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To evaluate anti-anxiety activity of thymol

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ABSTRACT

Objective: To evaluate anti-anxiety activity of thymol (5, 10, 20 mg/kg. i.p.) in Swiss albino mice. **Methods:** Six group (n=5) of mice were used in this study. Drug was given to each animal intraperitonealy, behavior testing was performed in animal models after 30 min of all treatment, time spent in light area/open space was observed for 5 min duration (300 s). Significant increase in percentage of time spent in open arms of EPM and significant increase in percentage of time spent in light compartment of LDT indicate anxiolytic-like effect respectively. Significant decreased in above parameters indicates anxiogenic effect. **Results:** Thymol 20 mg/kg significantly increased percentage of time spent in open arms of EPM and light compartment of LDT as compared to their vehicle treated group. **Conclusions:** Thymol (20 mg/kg) produced significant anti-anxiety effect as compared to vehicle (0.01% ethanol) treated mice in both EPM and LDT behavioral models.

1. Introduction

Anxiety disorders are common mental disorders that share extreme or pathological anxiety as the primary disturbance in mood or emotional tone[1]. Common denomination of all anxiety disorders is a state of increased fear and exaggerated version of the acute stress response[2]. Literature revealed a link between anxiety and problems with the regulation of various neurotransmitters. The large numbers of neurotransmitters, peptides, hormones, and other neuromodulators have been implicated in fear and anxiety[3]. 5-HT pathway originating from the dorsal raphe nucleus (DRN) and innervating the amygdala and frontal cortex facilitates conditioned fear. Selective 5-HT reuptake inhibitors (SSRIs) and $5-HT_{1A}$ or $5-HT_3$ receptor-selective drugs can have anti-anxiety effects in certain anxiety disorders and animal models[4]. Several preclinical studies have shown that stress and anxiety cause a marked increase in NA release in several rat brain regions, including the hypothalamus, the amygdala, and the LC. γ -Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the brain. The $GABA_A$-benzodiazepine receptor is an important target for several anxiolytic drugs and may therefore play an important role in anxiety-related disorders[5]. It is well reported that Diazepam, $GABA_A$-benzodiazepine receptor agonist at the dose of 2 mg/kg, i.p. significantly showed anti-anxiety activity in various behavioural paradigms of animals[6]. Several studies have shown that positive allosteric modulators (which potentiate GABA action), such as progesterone and allopregnanolone, have anxiolytic effects in various animal models[7]. Several peptides, such as cholecystokinin (CCK), neuro-peptide Y (NPY), tachykinins (substance P, neuro-kinins A and B), and natriuretic peptides (atrial natriuretic peptide or C-type natriuretic peptide) may play important roles in fear and anxiety-related behaviors[8]. There is increasing evidence that NO may underlie anxiety in the elevated plus-maze test, an animal model of anxiety. NO analogues have been found to reduce GABA-gated current via cGMP-dependent pathways, leading to anxiety[9]. cGMP downregulates GABA receptor function in hippocampus, an important area involved in anxiety[10]. The elevated plus-maze (EPM) test, one of the most popular animal models for research on anxiety, is based on the natural aversion of rodents for open spaces and uses an elevated plus-maze with two open and

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two closed arms[11,12]. This test is rapid and was found to be sensitive to the effects of both anxiolytic and anxiogenic agents. The Light/Dark exploration test (LDT) is another commonly used model for anxiety[13], devised by Crawley[14], this test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, i.e. novel environment and light. The LDT has been widely adopted as an anxiolytic screening test in mice[15]. Thymol (2–isopropyl–5–methylphenol) is naturally occurring phenolic monoterpene derivative of cymene, which is found in the oils of thyme and plants such as *Thymus vulgaris*[16], *Thymus glandulosus*[17], *Thymus hyemalis*[18], *Thymus zygis*[19]. It exhibits multiple biological activities: antibacterial[20], antifungal[21], anti–oxidant[22], free radical scavenging[23] and anti–lipid peroxidative[24] properties. Thymol was also shown to have strong anti–inflammatory action by decreasing the release of inflammaroty metabolites like prostanoids, interleukins and leukotrienes[25,26]. Thymol acts as a $GABA_A$ receptor agonist/modulator[27].

2. Materials and methods

2.1. Animals

Swiss albino mice of either sex (20–30 g) were employed in the present study. Animals were procured from Disease Free Small Animal House, LLRUVAS, Hisar, Haryana, India. Animals were provided normal diet and water ad libitum and were exposed to natural light and dark cycle at controlled room temperature of 20–25 ℃. The animals were acclimatized to the laboratory condition before experiments. The animals were kept fasted 2 h before drug administration, all the behavioral paradigm ware performed during day time between 9 a.m. and 2 p.m.[28]. Experimental protocol was approved by Institutional Animal Ethics Committee (IAEC). Care of the animals was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

2.2. Drugs and chemicals

Thymol, Diazepam, Ethanol and Sodium chloride were used in this study. Thymol, was procured from Central Drug House (CDH) Ltd. India. Diazepam (Calmpose®); Ranbaxy Laboratories, Ltd., Gurgaon, India. Normal saline (0.9% NaCl) was used as vehicle for Diazepam while absolute ethanol solution (0.01%) was used as vehicle for Thymol. Volume of injection for mouse was 10 mL/kg.

2.3. Selection of doses

Doses were selected on the basis of literature, *i.e.*, Thymol

(5, 10, 20 mg/kg, i.p.), Diazepam (2 mg/kg, i.p.)[29].

2.4. Behavioral paradigms

2.4.1. Elevated plus maze

The elevated plus maze (EPM) was first proposed as an animal model of anxiety by Handley and Mithani[30]. Elevated plus maze consisted of two close arms, 16 cm×5 cm×12 cm, and two open arm, 16 cm×5 cm, connected to a central platform (5 cm×5 cm). The maze was elevated to a height of 25 cm above the floor. During the experiment each mouse was placed in the central compartment face towards either of open arm, observed for 5 min to record the time spent in open arms, the total observation of experiment was 5 min (300 s). An entry was counted when all four paws of the mouse entered an open or closed arm. Arm entry was defined as all four paws having crossed the dividing line between and arm and the central area[28]. Maze was washed thoroughly with 5% ethanol after each observation to remove odor[31]. Percent of time spent by mice in open arms was calculated as follows:

$$\% = \frac{\text{Number of seconds spent in open arms}}{300 \text{ total sec (5 min observation time)}} \times 100$$

2.4.2. Light/ dark test

The light/dark test (LDT) model was first described by the Crawely and Goodwin[32]. The apparatus consisted of rectangular shaped box (45 cm×27 cm×27 cm) partition into two compartments connected by a 7.5 cm×7.5 cm opening in the wall between the compartments. An animal was placed in the centre of the light compartment face toward opening of the wall and observed for 5 min, for time spent in open (white/light) compartment. Apparatus was thoroughly washed with 5% ethanol after each observation to remove odor[31]. Percent of time spent by mice in light area was calculated as follows:

$$\% = \frac{\text{Number of seconds spent in light compartment}}{300 \text{ total sec (5 min observation time)}} \times 100$$

2.5. Experimental protocol

There were six group of mice used in this study. Each group consisted of five mice. Behavior testing was performed carefully in stepwise manner *i.e.* mice in each group were subjected to maximum two behavioral tests of anxiety, one followed by another to minimize the stress, that may arise from continuous exposure to these paradigms. Doses and routes of administration of drugs were selected according to previous studies as reported in the literature. All treatments (vehicle, 10 mL/kg; Thymol 5, 10 and 20 mg/kg; Daizepam 2 mg/kg) were administered intraperitoneally (i.p.) in a fixed volume of 1 mL/100 g body weight in separate groups of mice. Behavior testing was performed *i.e.* drug was given to

each animal, after 30 min behavior testing was performed in EPM and LDT.

2.6. Statistical analysis

All the results were expressed as mean±SEM. All statistical analysis was done using one way analysis of variance (ANOVA) followed by the Tukey's post hoc test. $P<0.05$ was considered as significant when compared to their respective control group.

3. Results

In elevated plus maze and light/dark test, significant increase in percentage of time spent in open arms and significant increase in percentage of time spent in light compartment indicate anxiolytic–like effect respectively. On the other hand, significant decreased in above parameters indicates anxiogenic effect.

3.1. Effect of different drug treatments on mice behavior in elevated plus maze

Diazepam significantly increased percentage of time spent in open arms of elevated plus maze. Thymol 20 mg/kg significantly increased percentage of time spent in open arms as compared to vehicle (0.01% ethanol) treated mice. Therefore thymol (20 mg/kg) produced significant anti–anxiety effect as compared to vehicle (0.01% ethanol) treated mice (Table 1; Figure 1).
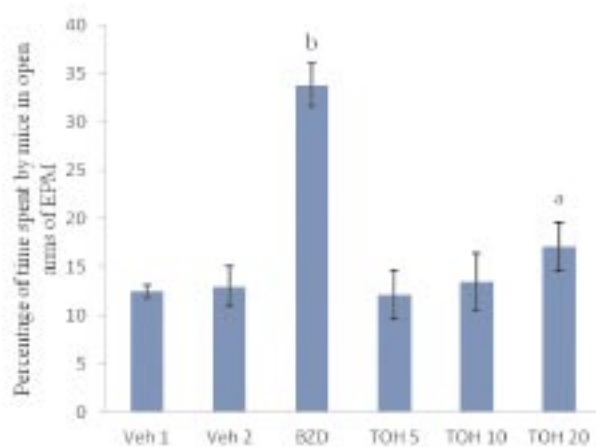
Figure 1. Values are expressed as Mean ± SEM, $n=5$ in each group. Data was analyzed by one–way ANOVA followed by Tukey's Post–hoc test. [a]$P<0.05$ significant difference from vehicle 1 treated group, [b]$P<0.001$ significant difference from vehicle 2 treated group.

3.2. Effect of different drug treatments on mice behavior in light/dark test

Diazepam significantly increased percentage of time spent

in light compartment of light/dark test. Thymol (20 mg/kg) significantly increased percentage of time spent in light compartment of light/dark test as compared to vehicle (0.01% ethanol) treated mice. Therefore thymol (20 mg/kg) produced significant anti–anxiety effect as compared to vehicle (0.01% ethanol) treated mice (Table 2; Figure 2).

Table 1
Effect of different treatments on percentage of time spent by mice in open arms of elevated plus maze.

| Treatments	Dose (mg/kg, i.p.)	% of time spent in open arms
Vehicle 1 (0.01% ethanol)	10 mL	12.46±0.75
Vehicle 2 (0.9% NaCl)	10 mL	13.01±2.07
Diazepam	2	33.80±2.29[b]
Thymol	5	12.13±2.56
Thymol	10	13.46±3.01
Thymol	20	17.13±2.50[a]

Values are expressed as Mean ± SEM, $n=5$ in each group. Data was analyzed by one–way ANOVA followed by Tukey's Post–hoc test. [a]$P<0.05$ significant difference from vehicle 1 treated group, b$P<0.001$ significant difference from vehicle 2 treated group.

Table 2
Effect of different treatments on percentage of time spent by mice in light compartment of light/dark test.

Treatments	Dose (mg/kg, i.p.)	% of time spent in light compartment
Vehicle 1 (0.01% ethanol)	10 mL	15.73±2.65
Vehicle 2 (0.9% NaCl)	10 mL	16.46±2.82
Diazepam	2	37.93±2.41[b]
Thymol	5	16.86±2.87
Thymol	10	16.86±2.50
Thymol	20	21.00±2.30[a]

Values are expressed as Mean ± SEM, $n=5$ in each group. Data was analyzed by one–way ANOVA followed by Tukey's Post–hoc test. [a]$P<0.05$ significant difference from vehicle 1 treated group, [b]$P<0.001$ significant difference from its vehicle 2 treated group.
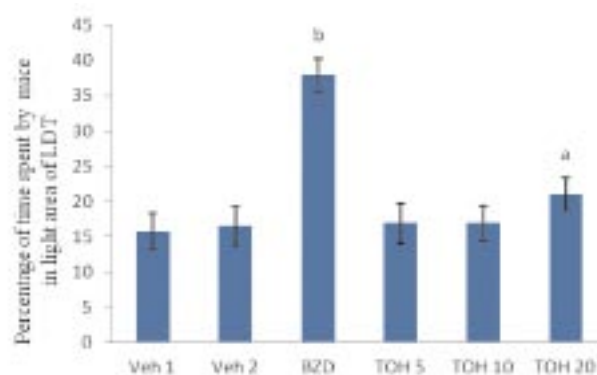
Figure 2. Values are expressed as Mean ± SEM, $n=5$ in each group. Data was analyzed by one–way ANOVA followed by Tukey's Post–hoc test. [a]$P<0.05$ significant difference from vehicle 1 treated group, [b]$P<0.001$ significant difference from its vehicle 2 treated group.

4. Conclusion

Elevated plus maze (EPM) and Light/dark test box (LDT) are two best models of anxiety. These both come under ethologically based animal models of fear and anxiety and involves the animal's spontaneous or natural reactions (*e.g.* flight, avoidance and freezing) to stress stimuli that do not explicitly involve pain or discomfort[33]. In the present study, only thymol 20 mg/kg dose produced significant anti–anxiety like activity among its three doses. Diazepam binds to a specific subunit on the $GABA_A$ receptor at a site distinct from the binding site of the endogenous GABA molecule, known as an allosteric site. The $GABA_A$ receptor is an inhibitory channel which, when activated, decreases neuronal activity. Benzodiazepines cause an increased opening of the chloride ion channel when GABA binds to its site on the $GABA_A$ receptor, leading to more chloride ions entering the neuron, which in turn leads to enhanced central nervous system depressant effects[34]. Thymol is a positive allosteric modulator of the $GABA_A$ receptor[35]. The GABA–modulating and GABA–mimetic activities of thymol on human $GABA_A$ and fruitfly (Drosophila melanogaster Meig.) homomeric RDLac $GABA_A$ receptors expressed in Xenopu oocytes. Thymol enhanced the GABA–dependent chloride currents in oocytes expressing various human $GABA_A$ receptor isoforms as well as the insect $GABA_A$ receptor[36]. It has been observed that NO donors produce 5–HT release in a biphasic way, with low concentrations of NO donors decreasing 5–HT release in the hypothalamus and high concentrations increasing it. Both effects are mediated by cGMP[37]. There is evidence suggesting the role of NO/cGMP signaling pathway in effect of NO on anxiety[38]. Inhibition of NO/cGMP signaling pathway by inhibiting of NOS has been reported to produce anti–anxiety effect[39]. Thymus vulgaris oil showed a significant decrease in aflatoxin–induced increased production of NO in liver and kidney proved its NO modulating anti–oxidant activity[40]. Thymus vulgaris extract significantly inhibit the enhanced production of NO, induced by LPS and INF–γ in a dose dependent manner[41]. Final conclusion of this study is that, the anti–anxiety like activity of thymol may through possible modulation of GABA pathway or/and NO–cGMP pathway or/and 5–HT pathway. Further study is needed to explore the precise mechanism in anxiety by the Thymol.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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