) of the apparatus. The AIMF significantly ($P<.05$) reduced the number of rearing dose dependently and the effect was more prominent on the assisted rearing indicating decrease in locomotion. A significant decrease in locomotion was also observed in the third parameter, i.e., decrease in the number of squares traversed. The anxiogenic agent *m*-CPP also reduced the number of rearing, assisted rearing, and the number of squares traversed. The observations are given in Table 3. TM also exhibited a similar decrease in rearing, assisted rearing, and the number of squares traversed. Both diazepam and ondansetron increased the number of rearing, assisted rearing, and the number of squares traversed significantly ($P<.05$). The observations are summarized in Table 4.

3.3. Hole-board apparatus

The decrease in the number of head poking is a simple yet reliable parameter to assess the anxiogenic activity of a test compound. The animals treated with vehicle exhibited 55.2 ± 2.55 head poking, whereas AIMF reduced the number of head poking significantly and dose dependently. A similar decrease was observed with TM also. The reference standard anxiogenic *m*-CPP also reduced head poking. The observations are given in Table 5.

Table 4
Effect of TM on rearing and locomotion in the open-field test in mice ($n=6$)

Treatment (dose: mg/kg)	Rearing (mean ± S.E.M.)	Assisted rearing (mean ± S.E.M.)	Number of squares traversed (mean ± S.E.M.)
Vehicle	10.2 ± 1.5	27.5 ± 3.9	118.0 ± 12.8
TM (10)	2.2 ± 0.4*	8.2 ± 2.6*	34.3 ± 4.6*
TM (30)	2.0 ± 0.9*	8.6 ± 1.2*	21.2 ± 4.6*
TM (100)	1.7 ± 0.2*	6.4 ± 0.9*	22.8 ± 3.6*
Diazepam (1)	16.2 ± 0.8*	38.2 ± 1.8*	155.0 ± 8.4*
Ondansetron (0.5)	17.2 ± 0.9*	36.5 ± 0.8*	169.0 ± 8.5*

* $P<.05$, compared to vehicle-treated group (Student's t test).

Table 5
Effect of AIMF and TM on the number of head poking in the hole-board apparatus in mice ($n=6$)

Treatment	Dose (mg/kg)	Number of head poking (mean ± S.E.M.)
Vehicle	–	55.2 ± 2.55
AIMF	30	36.4 ± 4.87*
	100	24.2 ± 3.53*
	300	17.8 ± 1.88*
TM	10	26.3 ± 2.46*
	30	12.0 ± 1.56*
	100	22.0 ± 2.56*
m-CPP	1	26.0 ± 3.9*

* $P<.05$ (Student's t test).

3.4. Acute toxicity

Mortality was not observed in any group that received 3.0 g/kg of AIMF or 1.0 g/kg of TM.

4. Discussion

The decrease in occupancy in open arm and reduction in the open-arm entries strongly indicate the anxiogenic activity of the *n*-hexane extract of MF seeds, the AIMF, and TM. TM was found to be more anxiogenic than AIMF. In the EPM test, decreased occupancy in the open arm and/or reduction in the open-arm entries in relation to the total arm entries, provides a measure of fear-induced inhibition of exploratory activity which is attenuated by anxiolytics and increased by anxiogenic agents (Pellow and File, 1986; Pellow et al., 1987). Different categories of anxiolytic agents such as diazepam, acting on GABAA receptor; buspirone, a 5-HT1A receptor agonist; and ondansetron, a 5-HT3 receptor antagonist were used to study the antagonism between these agents and the TM. Thus, TM induced a nonspecific anxiogenic activity as all the anxiolytic agents used in this study antagonized its effect. Many anxiolytic agents act differentially in the EPM with regard to the arm entries, time spent in the open arm suggesting nonspecific nature of anxiety or multiplicity of drug actions. Imaizumi et al. (1994) have shown that diazepam (4 and 8 mg/kg) increased the open-arm entries and time spent on the open arm without changes in the total arm entries; buspirone increased open-arm entries and the time spent in the open arm at high dose but decreased the total arm entries and the 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) decreased the total arm entries but did not changed the open-arm entries and time spent on the open arms. Researchers have reported different effects of buspirone on the EPM test, i.e., anxiolytic (Dunn et al., 1989; Kshama et al., 1990; Lee and Rodgers, 1991), noneffective (Moulton and Morinan, 1990;

Wada and Fukuda, 1991), anxiogenic (Klint, 1991; Moser, 1989; Rocha et al.,1994). Therefore, anxiety-modulating drugs must be carefully assessed on the EPM, especially those acting on 5-HT receptors. Therefore, in the present study, the EPM test was supplemented by other tests like the open-field test and the hole-board test. We noted significant decrease in the time spent in the open arm and the number of entries in the open arm and also the total number of entries. Thus, the observations suggested anxiogenic activity of both AIMF and TM in the elevated maze.

File and Wardil (1975) have assessed the anxiogenic or anxiolytic activity of some agents using the hole-board test. We observed that AIMF and TM reduced head poking (dipping) in the hole-board apparatus and a similar effect was produced by *m*-CPP, a 5-HT2 receptor agonist known to induce anxiety (Rodgers et al., 1992). In the open-field test, we noticed a decrease in rearing as well as locomotion by both AIMF and TM, whereas the anxiolytic agents increased both rearing as well as locomotion. We have assessed the effect of AIMF and TM on one more parameter, i.e., assisted rearing in which an animal rears taking support of walls of the apparatus. The vehicle-treated group showed increased tendency to reach to the walls and rear rather than rearing without support, which was substantially reduced by the anxiogenic and increased by the anxiolytics. This is also suggestive of the decreased locomotion by anxiogenic agents. Decrease in locomotion is indicative of diminished dopaminergic transmission, which may be secondary to the rise in 5-HT level caused by anxiogenic agents (Jones et al., 1992; Kahn et al., 1988).

In view of the multiple types of anxiety behavior (Salzman et al., 1993; Sibille et al., 2000; Tsuji et al., 2000), it is reasonable to believe that some anxiolytic agents will act differently to the same anxiogenic agents. However, TM inhibited anxiolytic activity of ondansetron (5-HT3 receptor antagonist), buspirone (5-HT1A receptor agonist), and diazepam (acting on GABAA receptor) suggesting a link between 5-HT and GABA systems. Thus, it is concluded that MF, AIMF, and TM possess anxiogenic activity. More investigations are necessary to identify the other anxiogenic principles of nutmeg, if any, and to know their mechanism of anxiogenic action.

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